Airway pathology in COPD : smoking cessation and pharmacological treatment intervention. Results from the GLUCOLD study

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De arts (in tegenstelling tot de natuurvorser) houdt zich bezig met een enkel organisme, het menselijk wezen, waarbij hij ernaar streeft diens identiteit onder moeilijke omstandigheden intact te laten.
(Ivy McKenzie)

Contents

Chapter 1

General introduction and aims of the study

Introduction

Chronic obstructive pulmonary disease (COPD) is a major cause of morbidity and mortality worldwide. Patients suffer from progressive dyspnea at rest or on exertion, chronic cough and sputum expectoration. The disease is characterized by progressive and largely irreversible airways obstruction, which is demonstrated by an accelerated decline of lung function (FEV_1: forced expiratory volume in 1 second) with age. In addition, a subgroup of patients experience frequent exacerbations, which may require hospital admission. The disease leads to impaired quality of life, disablement and eventually death. The main risk factor for development of COPD is cigarette smoking, and smoking-induced inflammation in the lung is thought to play a key role in the pathogenesis of COPD. In the studies described in this thesis we therefore focused on the role of airway inflammation in relation to lung function, smoking and therapeutic intervention.

Definition of COPD

COPD is defined by the Global Initiative for Chronic Obstructive Lung Disease (GOLD) as *"a preventable and treatable disease with some significant extrapulmonary effects that may contribute to the severity in individual patients. Its pulmonary component is characterized by airflow limitation that is not fully reversible. The airflow limitation is usually progressive and associated with an abnormal inflammatory response of the lung to noxious particles or gases"* (1;2). Consequently, spirometry is essential for the diagnosis and is used for classification of the severity of COPD (table 1). The characteristic symptoms of COPD are chronic and progressive dyspnea, cough and sputum production.

Table 1. GOLD classification of COPD severity based on post-bronchodilator FEV_1 (1;2).

Stage I: Mild COPD	$FEV_1/FVC < 0.70$ $FEV_1 \geq 80\%$ predicted
Stage II: Moderate COPD	$FEV_1/FVC < 0.70$ $50\% \leq FEV_1 < 80\%$ predicted
Stage III: Severe COPD	$FEV_1/FVC < 0.70$ $30\% \leq FEV_1 < 50\%$ predicted
Stage IV: Very Severe COPD	$FEV_1/FVC < 0.70$ $FEV_1 < 30\%$ predicted or FEV_1 < 50% predicted plus chronic respiratory failure

Abbreviations: FEV_1: Forced Expiratory Volume in one second; FVC: Forced Vital Capacity; respiratory failure: arterial pressure of oxygen (PaO_2) less than 8.0 kPa (60 mmHg) with or without arterial pressure of CO_2 ($PaCO_2$) greater than 6.7 kPa (50 mmHg) while breathing air at sea level.

Epidemiology of COPD

COPD is one of the leading causes of mortality and morbidity worldwide (3). The prevalence of COPD defined by lung function criteria in adults aged ≥40 years is ~9-10% (4). According to the latest WHO estimates (2007), currently 210 million people have COPD and 3 million people died of COPD in 2005 (5). In the Netherlands, the prevalence of COPD was 316,400 individuals in 2003, there were 18,500 hospital admissions due to COPD in 2004, and 5,662 people died of COPD in 2004 (6). However, underrecognition and underdiagnosis of COPD leads to significant underreporting, and therefore underestimation of prevalence data. The prevalence, morbidity, and mortality of COPD vary across countries and different groups within countries, but are directly related to tobacco smoking. In addition, these figures are projected to increase in the coming decades as smoking frequencies rise and the population ages (7;8). The WHO predicts that COPD will become the fourth leading cause of death worldwide by 2030 (5).

Risk factors for COPD

Cigarette smoking is the most common risk factor for COPD. Indeed, Fletcher *et al*. have shown that the decline in lung function is faster in smokers compared to non-smokers (9). However, only 15-20% of all smokers, and up to 50% of elderly (>75 years) smokers (10), developed COPD suggesting a role for age and individual susceptibility. Alpha-1 antitrypsin deficiency (SERPINE1 gene; a serine protease inhibitor protein) is the most important known genetic risk factor for COPD (11). Polymorphisms in another related gene, SERPINE2, has also been proposed as a potential risk factor (12;13). Other candidate genes have not been reliably replicated (14), such as tumor necrosis factor (TNF)-α gene (15), transforming growth factor (TGF)-β1 gene (16), microsomal epoxide hydrolase (mEPHX)1 gene (17), and A Disintegrin and Metalloprotease (ADAM) 33 gene (18). Further risk factors include: exposure to occupational dust and chemicals, air pollution, reduced lung growth and development, oxidative stress, female gender, infections, low socioeconomic status, inadequate nutrition, cooking and heating in poorly ventilated spaces, and asthma (1;2). Furthermore, it has been reported that increased airway hyperresponsiveness (19), elevated serum IgE and peripheral blood eosinophilia are associated with a more rapid decline of FEV_1 in smokers (20).

Heterogeneity of COPD

COPD has been recognized as a heterogeneous disorder in terms of clinical presentation, physiological and pathological characteristics (21;22). Clinical phenotypic distinctions in COPD include: symptoms of chronic bronchitis, frequent exacerbations, weight loss, rapid lung function decline, airways hyperresponsiveness,

impaired exercise tolerance, and in some patients perhaps features of asthma (23). This is accompanied by pathophysiological characteristics such as partial reversibility to bronchodilators, air trapping, impaired diffusing capacity, and airway hyperresponsiveness (24). Pathological findings include: features of emphysema, large and small airways disease, epithelial goblet cell hyperplasia, squamous cell metaplasia, and presence of eosinophilic airway inflammation in some patients with COPD (25). The presence and contribution of these features to the severity of COPD varies between patients and may be related to distinct pathophysiological mechanisms involved in development, clinical presentation, and course of the disease. It is increasingly recognized that disease heterogeneity provides opportunities for targeted interventions (23;26;27).

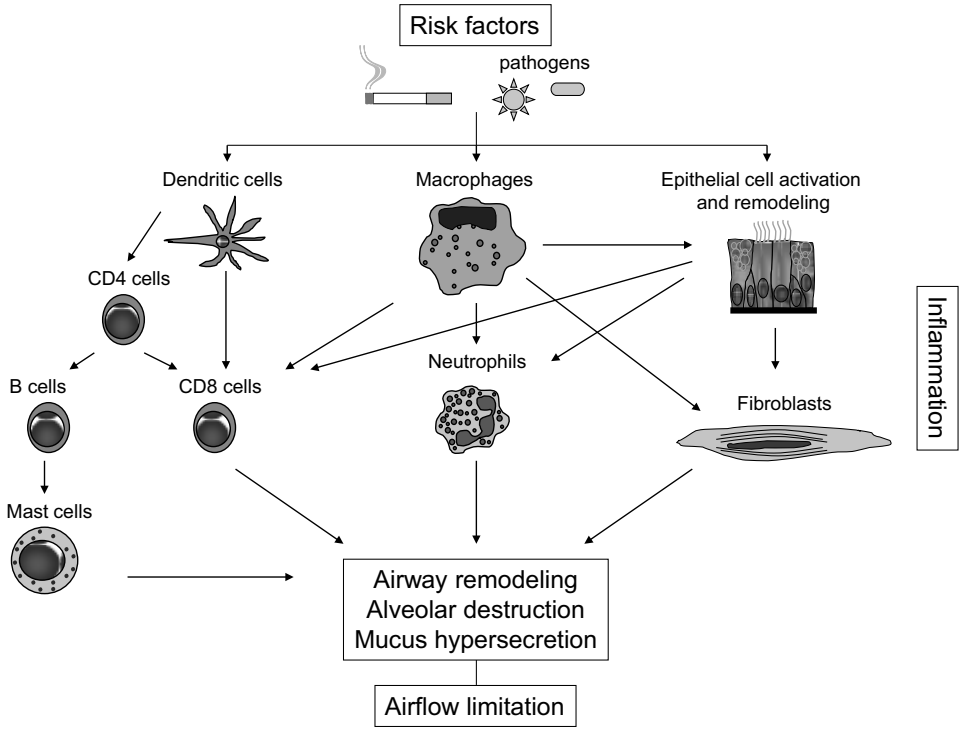
Pathology of COPD

Pathological changes are present at different levels and compartments of the lungs of patients with COPD: proximal airways, peripheral airways, lung parenchyma, and pulmonary vasculature. Induced sputum and endobronchial biopsies mainly represent the central airways. Bronchoalveolar lavage fluid (BAL) and surgical resection samples represent peripheral airways and lung parenchyma. Changes in the central airway wall include increased numbers of macrophages, $CD8^+$ T-lymphocytes and B-lymphocytes, increased epithelial goblet cell numbers and squamous cell metaplasia, and enlarged submucosal glands (28). The peripheral airways show increased numbers of macrophages, T-lymphocytes (predominantly $CD8^+$), B-lymphocytes, and mast cells (29). In addition, localization of neutrophils and $CD8^+$ cells has been observed in the airway smooth muscle layer in smokers with COPD (30). Structural changes of peripheral airways consist of airway wall thickening, with increased extracellular matrix components (31) and smooth muscle mass (32;33), peribronchial fibrosis, luminal inflammatory exudates, and airway narrowing (34). The lung parenchyma also demonstrates increased numbers of macrophages and $CD8^+$ T-lymphocytes, next to alveolar wall destruction, and apoptosis of epithelial and endothelial cells (35). The pulmonary vasculature shows increased numbers of macrophages and $CD8^+$ T-lymphocytes, thickening of the intima, endothelial cell dysfunction, and increased smooth muscle mass. Finally, the airway lumen of patients with COPD contains predominantly neutrophils (36) and increased numbers of $CD8^+$ T-lymphocytes (37) in larger airways, and increased numbers of macrophages and neutrophils in the periphery of the lung (38). In some studies increased numbers of eosinophils have been described in the airway lumen of (a subgroup of) COPD patients (39).

Pathogenesis of COPD

The pathogenesis of COPD is strongly linked to the effects of cigarette smoke on the lungs. Different important processes have been suggested to play a role in the development and progression of COPD. The key processes include pulmonary inflammation, oxidants-antioxidants imbalance, and protease-antiprotease imbalance (40-42). In addition, it is thought that the observed airway remodeling, including epithelial alterations and changes in the composition of the extracellular matrix, may be caused by an aberrant repair process following initial injury. The enhanced or abnormal inflammatory response to cigarette smoke is a characteristic feature of COPD and has the potential to produce lung injury (Figure 1). Both innate and adaptive inflammatory and immune responses are involved in pulmonary inflammation in COPD. Since inflammation and epithelial remodeling are the main topics of this thesis, the role of inflammatory cells and epithelial cells in COPD will be discussed more extensively below.

Figure 1. Inflammatory cascade in COPD.



Innate immune responses and COPD

Neutrophils

The role of neutrophils in the pathogenesis of COPD is not entirely clear. Multiple studies have shown increased neutrophil counts in sputum (36;43;44) and airway lavage fluid (38;45-47) from patients with COPD, whereas studies about their presence in the airway wall are inconsistent (30;48;49). Relationships have been shown between airflow limitation and sputum (36;43), BAL (46;47), and bronchial neutrophils (49), and between rate of FEV_1 decline and sputum neutrophils (50) in patients with COPD. Neutrophils can migrate to the respiratory tract under control of chemotactic factors, such as LTB4, IL-8, and other CXC chemokines, which are increased in COPD airways (51). Neutrophils are a source of reactive oxygen metabolites, inflammatory cytokines, lipid mediators, antimicrobial peptides, and tissue damaging proteinases, such as neutrophil elastase, cathepsin G, and proteinase 3, as well as matrix metalloproteinase (MMP)-8 and MMP-9. These compounds are implicated in the generation of mucous metaplasia in chronic bronchitis and the destruction of lung tissue in emphysema (52), and thereby may play a role in progression of airflow limitation in COPD.

Macrophages

Increased macrophage numbers have been observed in lavage fluid (38), the airway wall (48;53;54), the lung parenchyma (55), and bronchial glands (56) of patients with COPD. Macrophage numbers in the airways correlate with the severity of COPD (49). Macrophages play a central role in inflammation and host defense against microorganisms, but they also participate actively in the resolution of inflammation after alternative activation. It is unclear which of these macrophage sub-phenotypes predominates in the airways of COPD patients. Macrophages may release reactive oxygen species, chemotactic factors, inflammatory cytokines, smooth muscle constrictors, mucus gland activators, extracellular matrix proteins, and matrix metalloproteinase enzymes (MMPs). Particularly, the latter are thought to be involved in emphysema. *In vivo* studies have indeed observed increased expression of MMP-2, MMP-9, and MMP-12 in patients with COPD compared with healthy controls (57). In addition, alveolar macrophage number in lung parenchyma has been correlated to the severity of lung destruction (55), suggesting a role for macrophages in the development of emphysema.

Eosinophils

Airway eosinophilia can be observed in patients with stable COPD in sputum (39;58-60), BAL fluid (59), and the airway wall (45;59;61). In addition, sputum eosinophilia has been found to be associated with airways obstruction (39) and

with hyperresponsiveness to adenosine 5'-monophosphate (AMP) in COPD (62). During exacerbations of chronic bronchitis eosinophil counts can even be as high as in asthma (63-65). These findings suggest that airway eosinophilia is functionally important in patients with COPD. It has been suggested that eosinophilia in COPD is related to the intensity of the inflammatory process in the airways, leading to a nonspecific recruitment and activation of eosinophils (59). However, the eosinophilic inflammation seen in patients with COPD might also identify a subgroup of COPD patients that shares some characteristics with patients with asthma, including not only eosinophilia, but also a partial bronchodilator response to inhaled salbutamol (60).

Mast cells

A role for mast cells in the pathogenesis of COPD is yet unclear. Whereas increased numbers of mast cells have been found in the airways of COPD patients by our group (29), and in patients with chronic bronchitis by other investigators (66;67), most previous studies show no evidence of mast cell abundance in the airways or parenchyma of COPD patients (49;56;68;69). Mast cells and their secreted cytokines (IL-8, TNF-α) and enzymes (tryptase, chymase), have been shown to initiate and drive a variety of processes relevant to airway inflammation and remodeling. These include airway fibrosis and extracellular matrix turnover (70;71), angiogenesis, airway smooth muscle and epithelial cell hyperplasia, inflammation, alterations in bronchial tone, and mucus hypersecretion (72;73). It is yet unclear whether these mast cell induced mechanisms play a role in the pathogenesis of COPD. Interestingly, a more recent study demonstrated similar distributions of tryptase and chymase positive mast cells in the airways of COPD patients compared to controls (74). Moreover, higher numbers of these cells in peripheral airways were associated with less severe airflow limitation in COPD. It is unclear whether these results reflect a protective role of mast cells in the pathogenesis of COPD or increased degranulation of mast cells.

Dendritic cells

Dendritic cells initiate and regulate both innate and adaptive immune responses to inhaled antigens, viruses and bacteria. Recently, increased numbers of dendritic cells have been observed in small airways and induced sputum from patients with COPD compared to smokers without COPD, increasing with disease severity (75). CCL20, the most potent chemokine in attracting dendritic cell precursors to sites of inflammation, is also increased in the airways of COPD patients (75). The role of dendritic cells in de pathophysiology of COPD remains to be clarified.

Epithelial cells

The airway epithelium of patients with COPD undergoes alterations, including squamous cell metaplasia, and goblet and basal cell hyperplasia (76). Bronchial epithelial cells contribute to an adequate maintenance of lung homeostasis by mucus production, ciliary beating, secretion of antimicrobial products and adequate immunological drive in response to noxious stimuli. Therefore, epithelial remodelling in COPD may be involved in the pathogenesis of the disease. Goblet cell hyperplasia is more pronounced in smokers with COPD compared to those without airflow limitation (77). In addition, it contributes to mucus hypersecretion, which is associated with morbidity and mortality in COPD (78;79). Squamous cell metaplasia impairs mucociliary clearance and contributes to the increased risk of squamous cell carcinoma as observed in COPD (80). The mechanisms underlying epithelial alterations in COPD are incompletely understood. The epidermal growth factor receptor (EGFR) cascade has been shown to be involved in mucin production and goblet cell hyperplasia (81;82), repair of damaged epithelium (81;82), as well as development of squamous cell carcinoma (83). In addition to EGFR ligands, a wide variety of stimuli can induce EGFR activation *in vitro* and in animals, including cigarette smoke (81;82). Additionally, epithelial EGFR expression is increased in bronchial biopsies from smokers with (84;85) and without (85;86) COPD compared to non-smokers. Previously, we have observed higher epithelial EGFR expression in ex-smokers with COPD compared to non-COPD, but not in current smokers, suggesting that current smoking may obscure differences in EGFR expression (87). Therefore, EGFR activation may play a role in epithelial phenotypic alterations observed in COPD through active smoking.

Adaptive immune responses and COPD

T-Lymphocytes

In COPD the $CD8^+$ T-cell is the most prominent lymphocyte subtype. Increased $CD8^+$ T-lymphocyte numbers have been found in sputum (37), the airway wall (32;53;88), and lung parenchyma (69;89) of COPD patients. Moreover, a strong correlation has been found between severity of airflow limitation and the number of $CD8^+$ cells (53;88). Mucosal $CD8^+$ cells have also been associated with airway hyperresponsiveness to AMP (62). Additionally, sputum $CD8^+$ cells of COPD patients have elevated cytotoxic activity (90). A key function of $CD8^+$ cells is to combat viruses, which may consequently lead to collateral tissue damage via release of lytic substances such as perforins and granzymes (25). $CD8^+$ cells can induce structural cell apoptosis (25), as suggested for alveolar endothelial and epithelial cells (89). A more recent study demonstrated that patients with COPD had a blunted regulatory T-cell response to tobacco smoke, and higher $CD8^+CD45RA^+$ and lower

$CD8^+CD45R0^+$ than smokers with normal lung function, indicating a shift to more final maturation-activation state of CD8+ T-lymphocytes (91). In addition, It has been hypothesized that smoking and inflammation induced injury to the lung may result in structural alterations of cell- and tissue proteins into "autoantigens" that are recognized by T-lymphocytes leading to further lung injury (89). Alternatively, a persistent intracellular pathogen, such as adenovirus (92), may provide a foreign antigenic stimulus that activates T-lymphocytes.
The role of $CD4^+$ cells in COPD is unknown. $CD4^+$ cells are a diverse group of lymphocytes in which various subtypes are recognized that may contribute to COPD pathogenesis. These include not only Th1 and Th2 cells, but also regulatory and Th17 cells. $CD4^+$ cells may contribute to the inflammatory process by production of pro-inflammatory cytokines, providing help for B cell responses, and may be important by their actions as T-helper cells, priming $CD8^+$ cytotoxic responses, maintaining their memory and ensuring survival (25).

B-lymphocytes and plasma cells
It has been demonstrated that B-lymphocyte numbers and lymphoid follicles in the small airways of COPD patients are increased (34;93), and associated with disease severity (34). While previous studies observed similar B cell counts in large airways of COPD patients compared to controls (53;54), we have shown increased numbers in bronchial biopsies associated with severity of airflow limitation (94). These B cells can result from a local inflammatory process, an altered T-helper (Th)1-Th2 balance and/or can reflect an antigen-specific reaction to pathogens, components of cigarette smoke, or auto-antigens. In line with this, B-cell follicles with an oligoclonal, antigen-specific reaction were found in men and mice with emphysema (95). Furthermore, the presence of anti-elastin antibodies (96) and IgG anti-epithelial cell antibodies, and the potential to mediate cytotoxicity (97), support a role for auto-reactive adaptive immune responses in patients with COPD. Plasma cells are terminally differentiated effector B cells, and are the cellular source of mucosal immunoglobulin production. There is limited data on their role in COPD. One single study by Zhu *et al.* examined their numbers in patients with chronic bronchitis with or without airflow limitation (67). It was concluded that patients with chronic bronchitis have increased plasma cell counts in submucosal glands and subepithelial compartments compared to controls, which was not significant for patients with airflow limitation.

Pathophysiology of COPD
Irreversible airflow limitation, the main characteristic of impaired lung function in COPD, results from the loss of elastic recoil of the parenchyma and from the

increase in airway resistance. The pathological substrate of airflow limitation is predominantly located in the periphery of the lung (98). In the emphysematous lungs, parenchyma destruction and loss of alveolar integrity is observed, which leads to reduced recoil and collapsed small airway lumens. Additionally, due to destruction of the parenchyma, gas exchange may be impaired, resulting in reduced diffusing capacity. The current working hypothesis is that inflammatory cell infiltration of small airways, together with fibrosis and smooth muscle cell proliferation results in reduced diameter and increased resistance. In addition, mucus hypersecretion may also contribute to airflow limitation. The altered compliance and resistance of small airways may result in uneven distribution of ventilation and early airway closure, as can be measured by "small airways tests", such as the single-breath nitrogen test (sbN_2-test) (99). The physiological abnormalities observed in COPD are progressive and finally cause hypoxia.

Management of stable COPD

Current guidelines recommend smoking cessation and on demand use of short-acting bronchodilators for all GOLD severity stages of COPD (1;2). Addition of long-acting bronchodilators is advised for patients with at least GOLD stage II COPD, and treatment with inhaled corticosteroids for patients with at least GOLD stage III COPD and frequent exacerbations (1;2). In the following paragraphs, the effects of smoking cessation, bronchodilators, inhaled steroids, and combination therapy of inhaled steroids with long-acting bronchodilators on clinical and, in particular, inflammatory parameters in COPD patients will be discussed more extensively.

Smoking cessation

Smoking cessation is able to reduce COPD progression (100) and improve survival (101). Moreover, patients who quit smoking experience fewer respiratory symptoms and less hyperresponsiveness as compared to those who continue smoking (102;103). These beneficial clinical effects of smoking cessation do not appear to be accompanied by a simultaneous simple reduction of airway inflammation characteristic for COPD. Induced sputum from COPD smokers and ex-smokers reveal similar inflammatory cell counts in one study (44), and higher macrophage counts in smokers in another (104). There are only few cross-sectional studies comparing smokers and ex-smokers regarding bronchial inflammation in heterogeneous and relatively small groups of patients without an established diagnosis of COPD (105). Most of these previous studies were performed in patients with chronic bronchitis (66;106). In patients with symptoms of chronic cough and expectoration, ex-smokers tended to have lower mast cell numbers in the lamina propria

than current smokers (66), whereas the number of neutrophils, macrophages, eosinophils, and lymphocytes in bronchial biopsies have been reported to be similar (106). Recently, it was observed that in patients with established COPD there are also no differences in bronchial T-lymphocytes, neutrophils, macrophages and mast cells between current and ex-smokers (107), but that current smokers have lower numbers of dendritic cells (108). In contrast, another study observed a positive relation between macrophages in bronchial biopsies and current smoking in COPD (104). Finally, in a prospective study of COPD patients bronchial inflammation also persisted after smoking cessation, while the number of sputum neutrophils, lymphocytes, IL-8 and ECP levels significantly increased (109). It needs to be emphasized that the majority of studies did not take duration of smoking cessation into account when comparing current and ex-smokers. However, it has been shown that this may influence the inflammatory response in small airways (61). In summary, airway inflammation characteristic for COPD seems to persist after smoking cessation, in contrast to clinical beneficial effects on symptoms and decline of lung function. Possible explanations for this ongoing inflammatory response have been proposed, and include persistent presence of a microbial stimulus or the development of autoimmune disease or alternatively to represent the inflammatory component of tissue repair.

Pharmacological intervention

Bronchodilators

Inhaled bronchodilator medications (β_2-agonists and anticholinergics) are central to the symptomatic management of COPD (110;111). The principal action of β_2-agonists is to relax airway smooth muscle by β_2-adrenergic receptor stimulation, which increases cAMP and produces functional antagonism to bronchoconstriction. The most important effect of anticholinergics is to block the acetylcholine effect on muscarinic receptors. Long-acting β_2-agonists (salmeterol) and long-acting anticholinergics (tiotropium) are more effective than short-acting bronchodilators (112;113). Both salmeterol and tiotropium have beneficial effects on symptoms, exacerbation rates and lung function level in COPD patients (114-117). In addition, salmeterol has an effect on decline in lung function (118), which was not observed with tiotropium treatment (117). It has been suggested that bronchodilators also exhibit some anti-inflammatory effects. In fact, salmeterol inhibits inflammatory responses by neutrophils and mononuclear cells *in vitro* (119;120), and in mouse models of lung inflammation *in vivo* (121). In addition, salmeterol has anti-inflammatory effects in LPS exposed healthy volunteers (122) and in mild asthmatics (123;124). To our knowledge, the effects of salmeterol mono-therapy on airway inflammation in COPD have not been studied. Although tiotropium is

able to suppress acetylcholine-induced release of chemotactic mediators *in vitro* (125) and inhibit allergen-induced increase in MUC5AC-positive goblet cell numbers and eosinophil infiltration *in vivo* in guinea pigs (126), it does not seem to attenuate sputum and systemic inflammatory markers in COPD patients (127).

Inhaled steroids

Regular therapy with inhaled corticosteroids leads to clinical benefit in terms of symptoms, exacerbation rate, and initial improvement in FEV_1 (128;129). In addition, withdrawal of inhaled steroids in COPD results in deterioration of lung function, symptoms and exacerbation rate (130;131). So far, it has not been settled whether ICS alone or combined with LABA changes FEV_1-decline in COPD (132-137) and if so, in which COPD phenotypes. ICS may have the potential of 'disease modification' since long-term therapy improves airway hyperresponsiveness (138), a disease feature that is present in about 70% of the COPD population and by itself constitutes a risk factor for accelerated FEV_1 decline (139;140). Interestingly, a recent analysis of the TORCH study suggests that prolonged therapy with ICS and/or LABA attenuates FEV_1-decline in COPD (118). Several short-term studies have investigated the anti-inflammatory effects of ICS in patients with COPD. 2-3 Months treatment with inhaled steroids in patients with COPD reduces bronchial mast cells, but not $CD8^+$ cells, neutrophils or macrophages (141;142), and may reduce sputum total cell counts, neutrophils and lymphocytes (143). In addition, the presence of sputum eosinophilia predicts the response of airways obstruction to oral and inhaled corticosteroid treatment in COPD (58;144-146), and can even be used to guide it (27). Therefore, the clinical effects of long-term treatment with inhaled steroids in COPD may be mediated, at least partially, by anti-inflammatory effects. However, the longer-term anti-inflammatory effects of inhaled corticosteroids have not been studied before.

Combination therapy

Addition of LABA to inhaled corticosteroids treatment in COPD is more effective in improving lung function level (147;148), health status (114;149) and reducing exacerbations (114;150), although it does not have supplementary effects on FEV_1 decline (118). Combination treatment with a LABA for 3 months may have additional anti-inflammatory effects on bronchial $CD8^+$ and $CD68^+$ cells compared to mono-therapy with inhaled steroids (151), and reduces sputum neutrophils and eosinophils compared to placebo (152). To our knowledge, the longer-term anti-inflammatory effects of combination therapy have not been studied previously. Finally, although triple therapy with salmeterol/fluticasone propionate and tiotropium seems to have additional bronchodilator effect after 2 weeks treatment (153), long-term effects of this combination have not been studied so far.

Aims of the present studies

In summary, this thesis addresses airway pathology in relation to three different aspects of COPD: pathophysiology, smoking cessation and pharmacological treatment. The questions mentioned in the introduction have been addressed in cross-sectional and longitudinal analyses from the Groningen Leiden Universities Corticosteroids in Obstructive Lung Disease (GLUCOLD) study.

The GLUCOLD study

The GLUCOLD study is a two-centre, randomized, double-blind, four armed, placebo-controlled, parallel-group trial. It was designed to investigate the effect of long-term versus short-term treatment with inhaled corticosteroids, either or not combined with a long-acting β_2-agonist, on bronchial inflammation in patients with stable COPD. The in- and exclusion criteria are presented in table 2.
114 COPD patients were randomized to 30 months treatment with fluticasone propionate, fluticasone propionate/salmeterol combination therapy, placebo, and 6 months fluticasone propionate followed by 24 months placebo (figure 2). Patients visited the out-patient clinic every three months to perform spirometry and obtain symptoms- and health status questionnaires. At baseline, after 6, and 30 months treatment more extensive measurements were performed including: peripheral blood analysis, diffusion capacity, bodyplethysmography, methacholine provocation, sputum induction, and bronchoscopy (figure 3).

Figure 2. Design of the GLUCOLD study.

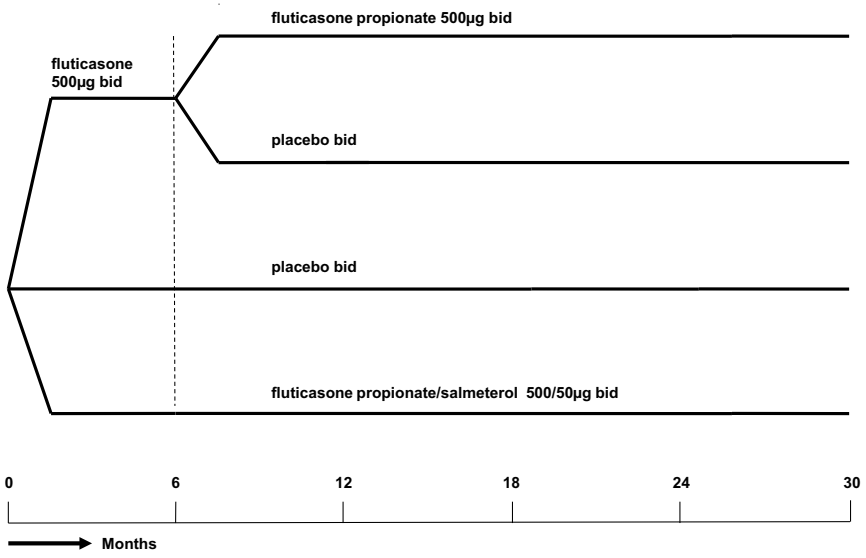
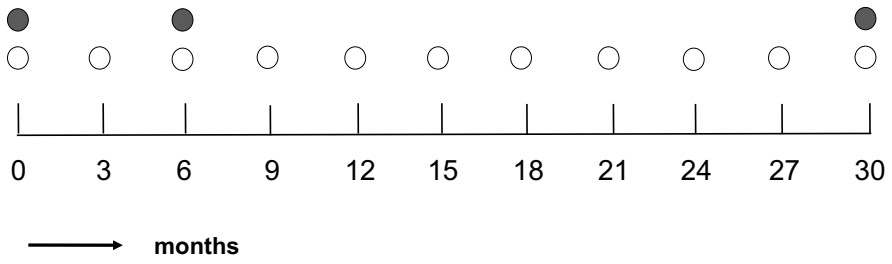


Table 2. In- and exclusion criteria of the GLUCOLD study.

Inclusion criteria
Age: 45-75 years
≥ 10 Packyears of smoking
≥ 1 of the following symptoms: chronic cough, chronic sputum production, frequent exacerbations, or dyspnoea on exertion
No course of oral corticosteroids during last 3 months, no maintenance treatment with inhaled or oral steroids during last 6 months
Postbronchodilator FEV_1 (after 400 μg of inhaled salbutamol) < 90% confidence interval (90% CI) of the predicted FEV_1, and postbronchodilator FEV_1/IVC ratio below the 90% CI of the predicted FEV_1/IVC ratio (154)
Postbronchodilator FEV_1 > 1.3 litre and > 20% of predicted value.
Exclusion criteria
Prior or concomitant history of asthma
Alpha-1 antitrypsin deficiency (SZ, ZZ, zero phenotype)
Other active lung disease, except for mild bronchiectasis
Contra-indications for elective bronchoscopy, such as: O_2 saturation <90%, abnormal coagulability, anti-coagulant therapy which cannot be temporarily withheld for performance of bronchoscopy, history of pneumothorax, uncontrolled angina pectoris
Other diseases likely to interfere with the purpose of the study.
Inability to keep diary and to understand written and oral instructions in Dutch

Figure 3. Measurements during the GLUCOLD study.



○ Symptoms, questionnaires, lung function

● CO diffusion, bodybox, peripheral blood, PC_{20} methacholine, sputum induction, bronchoscopy: bronchial biopsies

Research questions

Relation between airway inflammation and pathophysiology in COPD

1. *Are the various functional and inflammatory features of COPD separate, complementary domains of this heterogeneous disease?*
   In chapter 2 we applied a factor analysis including lung function indices and markers of inflammation in induced sputum and exhaled air from patients with clinically stable COPD.
2. *Is small airways dysfunction in patients with COPD associated with the inflammatory profile characteristic for COPD?*
   In chapter 3 we examined, in a cross-sectional study in 51 patients with COPD, the relationship of uneven ventilation and airway closure, measured by the single breath nitrogen (sbN_2)-test, with the number of inflammatory cells in bronchial biopsies, bronchoalveolar lavage (BAL) fluid, and induced sputum, together with the levels of neutrophil elastase (NE), IL-8, and secretory leukocyte proteinase inhibitor (SLPI) in BAL fluid.

Effect of smoking cessation on airway pathology in COPD

3. *Does bronchial inflammation in patients with established COPD differ between active smokers and patients who stopped smoking? Is airway inflammation associated with duration of smoking cessation?*
   In chapter 4 the number of inflammatory cells in bronchial biopsies of current and ex-smokers with COPD was investigated, and related to duration of smoking cessation.
4. *Is bronchial epithelial cell proliferation and differentiation in patients with COPD different between active smokers and those who stopped smoking, and is this difference influenced by the duration of smoking cessation? Are the epithelial changes associated with epithelial growth factor receptor (EGFR) expression?* In chapter 5 the extent of epithelial goblet cell hyperplasia, proliferation, squamous cell metaplasia, and EGFR expression in bronchial biopsies of current and ex-smokers with established COPD was measured and related to smoking cessation duration.

Treatment effects on airway pathology in COPD

5. *Are there additional anti-inflammatory effects of long-term treatment compared to short-term treatment with inhaled steroids in patients with COPD? Does discontinuation of inhaled steroids lead to a flare up of inflammation? Does addition of long-acting β_2-agonists to inhaled steroids lead to additional anti-inflammatory effects after long-term therapy?*
   In chapter 6 a longitudinal randomized trial was performed examining the effects of 30 months treatment with fluticasone propionate, fluticasone propionate/salmeterol combination therapy, placebo, or 6 months fluticasone propionate followed by 24 months placebo on sputum and bronchial inflammatory cells and bronchial epithelial remodelling.

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Chapter 2

Dissociation of lung function and airway inflammation in chronic obstructive pulmonary disease

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Abstract

Chronic obstructive pulmonary disease (COPD) is defined by progressive, irreversible airflow limitation and an inflammatory response of the lungs, usually to cigarette smoke. However, COPD is a heterogeneous disease in terms of clinical, physiological, and pathological presentation. We aimed to evaluate whether airflow limitation, airway responsiveness, and airway inflammation are separate entities underlying the pathophysiology of COPD by using factor analysis. A total of 114 patients (99 males/15 females, age 62 ± 8 years, 42 pack-years smoking, no inhaled or oral steroids >6 months) with irreversible airflow limitation (postbronchodilator FEV_1 63 ± 9% predicted, FEV_1/inspiratory vital capacity [IVC] 48 ± 9%) and symptoms of chronic bronchitis or dyspnea were studied in a cross-sectional design. Postbronchodilator FEV_1 and FEV_1/IVC, reversibility to inhaled β_2-agonists, diffusing capacity, provocative concentration of methacholine required to produce a 20% drop in FEV_1, total serum IgE, exhaled nitric oxide, and induced sputum cell counts (% eosinophils, % neutrophils) were collected. Factor analysis yielded 4 separate factors that accounted for 63.6% of the total variance. Factor 1 was comprised of FEV_1, FEV_1/IVC, and residual volume/total lung capacity. Factor 2 included reversibility, IgE, provocative concentration of methacholine required to produce a 20% drop in $FEV_{1,}$ and diffusing capacity. Factor 3 contained exhaled nitric oxide and factor 4 included sputum % neutrophils and % eosinophils. We conclude that airflow limitation, airway inflammation, and features commonly associated with asthma are separate and largely independent factors in the pathophysiology of COPD.

Introduction

Chronic obstructive pulmonary disease (COPD) is a disease characterized by progressive airflow limitation, which is not fully reversible (1). However, COPD has been recognized as a heterogeneous disorder (2), with components of chronic bronchitis, small airways disease, emphysema, and in some patients perhaps, features of asthma (3). This is accompanied by pathophysiological characteristics, such as partial reversibility to bronchodilators, air trapping, impaired diffusing capacity, and airway hyperresponsiveness (4). The presence and contribution of these features to the severity of COPD varies between patients and may reflect distinct pathophysiological mechanisms in development, clinical presentation, and course of the disease. It is increasingly recognized that such disease heterogeneity provides opportunities for targeted interventions (3;5).

Airway inflammation is thought to play an important role in the pathogenesis of COPD (6). The cellular inflammatory response is characterized by an increase in neutrophils, macrophages, and CD8-positive T-lymphocytes in small and large airways as well as in lung parenchyma (7). The major cell type in induced sputum is the neutrophil, the quantity of which is associated with the severity of airflow limitation (8;9). Although induced sputum does not cover all the inflammatory and structural changes of the lungs in patients with COPD, it does represent a non-invasive marker of inflammation that is potentially useful for disease monitoring. Sputum eosinophilia has also been observed in patients with stable COPD, but its relationship to airflow limitation is controversial (8;10;11). It has been argued that sputum eosinophilia is related to concomitant features of asthma (12). This link would indicate that the pathophysiological entities underlying the clinical phenotypes in COPD may be diverse and are still largely unknown.

The aim of this study was to objectively specify the heterogeneity of COPD by categorizing various functional and inflammatory features of COPD into separate, complementary domains without *a priori* assumptions. Factor analysis allows reducing multiple disease characteristics to a few independent factors, in which each factor groups associated parameters. Because this is essentially accomplished free of predetermined hypothesis on any interrelated parameters, this technique can be considered as a hypothesis-generating analysis.

Factor analysis has been applied previously in studies of patients with asthma (13-16) and COPD (17). In patients with asthma, it has demonstrated that lung function, baseline airway hyperresponsiveness, and eosinophilic inflammation in sputum are nonoverlapping dimensions (13), suggesting that evaluation of patients with asthma should include measurement of all these parameters. In COPD, factor analysis has been applied to study the relationship between dyspnea ratings, exer-

cise capacity, lung function and hyperinflation (17-23). However, these studies did not include inflammatory markers and were unable to study the interrelationships between airway inflammation and the functional features of COPD. Therefore, in the present study, we performed factor analysis, including lung function indices and markers of inflammation in induced sputum and exhaled air, in 114 patients with clinically stable COPD. Some of the results of this study have been previously reported in the form of an abstract (24).

Material and Methods

Patients

Hundred fourteen patients (15 women) aged between 45 and 75 years with COPD, participating in a multi-center trial (Groningen Leiden Universities and Corticosteroids in Obstructive Lung Disease; GLUCOLD study), were included in this study. Patients were recruited from our outpatient clinics, as well as by advertisements in local newspapers, and by screening lung functions from patients in general practice in and around Leiden and Groningen, The Netherlands. All patients were current or ex-smokers and had a history of at least 10 pack years of smoking. They had irreversible airflow limitation (postbronchodilator FEV_1 and FEV_1/IVC < 90% *confidence interval* (CI) of the predicted value (25), $FEV_1 \geq 1.3$ liter and > 20% of the predicted value) and at least one of the following symptoms: chronic cough, chronic sputum production, or dyspnea on exertion. In choosing the 90% CI of predicted values as selection criterion, as opposed to percentage of predicted values per se, we followed the recommendation by the European Respiratory Society (25). Patients with a prior or concomitant history of asthma, alpha-1 antitrypsin deficiency (SZ, ZZ, zero phenotype), or other active lung disease except for mild bronchiectasis were not permitted to the study. The patients did not use a course of oral or inhaled steroids during the last three months, and did not have maintenance treatment with inhaled or oral steroids during the last six months. Maintenance drug therapy of non-selective beta-antagonists, long-acting bronchodilators, methylxanthines, N-acetylcysteine or NSAID's was not permitted. Patients were allowed to use short acting β_2-agonists and ipratropium bromide as rescue medication. The patients were in clinically stable condition and had no symptoms of respiratory tract infection for at least two weeks prior to the study. The Medical Ethics Committees of each center approved the study and all patients gave their written informed consent.

Study Design

The present study had a cross-sectional design, consisting of baseline measure-

ments of the GLUCOLD Study. At visit 1 spirometry was performed before and after inhalation of salbutamol and blood was collected for measurement of total IgE. Body plethysmography, CO-diffusing capacity, and bronchial responsiveness to methacholine were obtained at a second visit. Finally, at the third visit exhaled NO was measured and hypertonic saline-induced sputum was collected. Inhaled bronchodilators were withheld for at least 8 hrs prior to visit day 1 and 2. All visits were performed within six weeks.

Lung Function

Spirometry was performed according to international guidelines (26). A daily-calibrated pneumotachograph (Masterscreen Pneumo or Masterscreen IOS; Jaeger, Würzburg, Germany) was used throughout the study. First, 3 slow inspiratory vital capacity maneuvers (IVC, largest value used) were carried out. Second, maximally 8 forced expiratory vital capacity (FVC) maneuvers were performed to obtain at least 3 technically satisfactory expiratory flow-volume curves from which the forced expiratory volume in 1 second (FEV_1) and FVC did not deviate > 5% or 100 ml from the largest FEV_1 and FVC. From these curves, we used values of the curve with the largest sum of FEV_1 and FVC (26).

Reversibility of airflow limitation (ΔFEV_1) was measured 15 min after administration of 400 μg salbutamol per metered dose-inhaler connected to a spacer (27). The response was expressed as change in FEV_1 as percentage of predicted value (27).

Total lung capacity (TLC) and residual volume (RV) were measured using a constant volume body plethysmograph (Masterscreen body or Masterlab body; Jaeger, Würzburg, Germany), using panting at 0.5 Hz (25).

The diffusing capacity (transfer factor) for carbon monoxide (TL_{CO}) and TL_{CO} per liter alveolar volume (K_{CO}) were measured using the single breath holding method with a rolling seal closed system (Masterscreen MS-CS-FRC, Masterlab transfer or Compactlab transfer; Jaeger, Würzburg, Germany) (28). Reference values for all lung function measurements were obtained from Quanjer *et al.* (25).

Methacholine challenge tests were performed according to the tidal breathing method (29), using serial doubling concentrations methacholine-bromide (0.038 to 39.2 mg/ml) in saline. Methacholine was aerosolized (DeVilbiss 646, Somerset, PA) and inhaled by the 2-min tidal breathing method at 5-minute intervals until FEV_1 dropped by ≥ 20% (lowest of two FEV_1 values; at 30 and 90 seconds) from baseline. The response was expressed as the provocative concentration causing 20% fall in FEV_1 (PC_{20}).

Sputum induction and processing

Sputum was induced and processed according to a validated technique (30), with

some modifications. Prior to sputum induction, 200 μg salbutamol was administered and baseline FEV_1 was recorded. Hypertonic sodium chloride aerosols (4.5 w/v %) were generated by a DeVilbiss Ultraneb 2000 ultrasonic nebulizer with a calibrated particle size (MMAD 4.5 μm) at maximal output (2.5 ml/min). The aerosols were inhaled via the mouth through a two-way valve (No. 2700; Hans-Rudolph, Kansas City, MO, USA), with the nose clipped. Subsequently, the patients inhaled hypertonic saline aerosols during 3 periods of 5 min and sputum was expectorated after each inhalation. Salbutamol was administered when FEV_1 dropped by > 10% from post-salbutamol baseline value and the procedure was interrupted when FEV_1 dropped by > 20%.
Whole sputum samples were processed. The sample was then mixed with an equal volume of dithiothreitol 0.1% w/v (Sputolysin, Calbiochem, USA) and placed in a shaking water bath at 37°C for 15 min. Then sputum was filtered through a nylon gauze (pore-size approximately 48 μm) and centrifuged (450 x g) for 10 minutes at 20°C. The cell pellet was then resuspended in phosphate-buffered saline (PBS) containing 1 % (w/v) human serum albumin (HSA), pH 7.4. Cell viability and total cell counts were performed by Trypan blue exclusion using a hemacytometer. Subsequently, cytocentrifuge slides were prepared (450 rpm, 6 minutes, 100 μl/slide, Cytospin 3, Shandon, Life Sciences International, Veldhoven, NL) and differential cell counts were performed on May-Grünwald-Giemsa-stained cytospins by a qualified technician. Differential leucocyte and cylindric epithelial cell counts were expressed as a percentage of nucleated cells excluding squamous cells. A sputum sample was considered adequate when the percentage squamous cells was less than 80% (30).

Exhaled nitric oxide

Exhaled NO (eNO) levels were determined according to a standardized procedure (31) with some modifications, using a chemiluminescence analyzer (Sievers NOA 270B or ECO physics CLD 700 AL). Patients were asked not to smoke for at least 1 hour prior to the test. A slow vital expiratory capacity maneuver with a constant expiratory flow of 100 mL/sec against an expiratory resistance of 5 cm H_2O was performed. Expiratory NO concentration was sampled continuously from the center of the mouthpiece and the average concentration was determined during a period of 10 seconds. Three reproducible successive recordings were made at 30-s intervals, from which the mean values of exhaled NO were used in the analysis.

Peripheral blood measurements

Total serum IgE concentrations were measured by fluoroimmunoassay (FEIA) using the Pharmacia CAP system (Pharmacia Diagnostics, Uppsula, Sweden).

Statistical analysis

Mean values and SD were computed. When appropriate variables were logarithmically transformed before statistical analysis and presented as median with interquartile range.

Exploratory factor analysis included the following variables: postbronchodilator FEV_1 (%pred), postbronchodilator FEV_1/IVC (%), ΔFEV_1 (%pred), K_{CO} (%pred), PC_{20} (mg/ml), RV/TLC (%), eNO (ppb), sputum % neutrophils and % eosinophils and total serum IgE (IU/ml). The possibility to perform factor analysis was tested by Bartlett's test of sphericity. The Kaiser-Meyer-Olkin (KMO), a measure of sampling adequacy based on correlation and partial correlation, was also evaluated. A high KMO (maximum 1.0) indicates that data are likely to factor well since correlations between pairs of variables can be explained by the other variables (low partial correlation coefficients). Correlation coefficients were analyzed by principal component factor analysis and subsequent rotation according to the standard Varimax criterion (32). In this type of analysis, the correlation between parameters is attributed to their common dependence on independent entities called "factors". The parameters are separated into independent subgroups, and the correlation of parameters within subgroups is due to their common factor. The coefficients that link parameters to factors are called "factor loadings", and are the correlation coefficient between parameters and factors. The number of factors is chosen to be as small as possible but large enough to account for most of the variation within the data. The number of factors was determined by the number of eigenvalues, an index of the proportion of variance explained by successive factors, whose magnitude was ≥ 1 on the Scree plot. However, also the percentage of total variance explained by the factors was taken into account. The Varimax rotation procedure aims to increase the interpretability of the factors by rotation to a simple structure with optimal loadings, such that they are high or low. Ideally, each variable would have a high loading on one factor, whereas its loadings on other factors would be low. In the Varimax rotation this is technically achieved by maximalizing the variation within a factor. Finally, we conducted three additional factor analyses to determine the stability of the factor structures, and thus the robustness of our findings. First, we excluded outliers from the data set, defined as data outside the range of mean $\pm$ 3 x SD, and repeated the factor analysis. Next, the analysis was repeated with number of neutrophils and eosinophils per ml sputum, instead of % neutrophils and eosinophils. Since smoking is the main risk factor for development of COPD, an additional factor analysis was performed in which the number of pack years was added. Univariate correlations were evaluated using Pearson correlation coefficient. All analyses were performed with the Statistical Package of Social Sciences (SPSS 11.0).

Results

Patient characteristics

Patient characteristics of the 114 participants are presented in Table 1. The mean (SD) postbronchodilator FEV_1 was 63.0 (8.8) %pred, with a range from 40.8 to 77.7 %pred. This result indicates that all patients were classified as having moderate to severe COPD according to Global Initiative for Chronic Obstructive Lung Disease (GOLD) criteria (GOLD stage II and III) (1). The patients were heavy smokers with a median of 41.8 pack-years and most of them were current smokers (63.2%).

Table 1. Patient characteristics.

	n	Mean (SD or IQR)
Clinical characteristics		
Sex, male/female	114	99 / 15
Age, yr	114	62 (8)
Smoking history, pack-years *	114	41.8 (31.2-54.8)
Current smoker, yes/no, n	114	72 / 42
IgE, IU/ml*	113	40 (11.5-125)
Lung Function		
Post FEV_1, %pred	114	63.0 (8.8)
Post FEV_1/IVC, %	114	48.2 (8.5)
ΔFEV_1, %pred	113	6.9 (4.9)
K_{CO}, %pred	112	75.9 (25.5)
PC_{20}, mg/ml†	110	0.60 (2.76)
RV/TLC, %	113	48.5 (8.8)
Airway inflammation		
eNO, ppm	92	13.1 (12.7)
Sputum eosinophils, %*	106	1.1 (0.3-2.2)
Sputum neutrophils, %	106	69.4 (16.0)
Sputum eosinophils, n (x 10^4/ml)*	106	1.4 (0.4-4.5)
Sputum neutrophils, n (x 10^4/ml)*	106	102 (47-229)

Definition and abbreviations: ΔFEV_1 = change in FEV_1 as percentage of predicted (reversibility to salbutamol); eNO = exhaled nitric oxide; IQR = interquartile range (25^{th} and 75^{th} percentile"); IVC = inspiratory vital capacity; K_{CO} = diffusing capacity for carbon monoxide per liter alveolar volume; %pred = percentage of predicted; PC_{20} = provocative concentration of methacholine causing a 20% fall in FEV_1; RV = residual volume, TLC = total lung capacity. * Median (IQR). † Geometric mean ± doubling dose.

Table 2. Varimax Rotated Factor-loading Matrix from Factor Analysis.

	Factor 1	Factor 2	Factor 3	Factor 4
Postbr. FEV_1, %pred	**0.89**	-0.16	-0.09	-0.07
Postbr. FEV_1/IVC, %	**0.82**	0.04	-0.23	0.22
RV/TLC, %	**-0.59**	-0.36	-0.03	0.15
ΔFEV_1, %pred	-0.06	**-0.72**	0.08	0.11
IgE, IU/ml	-0.09	**0.61**	0.10	-0.07
PC_{20}, mg/ml	0.36	**0.51**	-0.35	0.14
K_{CO}, %pred	0.44	**0.49**	0.25	0.31
eNO, ppb	-0.09	0.10	**0.89**	-0.11
Sputum eosinophils, %	-0.09	0.02	0.19	**-0.84**
Sputum neutrophils, %	-0.18	-0.25	0.47	**0.54**
Eigenvalue	2.67	1.50	1.20	0.99
Total variance explained, %	26.7	15.0	12.0	9.9

Definition and abbreviations: ΔFEV_1 = change in FEV_1 as percentage of predicted (reversibility to salbutamol); eNO = exhaled nitric oxide; IVC = inspiratory vital capacity; K_{CO} = diffusing capacity for carbon monoxide per liter alveolar volume; %pred = percentage of predicted; PC_{20} = provocative concentration of methacholine causing a 20% fall in FEV_1; RV = residual volume, TLC = total lung capacity. Bold values represent the highest loadings of a variable.

Factor analysis

Bartlett's test of sphericity indicated a correlation between the presently used variables because the correlation matrix was statistically different from an identity matrix (approximate χ^2 = 165.864, degrees of freedom = 45, $p<0.001$). The Kaiser-Meyer-Olkin value was 0.594. Factor analysis yielded 3 separate factors, explaining only 53.7% of the total variance in the data set when the eigenvalue 1 criterion was applied. Therefore, an additional factor analysis was performed with the same data in which 4 factors were selected. This resulted in an increase of total explained variance to 63.6%.

The correlations with the original variables obtained for each Varimax-rotated factor (called factor loadings) and the eigenvalues are displayed in Table 2. FEV_1, FEV_1/IVC, and RV/TLC loaded significantly on factor 1. Factor 2 included ΔFEV_1, total IgE, $PC_{20,}$ and K_{CO}. eNO loaded on factor 3, whereas sputum % neutrophils and eosinophils loaded on factor 4. Interestingly, K_{CO} contributed also to factor 1, and % neutrophils contributed also to factor 3.

Additional Factor Analyses

Factor analysis without outliers in the data set resulted in a similar four-factor struc-

ture as the original one, accounting for 63.4% of the total variance, with the exception that % neutrophils loaded on factor 3 with eNO and not on factor 4.
Factor analysis with number of neutrophils and eosinophils per ml sputum, instead of % neutrophils and eosinophils, did not change the contents of the factors essentially. Again four factors, accounting for 66.0% of the total variance, were found using the eigenvalue 1 criterion. Factor 1 was the same as in the original analysis. Factor 2 included numbers of neutrophils and eosinophils, both with a positive factor loading. Factor 3 contained the variables that originally loaded on factor 2: ΔFEV_1, total IgE, and PC_{20}. Finally, factor 4 included eNO and K_{CO}.
Factor analysis with inclusion of number of pack-years as an additional variable revealed four factors explaining 59.1% of total variance, according to the eigenvalue 1 criterion. All factors were similar to the original analysis described previously, with the exception that K_{CO} and PC_{20} switched from factor 2 to factor 1. Number of pack-years smoked loaded on factor 2 together with ΔFEV_1 and total IgE (Table 3).

Table 3. Varimax Rotated Factor-loading Matrix from Factor Analysis with pack years.

	Factor 1	Factor 2	Factor 3	Factor 4
Postbr. FEV_1/IVC, %	**0.77**	-0.0002	-0.33	0.20
Postbr. FEV_1, %pred	**0.72**	-0.19	-0.24	-0.03
RV/TLC, %	**-0.68**	-0.05	-0.20	0.25
K_{CO}, %pred	**0.59**	0.25	0.37	0.24
PC_{20}, mg/ml	**0.59**	0.28	-0.13	-0.03
Pack-years	-0.22	**0.70**	-0.32	0.06
ΔFEV_1, %pred	-0.29	**-0.62**	-0.11	0.20
IgE, IU/ml	0.07	**0.61**	0.18	-0.05
eNO, ppb	-0.18	0.05	**0.84**	0.02
Sputum eosinophils, %	-0.23	0.09	0.16	**-0.74**
Sputum neutrophils, %	-0.28	-0.08	0.25	**0.68**
Eigenvalue	2.67	1.56	1.22	1.06
Total variance explained, %	24.2	14.2	11.1	9.6

Definition and abbreviations: ΔFEV_1 = change in FEV_1 as percentage of predicted (reversibility to salbutamol); eNO = exhaled nitric oxide; IVC = inspiratory vital capacity; K_{CO} = diffusing capacity for carbon monoxide per liter alveolar volume; %pred = percentage of predicted; PC_{20} = provocative concentration of methacholine causing a 20% fall in FEV_1; RV = residual volume, TLC = total lung capacity. Bold values represent the highest loadings of a variable.

Univariate correlations

The univariate relationships among physiological and inflammatory parameters (sputum neutrophils, eosinophils, and eNO) were as follows. ΔFEV_1 was not associated with inflammatory parameters; however, RV/TLC was associated with sputum % neutrophils (r=0.203, p=0.04), and postbronchodilator FEV_1, RV/TLC, and PC_{20} were associated with number of neutrophils/ml sputum (r=-0.246, p=0.01; r=0.213, p=0.03 and r=-0.338, p<0.001, respectively). Postbronchodilator FEV_1, PC_{20}, and FEV_1/IVC were associated with eNO levels (r=-0.203, p=0.05; r=-0.207, p=0.05 and r=-0.304, p=0.003, respectively). FEV_1/IVC was also related to sputum % eosinophils (r=-0.219, p=0.02), while postbronchodilator FEV_1, K_{CO}, $PC_{20,}$ and FEV_1/IVC were also associated with number of eosinophils/ml sputum (r=-0.207, p=0.03; r=-0.204, p=0.04; r=-0.243, p=0.01 and r=-0.242, p=0.01, respectively).

Discussion

The aim of this study was to objectively specify the heterogeneity of COPD, by categorizing the various functional and inflammatory features of COPD into separate, complementary domains without *a priori* assumptions. Therefore, we performed a factor analysis using physiological and inflammatory data of 114 patients with moderate to severe COPD, not treated with inhaled steroids. This resulted in a four-factor structure, explaining 63.6% of the total variance. Factor 1 included: FEV_1, FEV_1/IVC and hyperinflation; factor 2: β_2-response, total serum IgE, airway hyperresponsiveness and K_{CO}; factor 3 included: eNO, and factor 4 included: sputum % neutrophils and eosinophils. These four factors indicate that airflow limitation, features commonly associated with asthma, and airway inflammation are separate, largely independent dimensions that characterize patients with COPD.

To our knowledge, this is the first study in patients with COPD combining functional parameters and markers of airway inflammation in a factor analysis. Previous studies have applied factor analysis on quality-of-life, symptoms scores, exercise capacity, and lung function parameters in patients with stable COPD, without evaluating inflammatory indices (17-23). However, the latter have been part of factor analysis in recent asthma research (13;15;16). In patients with mild to moderate asthma, Rosi and colleagues demonstrated by factor analysis that airway function, bronchial responsiveness with reversibility, and eosinophilic inflammation, as assessed in sputum, are independent dimensions (13). Our current results demonstrate that airway function, bronchial responsiveness with reversibility, and inflammation as assessed in sputum or exhaled are predominantly nonoverlapping dimensions in patients with COPD as well.

Interestingly, this study showed that measurements of airflow limitation, traditionally used to determine disease severity in COPD (33), and hyperinflation, a measure of air trapping, were combined in the first factor. According to the statistical method of factor analysis, these measurements represent an important, separate dimension in the assessment of patients with stable COPD. This result is consistent with some (17;23), but not all (21;22) previous factor analyses of lung function parameters in COPD. The second factor extracted from the data included reversibility of FEV_1, airway hyperresponsiveness, total serum IgE, and diffusing capacity. Similarly, Ries and colleagues reported that bronchodilator response and diffusing capacity of COPD patients were grouped into separate factors from expiratory flow rates and hyperinflation (17). To our knowledge, there are no other studies in patients with COPD that have included these variables in a factor analysis. Finally, the third and fourth factor included eNO and sputum % neutrophils and eosinophils, respectively. This is a novel finding, illustrating the partial independency of these markers of inflammation from the traditionally used functional disease markers in COPD.

We included a large (n=114) group of patients with stable COPD, not using inhaled steroids for at least 6 months and without a clinical diagnosis of asthma. In terms of disease severity, patient characteristics included COPD patients of GOLD Stage II and III. Inclusion of very mild or very severe patients could have produced different results, and therefore our results potentially lack generalizability. In contrast to some other studies, we did not exclude patients with COPD who were partially reversible to a bronchodilator, because the selection of non-reversible patients only would have excluded a large group of patients with COPD (34-36). Furthermore, it needs to be emphasized that this is a cross-sectional analysis of patients with stable COPD, and that the results do not account for exacerbations and other temporal events. Sputum cell counts and eNO were measured as noninvasive markers of inflammation. Obviously, this does not cover all the inflammatory and structural changes of the airways in COPD, but it does represent the markers that are potentially useful for disease monitoring.

Factor analysis is not an approach that is widely applied, presumably because of its complexity. A simple example that clarifies its value and interpretation has recently been described by Juniper and colleagues (14). The purpose of this procedure is to reduce a large set of variables and to clarify (absence of) relationships between various parameters without reference to a specific criterion. Factor analysis does not regroup variables that are highly correlated, but factors are created based on calculated estimates of shared variance among variables, with the restriction that the factors reflect independent sources of variation. This procedure allows the user to determine whether associations between parameters are

attributable to noise of measurements. In clinical research, factor analysis allows the many parameters that characterize the disease to be reduced to a few, relatively independent factors. We applied standard procedures of exploratory factor analysis with respect to the number of variables used in the analysis, the selection of number of factors, and the factor rotation (32). Additional factor analyses with replacement of % neutrophils and % eosinophils by cell counts per ml sputum, exclusion of outliers from the data set, and addition of the cumulative amount of smoking resulted in similar factor structures as the original analysis. This demonstrates the robustness of the current findings.

The disease heterogeneity in COPD in terms of lung function and inflammation suggests that distinct pathophysiological pathways contribute to COPD. In agreement with this concept, we observed that multiple functional and inflammatory characteristics were categorized into four independent dimensions. Interestingly, none of the parameters of factors 1 or 2 showed significant additional loadings on factors 3 or 4, and vice versa, which strengthens the independence of functional and inflammatory dimensions. One exception to this was K_{CO}, which loaded together with eNO in the additional factor analysis using number of sputum cells instead of cell percentages. The value of using factor analysis in mapping disease heterogeneity is illustrated by our finding that some of the parameters were found to provide complementary information (loading on different factors) despite the existence of mutual correlation in univariate analyses.

The first factor can be interpreted as irreversible airflow limitation. The fact that diffusing capacity also had modest loading on this factor confirms earlier findings that the destruction of lung tissue is associated with increased airflow limitation and hyperinflation (37). Alternatively, diffusing capacity could also be a descriptor of the status of altered pulmonary circulation in COPD: another structural component of COPD. Hence, restructuring of airways as well as lung tissue seems to be an important mechanism resulting in airflow limitation. Interestingly, our data suggest that this process is greatly independent of neutrophilic and eosinophilic inflammation in the larger airways (grouped into factor 4). Neutrophils are able to induce tissue damage through the release of serine proteases and oxidants. However, this is not a prominent feature of other pulmonary diseases where chronic airway neutrophilia is even more prominent, such as cystic fibrosis and bronchiectasis (38). This suggests that other factors are involved in the generation of emphysema. In addition, increased neutrophil numbers are found in the airway lumen, but they are not a prominent feature of the airway wall or parenchyma in patients with COPD (7). Furthermore, the presence and role of eosinophils in COPD are uncertain (38). The observed increased levels of eosinophil cationic protein and eosinophil peroxidase in induced sputum from COPD

patients suggest the eosinophils are degranulated (39), which may be the result of the high neutrophil elastase levels in COPD (40). Our data supports this close relationship between neutrophils and eosinophils in COPD, but apparently, airflow limitation requires more than the presence of these granulocytes *per se*.

Parameters that are known to be associated with asthma predominantly grouped into the second factor. This may not be unexpected, since airway hyperresponsiveness, partial reversibility of airflow limitation, and increased serum IgE levels are not uncommon in COPD (41). An alternative interpretation would be that this second factor represents risk factors for progression of COPD, because bronchodilator response, airway hyperresponsiveness, and serum IgE levels have been associated with lung function decline (42). The finding that number of pack-years also loaded on factor 2 strengthens this concept, because smoking is known to be the main risk factor for progression of COPD (43). Although factor 2 also included K_{CO}, its parallel loading on factor 1 suggests that gas exchange impairment is associated with diverse pathophysiology.

The current separation of airway hyperresponsiveness and FEV_1 into different factors supports epidemiological evidence that these disease characteristics provide complementary information on COPD (44), the PC_{20} in COPD not simply being a result of airflow limitation *per se*. However, the fact that PC_{20} also had moderate loading on the first factor and even highest loading the first factor in some of the additional factor analyses on, is in agreement with previous studies that suggest that PC_{20} is to some extent dependent on airway caliber in COPD (45). Although it has been reported previously that partial reversibility of airflow limitation is associated with sputum eosinophilia and elevated eNO in COPD (34), our factor analysis suggests that these features are largely independent. This confirms a previous factor analysis in asthma (13) and again may challenge the concept that eosinophilic airways inflammation is closely related to the "twitchiness" of the airways. Interestingly, we found that number of pack-years also loaded on the second factor with IgE and β_2-response, and not with airflow limitation or sputum neutrophils. A significant relationship between total IgE and the degree of tobacco smoking has been reported previously (46), suggesting that the increase in IgE may be partly due to tobacco smoking. Although, in this study, univariate analysis revealed significant correlations between eNO and FEV_1, FEV_1/IVC, and PC_{20}, eNO was extracted into a factor independent from functional parameters as well as sputum cell counts. This indicates that this exhaled marker might be a rather autonomic phenomenon and bodes ill for the use of eNO in monitoring of disease activity.

In conclusion, this analysis has categorized multiple disease features of COPD without *a priori* assumptions on their interrelationships. Our data suggest that

airflow limitation, asthma-like components, eNO and sputum inflammatory cell counts offer separate and additive information about the pathophysiological condition of COPD patients. This confirms the complex heterogeneity of the disease and may change some of the current concepts on the distinct pathophysiological pathways involved. Accordingly, it needs to be examined whether the clinical evaluation of patients with COPD should include each of these complementary entities.

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Chapter 3

Small airways dysfunction and neutrophilic inflammation in bronchial biopsies and BAL in COPD

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Abstract

Background: The single-breath N_2 test (sbN_2-test) is closely related to small airways pathology in resected lung specimens of smokers. We investigated whether uneven ventilation and airway closure are associated with specific markers of airway inflammation as obtained by bronchial biopsies, bronchoalveolar lavage (BAL), and induced sputum in patients with manifest chronic obstructive pulmonary disease (COPD).

Methods: Fifty-one patients with stable COPD not receiving corticosteroids were examined in a cross-sectional study (43 men; mean [SD] age, 63 ± 8 years; ex-smokers and smokers; median pack-years, 41 [interquartile range, 31 to 51 pack-years]). Postbronchodilator spirometry (FEV_1, 63 ± 8% of predicted) and sbN_2-test (slope of phase III [ΔN_2], closing capacity [CC]/total lung capacity [TLC] % of predicted) were performed. Inflammatory cell counts were assessed in bronchial biopsies, BAL (only in the first half of patients), and induced sputum. Neutrophil elastase (NE), secretory leukocyte proteinase inhibitor (SLPI), and interleukin-8 levels were determined in BAL fluid.

Results: ΔN_2 increased with subepithelial neutrophil numbers in bronchial biopsies (r_s=0.337, p=0.017), and with NE levels (r_s=0.443, p=0.039), NE/neutrophil ratio (r_s=0.575, p=0.005), and SLPI levels (r_s=0.484, p=0.022) in BAL. CC/TLC was associated with BAL neutrophil numbers (r_s=0.448, p=0.048). The sbN_2-test was not associated with any other inflammatory cell type in BAL or biopsies, nor with inflammatory cell counts in sputum. Of importance, the correlations between ΔN_2 and BAL NE/neutrophil ratio, and between CC/TLC and BAL neutrophil numbers, remained significant when adjusting for FEV_1 % of predicted.

Conclusions: The results of the sbN_2-test are associated with neutrophilic inflammation in bronchial biopsies and BAL in patients with COPD. Our findings support a role of neutrophilic inflammation in the pathogenesis of small airways dysfunction in COPD.

Introduction

COPD is characterized by progressive airflow limitation that is not fully reversible (1). The pathological substrate of airflow limitation is predominantly located in the small airways and lung parenchyma (2). The bronchi and bronchioli exhibit accumulation of inflammatory cells, mucus and plasma exudates, which are accompanied with fibrosis and proliferation of smooth-muscle cells, resulting in airway wall thickening. Together with destruction of lung parenchyma, these multiple features are implicated in the development of irreversible narrowing of the airways (2;3).

The pathological changes in the periphery of the lung are reflected by functional impairment as measured by physiological tests (4). Amongst these so-called "small airways tests", the single-breath N_2 test (sbN_2-test) has been used to evaluate the presence of uneven distribution of ventilation (slope of nitrogen alveolar plateau; phase III) and airway closure (phase IV) (5). Phase III and phase IV reflect differences in time constants, which are dependent on both local resistances (small airways disease) and local compliances (emphysema) (6). The sbN_2-test is not specific for the periphery of the lung, but it has been validated against small airways pathology scores (7). Because of this landmark study, the sbN_2-test is still one of the few physiological measures that have been anchored to small airways pathology in smokers. Furthermore, the combination of spirometry and the sbN_2-test has been suggested to be predictive of the annual decline of FEV_1 in smokers (8). Therefore, the sbN_2-test might be a complementary noninvasive tool to monitor pathology in the periphery of the lungs in patients with COPD.

The airway inflammation in COPD is characterized by influx of neutrophils and macrophages in the airway lumen (9-11), as well as elevated macrophage(12;13) and T-lymphocyte and B-lymphocyte (3;12;14;15) numbers in the airway wall. In addition, both interleukin (IL)-8 and neutrophil elastase (NE) are elevated in BAL fluid of smokers who acquire COPD (16;17). IL-8 is a main mediator of neutrophil chemotaxis in the airways, and NE is a neutrophil-derived serine proteinase that is able to cause structural changes in the lung, impairment of mucociliary clearance and host defense, and induction of mucus secretion (18). Secretory leukocyte proteinase inhibitor (SLPI) is a locally produced inhibitor of NE, and it has been suggested that SLPI plays a role in maintaining the protease-antiprotease balance in the lung, regulation of leukocyte function, host defense against infection, tissue repair, and matrix production (19).

Previous studies investigating the sbN_2-test in smokers in relation to small airways disease have examined pathology scores, including scores of the degree of inflammatory cell infiltration in resected lung tissue (7;20-22) or autopsy (23;24)

material without further characterization of the inflammatory profile because of lack of specific stainings at that time. In addition, measuring inflammation with less invasive tools, such as induced sputum and bronchoscopy with BAL and biopsies, has not been examined in relation to the sbN_2-test in patients with COPD. Therefore, we postulated that small airways function in patients with COPD, as reflected by the sbN_2-test, is associated with the inflammatory profile characteristic for COPD. To that end, we investigated the number of inflammatory cells in bronchial biopsies, BAL fluid, and induced sputum, together with the levels of NE, IL-8, and SLPI in BAL fluid in a cross-sectional study in 51 patients with COPD.

Materials and methods

Subjects

Fifty-one patients with COPD, participating in the Groningen Leiden Universities and Corticosteroids in Obstructive Lung Disease (GLUCOLD) study were included in this study. The sbN_2-test was performed in the Leiden center only. Patient characteristics and methods have been described in detail previously (25;26). In short, all patients had irreversible airflow limitation (postbronchodilator FEV_1 and FEV_1/inspiratory vital capacity [IVC] < 90% confidence interval [CI] of predicted value [comparable with Global Initiative for Chronic Obstructive Lung Disease (GOLD) stages II and III], $FEV_1 \geq 1.3$ L and > 20% of predicted value) and respiratory symptoms. Patients with a history of asthma, α_1-antitrypsin deficiency, other active lung disease, or receiving maintenance treatment with inhaled or oral steroids during the last 6 months were excluded from the study. They were allowed to use short-acting bronchodilators and were in clinical stable condition (no symptoms of respiratory tract infection for at least 2 weeks prior to the study, no course of oral or inhaled steroids during the last 3 months). Patients were (ex-) smokers, with at least 10 pack-years of smoking. The medical ethics committees of each center approved the study and all patients gave written informed consent.

Design and sbN_2-test

The study had a cross-sectional design including four visits. The sbN_2-test was performed 15 min after administration of 400 μg salbutamol per metered-dose inhaler connected to a spacer, in order to minimize contribution of smooth-muscle contraction. The measurement was performed using a dry rolling-seal spirometer (Spiroflow; Morgan; Kent, UK) filled with 100 % oxygen and equipped with a N_2 meter (Morgan) connected to the mouthpiece allowing continuous sampling as described previously (5;27;28). During this test, seated patients performed a slow full inspira-

tory and expiratory slow vital capacity (VC) maneuver at inspiratory and expiratory flow rates of approximately 0.5 L/s, which was controlled by a flow restrictor. The expiratory N_2 concentration was plotted against volume changes between total lung capacity (TLC) and residual volume (RV), and the slope of the nitrogen alveolar plateau (slope of phase III [ΔN_2]) was calculated by drawing the best-fit line through phase III of the expiratory volume concentration curve by a blinded observer (29). The first departure from this straight line was considered as indicative of airway closure, and closing volume (CV) [phase IV] and closing capacity (CC) [RV+CV] were calculated (5). This procedure has previously been validated in our laboratory (27;29), and the within-observer and between observer reproducibility (determined by calculation of the intraclass correlation coefficient, Ri) of determining ΔN_2 (Ri = 0.940, Ri = 0.995, respectively) and CV (Ri = 0.929, Ri = 0.935, respectively) were high. Two measurements were performed, of which the one with the highest VC was selected for further analysis. The measurements were only accepted if the IVC and expiratory VC during the sbN_2-test did not differ > 15% or 0.5 L from each other. All volumes from the sbN_2-test were corrected for body temperature and pressure, saturated with water vapor (BTPS), and the parameters derived from the sbN_2-test (ΔN_2, CV, CC) were expressed as percentage of predicted value (5;30).

Lung function and sputum induction

Postbronchodilator spirometry, reversibility to 400 μg salbutamol, bodyplethysmography, diffusing capacity, and sputum induction (full-sample method) were performed according to previously described methods (25).

Bronchoscopy and biopsy analysis

Fiberoptic bronchoscopy was performed using a previously described standardized protocol (26) according to recent recommendations (31). Biopsy processing, staining, and analysis have also been described in detail previously (26). In short, 4-μm thick paraffin-embedded sections were stained using specific antibodies against T-lymphocytes (CD3, CD4, CD8), macrophages (CD68), neutrophil elastase (NE), mast cell tryptase (AA1), eosinophils (EG2), and plasma cells (CD138). Digital images per coded biopsy section were prepared, and fully automated inflammatory cell counting procedures were performed according to previously described validated methods (32). The number of subepithelial positively staining inflammatory cells was counted within the largest possible area, of maximal 125-μm deep beneath the basement membrane, per biopsy section, and expressed as the mean number of cells per 0.1 mm^2 of two tissue samples per patient.

Bronchoalveolar lavage

We also performed BAL before biopsy samples were obtained. Because of ethical considerations, the BAL procedure was stopped during the course of the study because four patients had a serious adverse event that was considered to be possibly related to the BAL (pleural pain, fever, pneumonia, short-term cardiac ischemia). BAL was performed and processed according to previously described recommendations (33;34). First, 50 mL of NaCl 0.9% at 30°C was instilled, which was retrieved after 10 s of dwelling with gentile suction at ≤ 20 cm H_2O pressure. This portion was not used for analysis. Thereafter, three times 50 mL of saline solution was instilled with dwelling times of 10 s. BAL processing and differential cell counts were performed analogous to the methods described for sputum processing (25), with the major exception that no dithiothreitol (DTT, Sputolysin) was used for homogenization. In addition, if required, lysis of red blood cells was performed before processing. A BAL sample was considered adequate when the amount of fluid instilled was at least 100 mL, and recovered BAL fluid was at least 10 mL (excluding the first portion). The levels of soluble NE, SLPI, and IL-8 were determined in BAL using enzyme-linked immunosorbent assay (IL-8; CLB; Amsterdam, The Netherlands; NE and SLPI enzyme-linked immunosorbent assays developed in our laboratory) (35;36). For IL-8 measurements, BAL supernatant was first concentrated using filters (Centricon-3; Millipore; Bedford, MA) (17).

Statistical analysis

Mean values and SD were computed. When appropriate, variables were logarithmically transformed before statistical analysis and presented as median with interquartile range (IQR). Univariate correlations between postbronchodilator sbN_2-test parameters (% predicted) (5;30) and inflammatory parameters were evaluated using Spearman rank correlation coefficient (r_s). Multivariate linear regression analysis was used to adjust for FEV_1 to evaluate whether contribution of inflammation to small airways function was independent of the degree of airflow limitation. Statistical significance was assumed at $p< 0.05$.

Results

Patient characteristics

Table 1 shows the characteristics of the 51 patients who performed the sbN_2-test after bronchodilation. The patients had moderate-to-severe COPD (GOLD stages II and III) based on a postbronchodilator FEV_1 of 63.3 ± 8.4% of predicted, and had a median smoking history of 41 pack-years (IQR, 31 to 51 pack-years). They demonstrated elevated values of ΔN_2 and early airway closure (Table 1). In two patients, we were unable to determine a CV. Induced sputum and BAL cell counts are presented in Table 2, and bronchial inflammatory cell numbers are shown in Table 3. BAL was performed only in the first half of patients (n=22).

Table 1. Patient Characteristics (n=51).

General characteristics	
Male/female gender, No.	43/8
Age, yr	62.7 (8.1)
Smoking history, pack-yr	41 (31-51)
Current/not current smoker, No.	30/21
Lung Function	
Postbronchodilator FEV_1, % predicted	63.3 (8.4)
Postbronchodilator FEV_1/IVC, %	48.5 (8.5)
ΔFEV_1, % predicted	7.4 (5.7)
K_{CO}, % predicted	73.5 (23.7)
RV/TLC, %	48.2 (9.2)
Postbronchodilator sbN_2-test	
$\Delta N2$, % N_2/L)	4.3 (2.9-6.6)
CV, L	1.1 (0.45)
CV/VC, %	25.6 (9.2)
CC/TLC, %	62.5 (9.7)
ΔN_2, % predicted	314 (220-447)
CV/VC, % predicted	114 (40.3)
CC/TLC, % predicted	135 (19.7)

Data represent mean (SD) or median (IQR: 25th - 75th percentile). FEV_1= Forced expiratory volume in one second, IVC= Inspiratory vital capacity, ΔFEV_1= Reversibility to salbutamol (change in FEV_1 as percentage of predicted), K_{CO}= Diffusing capacity for carbon monoxide per liter alveolar volume, RV= Residual volume, TLC= Total lung capacity, ΔN_2= slope of phase III, CV= Closing Volume, VC= Vital Capacity, CC= Closing Capacity. %Predicted values calculated according Buist et al.(5;30)

Table 2. Cell counts in induced sputum and BAL.

	Sputum	BAL
No.	50	22
Volume recovered, mL	–	64.1 (26.8)
Recovery, %	–	44.2 (16.5)
Epithelial cells, %	1.3 (0.3-3.1)	1.9 (0.8-7.9)
Neutrophils, %	68.9 (15.1)	3.5 (1.8-6.7)
Macrophages, %	23.7 (18.2-31.2)	89.5 (76.1-94.8)
Eosinophils, %	1.0 (0.2-1.8)	0.2 (0-0.6)
Lymphocytes, %	1.9 (1.3-2.6)	2.6 (1.0-5.3)
Total cell counts, x 10^4/mL	182 (101-333)	30.9 (13.7-66.4)

Data represent mean (SD) or median (IQR: 25th - 75th percentile). BAL= Bronchoalveolar Lavage.

Table 3. Bronchial inflammatory cell counts (n=50).

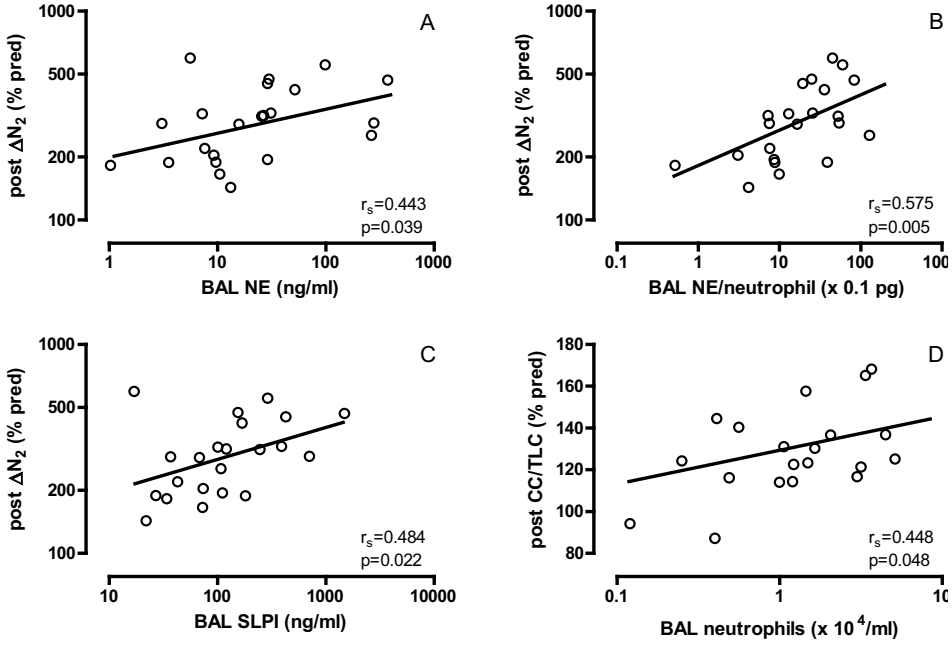
CD3	141 (59-204)
CD4	51 (29-79)
CD8	19 (8.0-32)
NE (neutrophils)	4.0 (1.9-8.5)
CD68 (macrophages)	8.5 (5.4-12)
EG2 (eosinophils)	2.0 (1.0-6.3)
AA1 (mast cells)	26 (21-34)
CD138 (plasma cells)	8.5 (3.5-12)

Data are presented as median cell number / 0.1 mm^2 (IQR: 25th - 75th percentile).

Relation between small airways function and airway inflammation

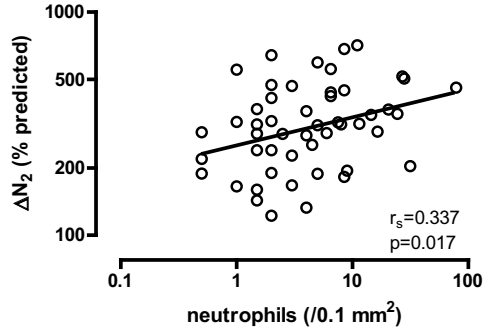
Small airway parameters as assessed with the sbN_2-test were predominantly associated with neutrophilic inflammation in BAL (Figure 1). Postbronchodilator ΔN_2 % of predicted increased with NE levels (median, 20.7 ng/mL; IQR 7.5 to 36.4 ng/mL], NE/neutrophil number ratio, and SLPI levels (median, 110 ng/mL; IQR 41.5 to 258 ng/mL) in BAL (r_s=0.443, p=0.039; r_s=0.575, p=0.005; r_s=0.484, p=0.022; respectively; Figure 1). However, ΔN_2 % of predicted was not related with IL-8 levels (median 64.5 pg/mL; IQR 28.4 to 192 pg/mL) or other inflammatory cell counts in BAL. Furthermore, postbronchodilator CC/TLC % of predicted was also associated with BAL neutrophil numbers (r_s=0.448, p=0.048; Figure 1). Postbronchodilator CV/VC % of predicted was not related with any of the BAL inflammatory markers. Postbronchodilator FEV_1 % of predicted was inversely associ-

Figure 1. Correlation between small airways dysfunction and inflammatory markers in BAL.



Correlations between postbronchodilator ΔN_2 % of predicted and NE levels in BAL (r_s=0.443, p=0.039) [*top left*], NE/neutrophil ratio in BAL (r_s=0.575, p=0.005) [*top right*], and SLPI in BAL (r_s=0.484, p=0.022) [*bottom left*]. Correlation between postbronchodilator CC/TLC % of predicted and neutrophil numbers in BAL (r_s=0.448, p=0.048) [*Bottom right*].

Figure 2. Correlation between small airways dysfunction and bronchial inflammation.



Correlation between postbronchodilator ΔN_2 % of predicted and neutrophil numbers in the lamina propria of bronchial biopsies, presented as the number of cells / 0.1 mm^2 tissue examined (r_s =0.337, p=0.017)

ated with ΔN_2 (R=-0.522, p<0.001). Interestingly, the correlations between ΔN_2 and BAL NE/neutrophil ratio, and between CC/TLC and BAL neutrophil numbers remained significant when adjusting for FEV_1 % of predicted (p=0.008, p=0.041; respectively).

Bronchial biopsies showed associations with sbN_2-test that were largely consistent with those found in BAL. Again, postbronchodilator ΔN_2 % of predicted was positively associated with subepithelial neutrophil numbers in bronchial biopsies (r_s =0.337, p=0.017; Figure 2), but not with other bronchial inflammatory cell counts. Significance of this latter correlation was lost when adjusting for FEV_1 % of predicted. The sbN_2-test was not significantly correlated with inflammatory cell counts in induced sputum.

Discussion

This study shows that ΔN_2 (nitrogen alveolar plateau) is associated with NE, NE/neutrophil ratio, and SLPI levels in BAL, as well as with neutrophil numbers in bronchial biopsies in COPD. In addition, closing capacity appeared to be associated with neutrophil numbers in BAL. These relations were independent of the degree of airflow limitation. Our results indicate that uneven ventilation and airway closure in COPD indirectly reflect neutrophilic inflammation in the wall of large airways as well as in the lumen of peripheral airways and alveoli as mirrored by BAL.

To our knowledge, this is the first study in patients with COPD examining the relation between the sbN_2-test and airway inflammation in bronchial biopsies, BAL, and induced sputum. Multiple studies have focused on the relation between indexes of the sbN_2-test and small airways pathology, including inflammation scores, in resected lung tissue (7;20-22) or autopsy material (23;24). However, the type of inflammatory cells was not specified in these pathology studies. It has been demonstrated that peripheral airways dysfunction in COPD, as determined by quantitative high-resolution CT, is associated with sputum neutrophilia (37). Our results extend these previous results by showing that neutrophilic inflammation in the airway wall of large airways, as well as in the lumen of peripheral airways and alveoli as reflected by BAL, correlates with uneven ventilation and airway closure as revealed by a relatively simple single-breath test in patients with COPD.

There are a few considerations when interpreting our results. First, the patients represented COPD patients of GOLD stages II and III (1). Because of ethical considerations, the BAL procedure was discontinued (*ie*, a few patients reported side

effects in relation to the BAL), and therefore BAL was performed only in the first half of patients. Since this was not anticipated, it is unlikely that this has introduced selection bias for the BAL-results. Second, we used % of predicted values and post-bronchodilator sbN_2-tests for exploration of relations with inflammatory markers in order to prevent possible variability by age, sex, and smooth-muscle tone. Finally, we chose to use the sbN_2-test to evaluate the presence of uneven distribution of ventilation (ΔN_2, phase III) and airway closure (phase IV). Phase III and phase IV reflect differences in time-constants, which are dependent on both local resistances (small airways disease) and local compliances (emphysema) (6). It is known that besides small airways, other factors contribute to the outcomes of the sbN_2-test test, such as topographical distribution of ventilation (38;39). In addition, there is evidence that multiple breath washout curves can distinguish uneven ventilation in the conducting small airways from the acinar lung zones (40). However, the latter technique still lacks external validation by relating it to small airways pathology. Therefore, we gave preference to the sbN_2-test based on its well-described relationship with small airways pathology in resected lung tissue (7).

How can the present results be interpreted? The association of the sbN_2-test with neutrophil numbers in BAL and bronchial biopsies, and with NE and NE/neutrophil ratio supports a role for airway neutrophil accumulation and activation in the pathogenesis of small airways and/or alveolar dysfunction in COPD. Neutrophils migrate into the lung in response to the presence or release of chemoattractants, such as IL-8. It is therefore tempting to speculate that accumulation of neutrophils in the airway wall of small airways also contributes to small airways dysfunction in COPD. However, we have recently demonstrated that the number of neutrophils in the lamina propria of the small airway is larger than in the lamina propria of the large airways in smokers (41). This suggests that the distribution of neutrophils along the tracheobronchial tree may not be uniform. This should be addressed in studies directly examining peripheral lung tissue.

The recruited neutrophils in the airways can release NE, which is able to cause tissue destruction, but also plays a role in mucus hypersecretion (18). Consequently, NE may contribute to loss of alveolar attachments and/or mucus hypersecretion, which both may lead to small airways narrowing and accordingly to inhomogeneous distribution of ventilation and early airway closure in patients with COPD (3;42). It has been shown previously in our laboratory that numbers of SLPI-containing epithelial cells increase with more severe small airways disease and destruction of alveolar attachments (43), which may possibly be part of the defense against inflammatory and destructive processes in small airways. This may explain the observed association between small airways function and

SLPI levels in BAL in the present study. Furthermore, the lack of an association between small airways function and inflammatory cells in induced sputum may not be unexpected because induced sputum is likely to represent a different compartment than BAL and bronchial biopsies in COPD(44), presumed to originate from large airways (45). Finally, lymphocytes, especially $CD8^+$ T-cells, have been implicated to play a role in the pathogenesis of COPD. In the present study, we did not observe a relation between these cells and the sbN_2-test. This may be due to non-uniform distribution of inflammatory cells along the tracheobronchial tree, even though $CD8^+$ cell numbers do not appear to be different between large and small airways in smokers (41).

What is the clinical relevance of our results? It appears from our results that the sbN_2-test is a non-invasive tool, complementary to spirometry, which is associated with neutrophilic airway inflammation in COPD. It has been observed that the sbN_2-test contributes to prediction of the decline in FEV_1 in patients with established airflow limitation (8). Therefore, we may speculate that neutrophilic inflammation is involved in progression of COPD by contributing to small airways and/or alveolar pathology. Consequently, monitoring the sbN_2-test might add to the management of patients with COPD.

In conclusion, uneven ventilation and airway closure in patients with stable COPD are associated with neutrophil numbers in bronchial biopsies and BAL, and with NE and its local inhibitor SLPI in BAL. These findings suggest a role for neutrophilic inflammation in small airways dysfunction in COPD.

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Chapter 4

Relation between duration of smoking cessation and bronchial inflammation in COPD

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Abstract

Background: Chronic obstructive pulmonary disease (COPD) is associated with airway inflammation. Although smoking cessation improves symptoms and decline in lung function in COPD, it is unknown whether bronchial inflammation in patients with established COPD varies with the duration of smoking cessation.

Methods: 114 patients (99 men) with COPD of mean (SD) age 62 (8) years, a median (IQR) smoking history of 42 (31-55) pack-years, no inhaled or oral corticosteroids, all current or ex-smokers (n=42, quit >1 month, median cessation duration 3.5 years), postbronchodilator FEV_1 63 (9)% predicted, and FEV_1/IVC 48 (9)% were studied cross sectionally. The number of subepithelial T-lymphocytes (CD3, CD4, CD8), neutrophils, macrophages, eosinophils, mast cells, and plasma cells were measured in bronchial biopsy specimens [median (IQR) /0.1 mm^2] using fully automated image analysis.

Results: Ex-smokers with COPD had higher $CD3^+$, $CD4^+$, and plasma cell numbers than current smokers with COPD [149 (88-225) v 108 (61-164), p=0.036; 58 (32-90) v 40 (25-66), p=0.023; 9.0 (5.5-20) v 7.5 (3.1-14), p=0.044, respectively], but no difference in other inflammatory cells. Short-term ex-smokers (<3.5 years) had higher $CD4^+$ and $CD8^+$ cell numbers than current smokers (p=0.017, p=0.023; respectively). Conversely, long-term ex-smokers (quit ≥3.5 years) had lower $CD8^+$ cell numbers than short-term ex-smokers (p=0.009), lower CD8/CD3 ratios than both current smokers and short-term ex-smokers (p=0.012, p=0.003; respectively), and higher plasma cell numbers than current smokers (p=0.003).

Conclusions: With longer duration of smoking cessation, CD8 cell numbers decrease and plasma cell numbers increase. This indicates that bronchial T-lymphocyte and plasma cell counts, but not other inflammatory cells, are related to duration of smoking cessation in patients with COPD.

Introduction

Chronic obstructive pulmonary disease (COPD) is characterised by progressive airflow limitation and abnormal inflammatory responses in the airways (1). Persistent smoking-induced inflammation is thought to play an important role in the pathogenesis of COPD. It is characterised by influx of neutrophils into the airway lumen and increased macrophage and T-lymphocyte numbers in the airway wall (2;3). In particular, bronchial $CD8^+$ T-lymphocytes appear to be associated with the severity of the disease (3;4). Moreover, sputum $CD8^+$ cells of patients with COPD have increased cytotoxic activity (5) which may contribute to the tissue damage which occurs in these patients. It has recently been shown that B cell numbers in the small airways of patients with COPD are also increased (3). Plasma cells (terminally differentiated effector B cells) are the cellular source of mucosal immunoglobulin production and consequently play a central role in host defence against infection. However, there are limited data on their role in COPD, and their presence in the lung has not been investigated in relation to smoking status.

Smoking cessation is the only intervention able to reduce the progression of COPD (6). Moreover, patients who quit smoking experience less respiratory symptoms and hyperresponsiveness as compared to those who continue smoking (7;8). The largest improvements in lung function and symptoms occur within the first year after cessation. It is not yet clear whether these beneficial effects are accompanied by reversal of smoking induced pathology.

At present there is insufficient evidence that smoking cessation reduces inflammation in COPD. Few cross sectional studies have compared smokers and ex-smokers with regard to bronchial inflammation in heterogeneous and relatively small groups of patients without an established diagnosis of COPD (9). Most previous studies have been performed in patients with chronic bronchitis (10;11). In patients with symptoms of chronic cough and expectoration, ex-smokers tend to have lower mast cell numbers in the lamina propria than current smokers (10), whereas the number of neutrophils, macrophages, eosinophils, and lymphocytes in bronchial biopsies have been reported to be similar (11). Most studies did not take duration of smoking cessation into account when comparing current and ex-smokers. However, it has been shown that this may influence the inflammatory response in small airways (12). The available studies may not be representative of COPD since relatively low numbers of patients with airflow limitation were included. It therefore remains to be investigated whether bronchial inflammation varies with current smoking status and the duration of smoking cessation in patients with an established diagnosis of COPD.

In the current study we postulated that bronchial inflammation in patients with

established COPD differs between active smokers and patients who stopped smoking, and that this difference is influenced by the duration of smoking cessation. We therefore investigated the number of inflammatory cells (neutrophils, macrophages, eosinophils, mast cells, T-lymphocytes, plasma cells, and granzyme B^+ cells as a marker of activated cytotoxic cells) in bronchial biopsy specimens from current and ex-smokers with COPD in a large cross sectional study.

Methods

Subjects

One hundred and fourteen patients with COPD participating in the Groningen Leiden Universities and Corticosteroids in Obstructive Lung Disease (GLUCOLD) study were included in the study. Patient characteristics and methods have been described in detail previously (13). In short, all patients had irreversible airflow limitation compatible with GOLD stages II and III (postbronchodilator FEV_1 and FEV_1/IVC <90% confidence interval [CI] of the predicted value, FEV_1 ≥1.3 l and >20% predicted) and at least one of the following symptoms: chronic cough, chronic sputum production, or dyspnea on exertion. Patients with a history of asthma, α_1-antitrypsin deficiency, or other active lung disease were not included in the study. Study subjects had not taken a course of oral steroids during the previous 3 months and had not received maintenance treatment with inhaled or oral steroids during the previous 6 months. Patients were allowed to use short acting bronchodilators and were in a stable clinical condition. They were current or ex-smokers (quit smoking for at least 1 month) with a smoking history of at least 10 pack-years. A validated questionnaire was used to assess the smoking history (14). Those who had quit were asked at what age they stopped in order to calculate the duration of smoking cessation (years). The medical ethics committees of the Leiden University Medical Center and the Groningen University Medical Center approved the study, and all patients gave their written informed consent.

Study design and lung function

The study had a cross sectional design and consisted of four visits. Spirometry, reversibility to salbutamol, and carbon monoxide transfer coefficient were measured according to previously described methods in order to characterise the patients (13).

Bronchoscopy

Fiberoptic bronchoscopy was performed using a standardised protocol according

to recent recommendations (15). Smokers were requested to refrain from smoking on the day of the bronchoscopy. Patients were premedicated with 400 μg salbutamol p.i., 20 mg codeine p.o., and 0.5 mg atropine s.c., and local anesthesia with lidocaine (≤3 mg/kg). During the procedure 100% oxygen was delivered through a nasal canula (2 L/min) if required while transcutaneous oxygen was monitored continuously by oximeter with a finger probe. Bronchoscopic examinations were performed by experienced pulmonary physicians using a fiberoptic bronchoscope (18X, outer diameter 6 mm, Pentax Optical Co., Japan) and pairs of cup forceps (Reda, Tuttlingen, Germany). Six macroscopically adequate bronchial biopsy specimens were randomly taken from (sub) segmental carinae in the right or left lower lobe (left and right lungs were alternated per patient, all biopsies from one lung).

Biopsy processing and staining

Four biopsy specimens were immediately fixed in 4% neutral buffered formalin for 24 hours, then processed and embedded in paraffin, and two were immediately snap frozen and stored at –80°C. Paraffin embedded biopsies were cut in 4 μm thick sections and haematoxylin/eosin staining was used for evaluation and selection of the two morphological best biopsies per patient for analysis (without crushing artifacts, large blood clots, or only epithelial scrapings). If required, immunohistochemistry included antigen retrieval. Specific antibodies against T-lymphocytes (CD3, CD4, CD8), macrophages (CD68), neutrophil elastase (NE), mast cell tryptase (AA1), eosinophils (EG2), plasma cells (CD138), and granzyme B were used (Table 1). Besides staining the plasma cells, CD138 (syndecan-1) antibody also stains the

Table 1. Antibodies used for immunohistochemistry.

Antibody	Pretreatment	Species	Dilution	Clone	Origin*
CD3 (lymphocyte)	Citrate	Rabbit	1:500	Polyclonal	DAKO
CD4 (T helper)	Citrate	Mouse	1:300	CD4-368	Novocastra
CD8 (T cytotoxic)	Citrate	Mouse	1:50	CD8-144B	DAKO
Neutrophil elastase (neutrophil)	Protease	Mouse	1:3200	NP57	DAKO
CD68 (macrophage)	Citrate	Mouse	1:3000	KP1	DAKO
Tryptase (mast cell)	Citrate	Mouse	1:16000	AA1	DAKO
EG2 (eosinophil)	Trypsin	Mouse	1:150	EG-2	Pharmacia
CD138 (plasma cell)	EDTA	Mouse	1:6400	B-B4	IQ products
Granzyme B	Citrate	Mouse	1:200	CLB-GB7	CLB/Sanquin

*Dako, Glostrup, Denmark; Novocastra, Newcastle upon Tyne, UK; Pharmacia Diagnostics, Uppsala, Sweden; IQ products, Groningen, The Netherlands; CLB/Sanquin, Amsterdam, The Netherlands

bronchial epithelium and submucosal glands (16), but these structures were not present in the areas of subepithelial cell quantification. All stainings except for CD3 and CD4 were performed using an automatic staining machine (Dako) in two sessions, with one section per patient in each session. In short, the sections were incubated with an optimal dilution of the primary antibodies in 1% BSA/PBS at room temperature for 60 min. As a secondary antibody, the horseradish peroxidase conjugated anti-mouse or anti-rabbit EnVision system (DAKO, Glostrup, Denmark) was used, with NovaRED (Vector, Burlingame, CA) as the chromagen. The sections were counterstained with Mayer's haematoxylin (Klinipath, Duiven, the Netherlands). For negative controls, the first antibody was omitted from this procedure.

Analysis of bronchial biopsies

Multiple digital images per coded biopsy section were prepared using a colour camera (Basler A101fc-le) and a dedicated software program (RVC-software, Amersfoort, The Netherlands). These images were united into one large image that consisted of the entire biopsy section (100 μm = 115.7 pixels). Fully automated inflammatory cell counting procedures were performed according to previously described validated methods (17). The number of subepithelial positively staining inflammatory cells was counted within the largest possible area of maximal 125 μm deep beneath the basement membrane, per biopsy section, and expressed as the mean number of cells/0.1 mm^2 of the two biopsies. Because of very low numbers of granzyme B^+ cells, these were analysed using a semi-quantitative score of the entire biopsy section: 0 (absent staining), 1 (1-10 positive cells), or 2 (> 10 positive cells).

Statistical analysis

Mean values and standard deviations (SD) were computed for normally distributed variables. Cell counts and other variables that did not show a normal distribution were logarithmically transformed (square root in case of CD8/CD3 ratio) before statistical analysis and presented as medians with interquartile range (IQR). Normally distributed log transformed cell counts were analysed using parametric tests. EG2 data remained skewed after log transformation and were therefore analysed using non-parametric tests. Differences between smokers and ex-smokers were explored using χ^2-square tests, two tailed unpaired *t* tests, or Mann-Whitney tests. To study the influence of duration of smoking cessation on cell counts, we compared smokers with ex-smokers who quit <3.5 years and those who quit ≥3.5 years ago, since this was the median duration of smoking cessation, using one way ANOVA (Kruskal-Wallis tests in case of EG2). If these were statistically significant, two tailed unpaired *t* tests were applied for further exploration of between group differences. Multivariate linear regression analysis was applied

to adjust for significant differences in patient characteristics between the groups such as sex, age, pack-years, and FEV_1/IVC. Univariate correlations were evaluated using Spearman and Pearson's correlation coefficient. SPSS 12.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis.

Results

Patient characteristics

Table 2 shows the characteristics of the 72 smoking and 42 ex-smoking patients included in the study. Patients had moderate to severe COPD, based on a post-bronchodilator FEV_1 of 63.0 (8.8)% predicted, and a median smoking history of 42 pack-years. Median (IQR) duration of smoking cessation in ex-smokers was 3.5 (1-10) years. The differences in patient characteristics between current and ex-smokers, and between current smokers, short-term ex-smokers (quit <3.5 years ago), and long-term ex-smokers (quit ≥3.5 years) are shown in Table 2.

Table 2. Characteristics of current smokers and ex-smokers groups with COPD.

	Current smokers	Ex-smokers (combined group)	Ex-smokers (quit <3.5 years)	Ex-smokers (quit ≥3.5 years)
General				
Sex (M/F, n)	59 / 13	40 / 2 *	20 / 1	20 / 1
Age (years)	60 (8)	64 ± 7 *	61 (8)	67 (4) *†
Pack-years (years)	43 (32-56)	37 (28-53)	45 (29-65)	35 (26-41) *
Duration of smoking cessation (years)	–	3.5 (1-10)	1.0 (1.0-2.0)	10 (6.5-14.5)
Smoking duration (years)	44 (8)	41 (10)	43 (11)	39 (8)
Chronic bronchitis (%)	55.6	31.0 *	23.8 *	38.1
Lung Function				
Post-bronchodilator FEV_1 (% pred)	63.3 (8.3)	62.5 (9.6)	62.6 (10)	62.5 (9.4)
Post-bronchodilator FEV_1/IVC (%)	49.5 (8.5)	46.0 (8.3) *	45.3 (8.6) *	46.7 (8.1)
ΔFEV_1 (% pred)	6.9 (5.2)	6.8 (4.5)	6.9 (3.9)	6.8 (5.1)
K_{CO} (% pred)	73.3 (25.1)	80.4 (25.9)	75.3 (24.9)	85.7 (26.5) *

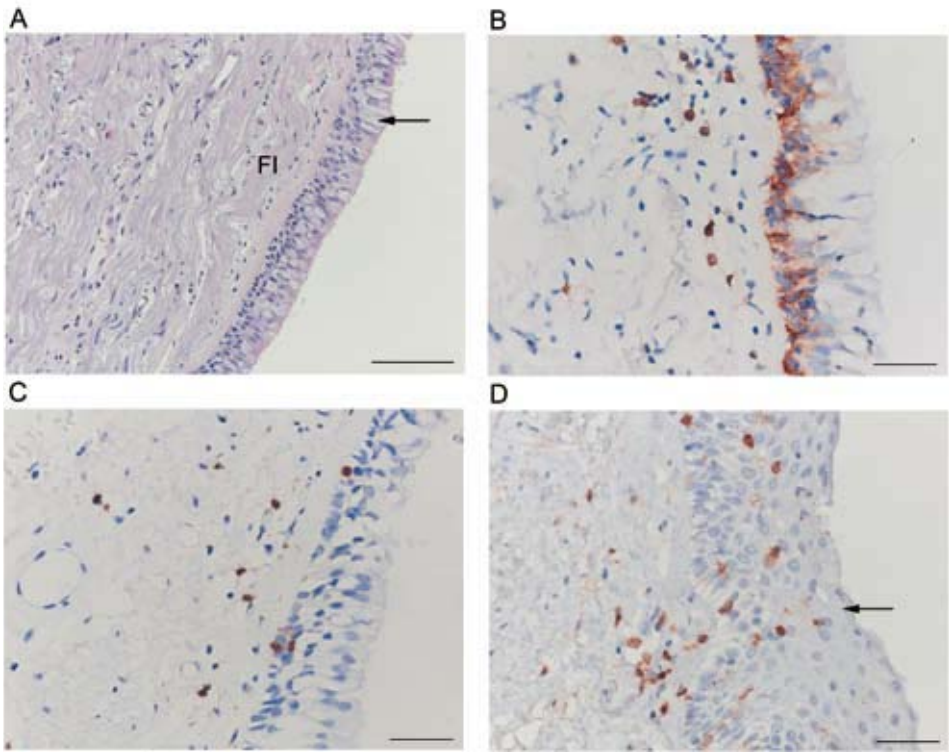
Data are presented as mean (SD) or median (IQR: 25-75th percentile). Ex-smokers are divided in two groups based on median duration of smoking cessation (3.5 years). FEV_1 = Forced expiratory volume in one second, IVC = Inspiratory vital capacity, ΔFEV_1 = Reversibility to salbutamol (change in FEV_1 as % of predicted), K_{CO}= Carbon monoxide transfer coefficient.

* $p<0.05$ compared with COPD current smokers (χ^2 tests for sex differences, two tailed unpaired *t* tests for other [log-transformed] data]. † $p<0.05$ compared with COPD ex-smokers who quit <3.5 years (two tailed unpaired *t* tests).

Bronchial inflammatory cell counts in smokers versus ex-smokers

All 114 patients underwent bronchoscopy; from one patient (ex-smoker) none of the biopsy specimens taken were adequate for analysis. Figure 1 shows examples of biopsy sections stained with haematoxylin/eosin, CD138, CD8, and CD4. The median analysed surface area of biopsy sections (mucosal area per patient, average of all antibodies, in which cells were counted, not corrected for shrinkage) was 0.35 (0.26-0.42) mm^2. Ex-smokers had higher $CD3^+$, $CD4^+$, and $CD138^+$ cell numbers than current smokers (p=0.036, p=0.023, p=0.044; respectively), but there was no significant difference in other inflammatory cell counts (Table 3). When differences in sex, age, and FEV_1/IVC were taken into account in multivariate linear regression analyses, the differences in CD3, CD4, and CD138 remained

Figure 1. Bronchial biopsy sections from patients with COPD.



(A) Bronchial biopsy section of a patient with COPD stained with haematoxylin/eosin showing goblet cell hyperplasia of the bronchial epithelium (*arrow*), fibrosis (*Fl*), and scattered inflammatory cells in the submucosa. (B) Plasma cell staining (CD138). The epithelial layer also stained positive but, for the analysis, only the subepithelial layer was taken into account. (C) $CD8^+$ T-lymphocyte staining. (D) $CD4^+$ T-lymphocyte staining also showing squamous cell metaplasia of the epithelium (*arrow*). Scale bars: (A) 100 μm, (B, C and D) 20 μm.

Table 3. Bronchial inflammatory cell counts of current smokers and ex-smokers with COPD.

Cell marker	Current smokers	Ex-smokers (combined group)	Ex-smokers (quit <3.5 yrs)	COPD Ex-smokers (quit ≥3.5 years)	p value ‡
CD3	108 (61-164)	149 (88-225) *	137 (93-229)	170 (62-221)	0.097
CD4	40 (25-66)	58 (32-90) *	64 (30-111) *	54 (32-75)	0.045
CD8	20 (11-37)	24 (8.8-41)	34 (18-54) *	16 (7.8-32) †	0.023
CD4/CD8	2.0 (1.1-3.7)	2.7 (1.3-5.0)	2.1 (1.2-3.3)	3.1 (1.8-6.4)	0.065
CD4/CD3	0.4 (0.3-0.6)	0.4 (0.3-0.8)	0.5 (0.3-0.8)	0.4 (0.3-0.6)	0.680
CD8/CD3	0.2 (0.1-0.3)	0.2 (0.1-0.3)	0.3 (0.2-0.4)	0.1 (0.1-0.2) *†	0.008
NE (neutrophils)	4.0 (2.0-7.8)	4.5 (2.0-9.0)	5.0 (2.6-9.0)	4.0 (1.8-9.8)	0.289
CD68 (macrophages)	8.5 (4.1-12)	11 (5.8-18)	11 (5.8-17)	8.5 (5.8-18)	0.124
EG2 (eosinophils)	1.3 (0.5-3.0)	2.0 (0.5-5.5)	1.5 (0.5-2.8)	3.5 (0.5-11)	0.183
AA1 (mast cells)	28 (20-34)	26 (18-35)	28 (22-36)	24 (14-33)	0.303
CD138 (plasma cells)	7.5 (3.1-14)	9.0 (5.5-20)*	7.8 (4.0-11)	12 (7.8-24) *	0.013

Data are presented as median cell number /0.1 mm^2 (IQR). Ex-smokers are divided in two groups based on median duration of smoking cessation (3.5 years).

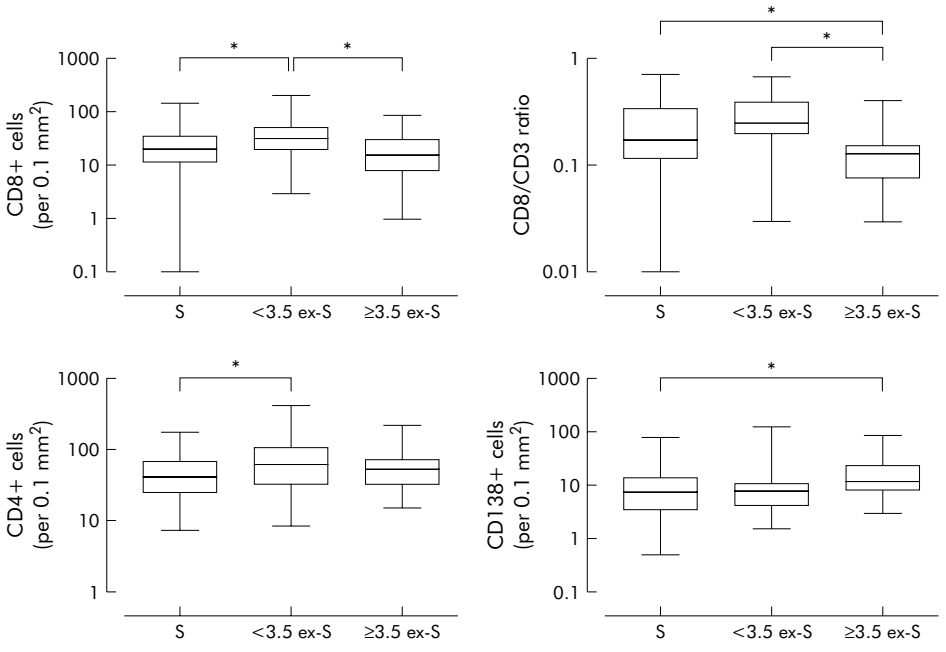
* $p<0.05$ compared with COPD current smokers (two tailed unpaired *t* tests of log transformed data, square root in case of CD8/CD3). † $p<0.05$ compared with COPD ex-smokers who quit < 3.5 years (two tailed unpaired *t* tests of log transformed data, square root in case of CD8/CD3). ‡ p value from one-way ANOVA (Kruskal-Wallis test in case of EG2) of log transformed data (square root in case of CD8/CD3) between current smokers and ex-smokers of <3.5 and ≥3.5 years with COPD.

significant. There were very few granzyme B$^+$ cells in most patients (median 0.5 [IQR: 0-1]), and the score was not different between current and ex-smokers with COPD.

Bronchial inflammatory cell counts and duration of smoking cessation

There were significant differences for CD4$^+$, CD8$^+$, CD8$^+$/CD3$^+$, and CD138$^+$ cells between current smokers, short-term ex-smokers, and long-term ex-smokers with COPD (Table 3). Short-term ex-smokers had higher CD4$^+$ and CD8$^+$ cell numbers than current smokers (p=0.017, p=0.023, respectively; Table 3, Figure 2). These differences persisted after adjustment for differences in postbronchodilator FEV_1/IVC between the groups. In contrast, long-term ex-smokers had lower CD8$^+$ cell numbers than short-term ex-smokers (p=0.009), lower CD8$^+$/CD3$^+$ ratios than both current smokers (p=0.012) and short-term ex-smokers (p=0.003), and

Figure 2. Bronchial inflammatory cell counts in smokers and ex-smokers with COPD.



Difference in CD4, CD8, CD8/CD3, and CD138 cell counts in the lamina propria of smokers (S), ex-smokers who quit <3.5 years ago (<3.5 ex-S), and who quit ≥3.5 years ago (≥3.5 ex-S) with COPD. Data are presented as box plots (median, IQR, range) of the number of cells /0.1 mm^2 tissue examined or ratios. * p<0.05 (two tailed unpaired *t* tests of log transformed data [square root in case of CD8/CD3]).

higher plasma cell numbers than current smokers (p=0.003; Table 3, Figure 2), and a trend towards significance versus short-term ex-smokers (p=0.069). When adjusting for differences in patient characteristics between the groups, all differences remained significant except for the difference in $CD8^+$ cells between short-term and long-term quitters. On the other hand, the difference in plasma cells between short-term and long-term quitters became significant when adjusting for age.

Correlations between bronchial inflammation and smoking behavior

When defining current smoking as 0 years stopped, longer duration of smoking cessation was associated with higher numbers of $CD3^+$ cells (r_s=0.221, p=0.019), $CD4^+$ cells (r_s=0.194, p=0.040), $CD138^+$ cells (r_s=0.217, p=0.021), and a trend with CD4/CD8 ratios (r_s=0.181, p=0.056). Excluding current smokers, longer duration of smoking cessation was associated with lower CD8/CD3 ratios (r_s=-0.395, p=0.011) and a trend with higher numbers of $CD138^+$ cells (r_s=0.307, p=0.051). The number of pack-years smoked was inversely correlated with $CD138^+$ cells (R=-0.295, p=0.002).

Discussion

This study aimed to determine whether the inflammatory cell profile in the bronchial mucosa is different between current smokers and ex-smokers with COPD, and whether this profile is influenced by duration of smoking cessation. Ex-smokers had higher numbers of $CD3^+$, $CD4^+$, and plasma cells, whereas numbers of neutrophils, macrophages, eosinophils, mast cells, and $CD8^+$ cells were not different from current smokers. Interestingly, short-term smoking cessation (below the median value of our cohort, that is <3.5 years) was associated with higher $CD4^+$ and $CD8^+$ T-lymphocytes whereas long-term smoking cessation (≥3.5 years) was associated with higher plasma cell numbers and lower CD8/CD3 ratios. These results indicate that the number of bronchial T-lymphocytes and plasma cells in patients with COPD is related to current smoking status and the duration of smoking cessation.

To our knowledge, this is the first study to compare bronchial inflammation in current and ex-smokers within a group of COPD patients and to examine the association with duration of smoking cessation. The observed higher numbers of $CD3^+$ and $CD4^+$ lymphocytes and plasma cells in ex-smokers with COPD compared to current smokers with COPD is novel. Consistent with our results, Mullen *et al* (18) also reported increased inflammation in peripheral airways of ex-smokers with mild COPD and mucus hypersecretion compared with current smokers,

although the type of cells was not specified. Our observation that large airway inflammation in COPD persists after smoking cessation is in line with previous results in smaller numbers of patients with chronic bronchitis (11). Rutgers *et al* (19) also reported ongoing airway inflammation in ex-smokers with COPD compared with healthy ex-smokers but, in contrast to the present study, they did not include current smokers with COPD in the analysis. Our results of an association between T-lymphocytes and plasma cells and duration of smoking cessation in COPD are not in line with a previous study by Wright *et al* (20) which found no difference in general peripheral airway inflammation between patients with COPD who had quit >2 years, <2 years, and current smokers. However, Lams *et al* (12) reported that CD8/CD3 ratios in peripheral airways are inversely associated with duration of smoking cessation in patients with and without airflow limitation. Taken together, it can now be inferred that, within a group of COPD patients, T-lymphocytes and plasma cell numbers are related to current smoking status and duration of smoking cessation while other inflammatory cells are not.

It seems unlikely that our results are affected by methodological errors. To our knowledge, this is the largest study to date to include bronchial biopsies of 114 well characterised patients with stable COPD of GOLD stages II and III, not using inhaled or oral steroids, and without a clinical diagnosis of asthma. It needs to be emphasised that this was a cross sectional study, and it cannot be ruled out that our ex-smoking group is a selected group of patients who quit smoking because they suffered more from smoking related symptoms and may already have had different cell numbers before quitting. Nevertheless, in the present study ex-smokers had significantly fewer respiratory symptoms than current smokers, while having similar pack-years and duration of smoking. In addition, in our analysis we did adjust for clinical differences between the groups. We did not confirm smoking status by laboratory tests and therefore cannot exclude the possibility that some ex-smokers were still smoking. However, this problem is comparable to those in other cross sectional studies in this area (10;11;19). A fully automated image analysis system was applied for cell counting in airway area sections (17). We are aware that counting cells in a two dimensional manner has limitations since it does not take into account the volume of the cell in a given sample; the smallest cells have the least chance of being counted in a single biopsy. Nevertheless, we were able to demonstrate differences in the smallest cells (LY) between the groups. There is still debate in the literature as to whether the theoretic basis of stereology fits well with the limitations of endobronchial biopsies (21). However, most of the present data in the literature is based on counting profiles/area which allows comparison between studies, although this is somewhat limited by other methodological factors. Because we observed no granzyme B^+ cells in most of

the biopsy sections analysed (56% of all sections) and no differences in a semi-quantitative score between the groups, we did not use digital image analysis for granzyme B^+ cell quantification which is a more time consuming procedure. We chose 3.5 years as a cut off time for short-term versus long-term smoking cessation groups because this was the median duration of smoking cessation, providing equal sample sizes in both groups.

How can we interpret these data? The ongoing inflammation in ex-smokers with COPD suggests the presence of a persistent stimulus that may act independently of cigarette smoking. There are several potential mechanisms - for example, chronic colonization of the airways with viral and/or bacterial pathogens in smokers with COPD (22) may be responsible for the inflammatory response (23;24) and persist after smoking cessation. In agreement with this, it has been observed that latent adenovirus could persist in ex-smokers with COPD which is associated with enhanced inflammation (25;26). Smoking may also trigger self-perpetuating inflammatory mechanisms by altering the balance between endogenous pro- inflammatory and anti-inflammatory mechanisms or, as recently suggested, it may induce autoimmune-like phenomena (27;28). In addition, it was recently observed that apoptosis of airway epithelial cells persists after smoking cessation in patients with COPD (29), which may induce persistence of inflammation. It has been suggested that, in smokers without COPD or chronic bronchitis who stopped smoking, lung inflammation is at least partially reversible (9) but persists in patients with COPD. It is therefore possible that the aforementioned stimuli may not persist in ex-smokers without COPD or chronic bronchitis.

The initial increase in $CD4^+$ and $CD8^+$ cells and later increase in plasma cells after smoking cessation might be explained by reversal of immunosuppression. Since both CD4 and CD8 cell numbers returned to similar levels in long-term quitters as in current smokers, this reversal of immunosuppression seems transient for lymphocytes but persistent for plasma cells. Smoking cessation may result in reversal of smoking induced harmful effects on airway epithelial cells (30) such as a reduction in metaplastic secretory cell numbers in small airways (20;31), leading to improved lung defence mechanisms. The effector mechanisms of immunity to environmental antigens could therefore be stimulated more efficiently in the absence of smoking, leading to a higher number of immunocompetent cells. Whereas the pro-inflammatory effects of active tobacco smoking have been extensively documented, it may also have selective anti-inflammatory effects as has been described for acute effects of smoking (32). Consistent with this and with our results, it was recently observed that cigarette smoke exposure reduced CD4 cell expansion following virus infection (33). In addition, tobacco smokers have decreased serum levels of immunoglobulins IgG and IgA (33;34), suggesting that cigarette

smoke modulates the humoral arm of adaptive immunity. Indeed, Soutar *et al* (35) described decreased numbers of IgA^+ cells in patients with fatal chronic bronchitis and suggested that these patients were deficient in plasma cells. These effects of decreased serum immunoglobulin levels appear to be reversible after smoking cessation (36). It is possible that smoking cessation leads to an improved capacity to produce immunoglobulins in the airway mucosa of patients with COPD by causing an increase in plasma cell numbers. Loss of these suppressive effects with smoking cessation may also explain our findings of increased numbers of inflammatory cells in ex-smokers, thereby ameliorating lung defence mechanisms.

The decreased CD8/CD3 ratio after quitting for ≥3.5 years compared with current smokers and short-term quitters, and the lower $CD8^+$ cell numbers in long-term compared with short-term quitters, suggest that smoking cessation may eventually result in decreased $CD8^+$ cell numbers. Since $CD8^+$ cells have been implicated in the pathogenesis of COPD, a decrease in the number of these cells or their cytotoxic activity may have beneficial effects. However, our findings of very low numbers of these cells that stain positive for the CD8 effector molecule granzyme B in the airways of patients with COPD, in the absence of differences between current and ex-smokers, suggest that these cells may not be cytotoxic. This may be because granzymes are involved in the pathogenesis of emphysema rather than in the conducting airways (37). In this respect, it is interesting to note that antigen-specific $CD8^+$ T-lymphocytes can persist in the lung long after clearance of a respiratory virus, and that these cells are highly activated and can be stimulated to proliferate, but do not express constitutive effector functions (38). In line with these findings, we did observe high numbers of granzyme B positive cells in lung tissue from children with childhood bronchiolitis obliterans, a disease that is thought to result from acute viral bronchiolitis (39). In vitro studies may provide additional information by examining more extensively the possible lack of cytotoxicity of $CD8^+$ T-lymphocytes in COPD.

What could be the clinical implications of our findings? There is good evidence that smoking cessation results in a decrease in respiratory symptoms (7), a lower decline in FEV_1 (6), and a reduction of airway hyperresponsiveness (40;41). Our data suggest that T-lymphocytes and plasma cell numbers change after smoking cessation in COPD while other inflammatory cells persist. Whether these relatively small changes in T-lymphocytes and plasma cell numbers contribute to the clinical benefits of smoking cessation in patients with COPD remains to be established in longitudinal studies. The results suggest that smoking cessation may result in an improvement in local humoral immunity which may result in less respiratory infections and exacerbations and thereby a reduced progression of COPD. However, the data also suggest that the clinical benefits of smoking cessation do not

simply result form a reduction in inflammatory cells. The mechanisms causing this sustained inflammatory pattern after smoking cessation in COPD remain to be clarified.

In conclusion, this study has shown that ex-smokers with COPD have higher numbers of bronchial $CD4^+$ and plasma cells than current smokers but the numbers of neutrophils, macrophages, and $CD8^+$ cells do not different between both groups. T-lymphocytes are higher in short-term quitters, whereas a longer duration of smoking cessation is associated with lower CD8/CD3 ratios and higher numbers of plasma cells. This suggests that changes in T-lymphocyte and plasma cell numbers may contribute to the clinical benefits of smoking cessation in COPD.

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Chapter 5

Smoking cessation and bronchial epithelial remodelling in COPD: a cross-sectional study

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Abstract

Background: Chronic Obstructive Pulmonary Disease (COPD) is associated with bronchial epithelial changes, including squamous cell metaplasia and goblet cell hyperplasia. These features are partially attributed to activation of the epidermal growth factor receptor (EGFR). Whereas smoking cessation reduces respiratory symptoms and lung function decline in COPD, inflammation persists. We determined epithelial proliferation and composition in bronchial biopsies from current and ex-smokers with COPD, and its relation to duration of smoking cessation.
Methods: 114 COPD patients were studied cross-sectionally: 99 males/15 females, age 62 ± 8 years, median 42 pack-years, no corticosteroids, current (n = 72) or ex-smokers (n = 42, median cessation duration 3.5 years), postbronchodilator FEV_1 63 ± 9% predicted. Squamous cell metaplasia (%), goblet cell (PAS/Alcian $Blue^+$) area (%), proliferating (Ki-67^+) cell numbers (/mm basement membrane), and EGFR expression (%) were measured in intact epithelium of bronchial biopsies.
Results: Ex-smokers with COPD had significantly less epithelial squamous cell metaplasia, proliferating cell numbers, and a trend towards reduced goblet cell area than current smokers with COPD (p=0.025, p=0.001, p=0.081, respectively), but no significant difference in EGFR expression. Epithelial features were not different between short-term quitters (<3.5 years) and current smokers. Long-term quitters (≥3.5 years) had less goblet cell area than both current smokers and short-term quitters (medians: 7.9% vs. 14.4%, p = 0.005; 7.9% vs. 13.5%, p = 0.008; respectively), and less proliferating cell numbers than current smokers (2.8% vs. 18.6%, p<0.001).
Conclusions: Ex-smokers with COPD had less bronchial epithelial remodelling than current smokers, which was only observed after long-term smoking cessation (>3.5 years).

Background

Chronic Obstructive Pulmonary Disease (COPD) is defined by progressive airflow limitation and airway inflammation (1), caused predominantly by cigarette smoking. Additionally, the airway epithelium undergoes alterations, including squamous cell metaplasia, goblet and basal cell hyperplasia (2). These findings are important for our understanding of the pathogenesis of COPD, since bronchial epithelial cells orchestrate an adequate maintenance of lung homeostasis by mucus production, ciliary beating, secretion of antimicrobial products and adequate immunological drive in response to noxious stimuli. Goblet cell hyperplasia is more pronounced in smokers with COPD compared to those without, suggesting a role in the development of airflow limitation (3). In addition, it contributes to mucus hypersecretion, which is associated with morbidity and mortality in COPD (4;5). Squamous cell metaplasia impairs mucociliary clearance and contributes to the increased risk of squamous cell carcinoma as observed in COPD (6).

The mechanisms underlying epithelial alterations in COPD are incompletely understood. The epidermal growth factor receptor (EGFR) cascade has been shown to be involved in mucin production and goblet cell hyperplasia (7;8), repair of damaged epithelium (7;8), as well as development of squamous cell carcinoma (9). A wide variety of stimuli can induce EGFR activation *in vitro* and in animals, including cigarette smoke (7;8). Additionally, epithelial EGFR expression is increased in bronchial biopsies from smokers with (10;11) and without (11;12) COPD compared to non-smokers. Previously, we have observed higher epithelial EGFR expression in ex-smokers with COPD compared to non-COPD, but not in current smokers, suggesting that current smoking may obscure differences in EGFR expression (13). Therefore, EGFR activation may play a role in epithelial phenotypic alterations observed in COPD through active smoking.

Smoking cessation improves respiratory symptoms and lung function decline in COPD, mostly within the first year after cessation (14;15), but interestingly bronchial airway inflammation persists or even worsens (16;17). To our knowledge, there are no studies comparing bronchial epithelial features between current and ex-smokers with established COPD. Possibly, smoking cessation contributes to restoration of epithelial characteristics in the large airways of COPD patients, which are directly and continuously exposed to the noxious substances present in cigarette smoke, thereby contributing to the clinical benefits observed after smoking cessation. Therefore, it needs to be addressed whether bronchial epithelial alterations and EGFR expression in large airways are reversible with smoking cessation and related to the duration of smoking cessation in COPD.

We hypothesised that bronchial epithelial cell proliferation and differentiation

in patients with COPD is more pronounced in active smokers than in those who stopped smoking, and that this difference is influenced by the duration of smoking cessation. Additionally, we questioned whether the epithelial changes are associated with EGFR expression. We therefore investigated the extent of epithelial goblet cell hyperplasia, proliferation, squamous cell metaplasia, and EGFR expression in bronchial biopsies of current and ex-smokers with established COPD in a large cross-sectional study.

Methods

Subjects

114 patients with COPD, who participated in a two-centre trial (Groningen Leiden Universities and Corticosteroids in Obstructive Lung Disease; GLUCOLD study), were included in this cross-sectional study. Patient characteristics and methods have been described in detail previously (17;18). In brief, all patients had irreversible airflow limitation (postbronchodilator FEV_1 and FEV_1/IVC < 90% *confidence interval* [CI] of predicted value) and chronic respiratory symptoms, they were all current or ex-smokers (quit ≥1 month), with at least 10 pack-years of smoking. Patients did not use a course of steroids during the last three months, and did not have maintenance treatment with inhaled or oral steroids during the last six months. They were allowed to use short-acting bronchodilators, and were in clinical stable condition. The medical ethics committees of each centre approved the study and all patients gave their written informed consent.

Lung function

Spirometry, reversibility to salbutamol, and diffusing capacity were measured according to previously described methods in order to characterise the patients (18).

Bronchoscopy

Fiberoptic bronchoscopy was performed using a standardised protocol according to recent recommendations (19) as described previously (17). Smokers were requested to refrain from smoking on the day of the bronchoscopy. Patients received premedication (400 μg salbutamol p.i., 20 mg codeine p.o., 0.5 mg atropine s.c.) and local anaesthesia (lidocaine). The bronchoscopies were performed by experienced pulmonary physicians using a fiberoptic bronchoscope (18X, outer diameter 6 mm, Pentax Optical Co., Japan) and pairs of cup forceps (Reda, Tuttlingen, Germany). Six macroscopically adequate bronchial biopsy specimens were taken from (sub) segmental carinae in the right or left lower lobe.

Biopsy processing and staining

Four paraffin-embedded biopsies were cut in 4 μm thick sections and haematoxylin/eosin staining was used for evaluation and selection of the two morphological best biopsies per patient for analysis (without crushing artefacts, large blood clots, or only epithelial scrapings). Sections were stained with Periodic acid-Schiff/Alcian blue (PAS/AB) and counterstained with Nuclear Fast Red to identify all secretory cells. Immunohistochemistry was performed as described previously for inflammatory cells (17), using specific antibodies against Ki-67 as a marker of proliferation (Dako M7240, dilution 1:100), and EGFR (Biogenex nr MU207-UC, dilution 1:50). Antigen retrieval was obtained using citrate for Ki-67 and pepsin for EGFR.

Analysis of bronchial biopsies

Total biopsy images were prepared with a 3-chip colour camera and analysed by means of image analysis software (Zeiss Vision KS400 system, Carl Zeiss, Göttingen, Germany) as follows. First, the length of the basement membrane was traced of all intact non-squamous metaplastic epithelium (A), intact squamous metaplastic epithelium (B), and damaged epithelium (C) in PAS/AB stained sections, in order to calculate the % intact epithelium ([A+B]/[A+B+C]) and the % metaplastic epithelium (B/[A+B]). In addition, the presence of metaplastic epithelium was also scored as absent (0) or present (1). Intact epithelium (A+B) was defined as a layer of both basal and columnar cells without detachment from the basement membrane, including areas of goblet cell hyperplasia or squamous metaplasia (20). Consequently, damaged epithelium (C) was defined as all remaining epithelium, including denuded basement membrane. Squamous cell metaplasia was defined as pseudostratisfied multilayered epithelium consisting of polygonal cells covered by flattened layer of squamous cells and absence of ciliated cells (21). Subsequently, the number of Ki-67 positively staining cells was counted in intact epithelium by a validated full automated procedure (22), and expressed as the number of Ki-67$^+$ cells/mm basement membrane. Densitometric analysis of PAS/AB and EGFR in intact epithelium (A+B) was also performed fully automated as follows (22). A linear combination of Red-, and Blue-filtered greyscale images was used, in order to derive a greyscale image (range 0-255) in which the "purple" staining (PAS/AB) and the "brown-red" staining (EGFR) highlighted above background. Results were expressed as the percentage of intact epithelium stained by PAS/AB and EGFR. In addition, EGFR staining intensity of positive areas was expressed as the average greyvalue, after normalization of the distribution towards the background peak (white: greyvalue 255) and subsequent inversion of the greyvalue distribution. Mean values of two biopsies analysed per patient were used for analysis.

Statistical analysis

Mean values and standard deviations (SD) were computed and presented, or median with interquartile range (IQR) in case of non-normal distributed variables. Since most epithelial markers were still non-normal distributed after log-transformation, these data were analysed using non-parametric tests. Differences between smokers and ex-smokers were explored using Chi-square tests or 2-tailed unpaired *t* tests for patient characteristics, and Mann Whitney tests for epithelial features. To study the association with duration of smoking cessation, we compared smokers with ex-smokers who quit <3.5 years and those who quit ≥3.5 years ago, since this was the median duration of smoking cessation, using Kruskal-Wallis tests. If these were significant, Mann Whitney tests were applied for further exploration of between-group differences. Univariate correlations were evaluated using Spearman rank correlation coefficient. To analyse correlations with years since smoking cessation, current smokers were included in the analysis as 0 years stopped. Multivariate linear regression analysis was applied to adjust for significant differences in patient characteristics between the groups, such as age, pack-years, and FEV_1/IVC. PAS/AB^+ area was measured in total intact epithelium, including squamous cell metaplasia, which by definition does not contain goblet cells. Therefore, linear regression analysis was also applied to adjust for % squamous cell metaplasia when analysing PAS/AB^+ differences between groups. SPSS 12.0 (SPSS Inc., Chicago, IL) software was used for statistical analysis.

Results

Patient characteristics

Patient characteristics of the 114 patients and subgroups of smokers and ex-smokers have been described in detail previously (17;18) (Table 1). Patients had moderate to severe COPD, based on a postbronchodilator FEV_1 of 63.0 (8.8)% predicted, and a median smoking history of 42 pack-years. Of the 114 COPD patients included in this study, 72 were current smokers and 42 were ex-smokers. Median duration of smoking cessation in ex-smokers was 3.5 years (IQR: 1-10 years). Differences in patient characteristics between the groups are presented in table 1.

Epithelial features in smokers versus ex-smokers with COPD

All 114 patients underwent bronchoscopy; from one patient (ex-smoker) none of the biopsies taken were adequate for analysis. The median analysed basement membrane length per biopsy was 5.03 (IQR: 3.64-6.14) mm, of which 1.12

Table 1. Patient characteristics.

	COPD smokers	COPD Ex-smokers		
		combined group	quit <3.5 yrs	quit ≥3.5 yrs
General				
Sex (M/F, n)	59 / 13	40 / 2 *	20 / 1	20 / 1
Age (yrs)	60 ± 8	64 ± 7 *	61 ± 8	67 ± 4 *†
Pack-years (yrs)	43 (32-56)	37 (28-53)	45 (29-65)	35 (26-41) *
Duration of smoking cessation (yrs)	–	3.5 (1-10)	1.0 (1.0-2.0)	10 (6.5-14.5)
Smoking duration (yrs)	44 ± 8	41 ± 10	43 ± 11	39 ± 8
Chronic bronchitis (%)	55.6	31.0 *	23.8 *	38.1
Lung Function				
Postbronchodilator FEV_1 (% pred)	63.3 ± 8.3	62.5 ± 9.6	62.6 ± 10	62.5 ± 9.4
Postbronchodilator FEV_1/IVC (%)	49.5 ± 8.5	46.0 ± 8.3 *	45.3 ± 8.6 *	46.7 ± 8.1
ΔFEV_1 (% pred)	6.9 ± 5.2	6.8 ± 4.5	6.9 ± 3.9	6.8 ± 5.1
K_{CO} (% pred)	73.3 ± 25.1	80.4 ± 25.9	75.3 ± 24.9	85.7 ± 26.5 *

Data are presented as mean ± standard deviation or median (IQR: 25th - 75th percentile), ex-smokers are divided in two groups based on median duration of smoking cessation (3.5 years). FEV_1 = Forced expiratory volume in one second, IVC = Inspiratory vital capacity, ΔFEV_1 = Reversibility to salbutamol (change in FEV_1 as percentage of predicted), K_{CO} = Diffusing capacity for carbon monoxide per liter alveolar volume, pred = predicted. Patient characteristics have been described before (17).
* p<0.05: compared to COPD smokers [Chi-square tests for sex differences, 2-tailed unpaired *t* tests for other (log-transformed) data].
† p<0.05: compared to COPD ex-smokers who quit <3.5 yrs (2-tailed unpaired *t* tests).

(0.59-2.11) mm was intact epithelium. Characteristics of intact epithelium in the total group of patients were: 9.74 (3.54-34.0) Ki-67$^+$ cells/mm BSM, 0 (0-19.7)% squamous cell metaplasia (37.3% of patients showed squamous cell metaplasia), 12.4 (4.69-18.9)% PAS/AB$^+$ area, and 10.4 (3.25-18.9)% EGFR$^+$ area.

Ex-smokers had significantly less Ki-67$^+$ cell numbers, and % of patients with squamous cell metaplasia as well as % of epithelium with squamous cell metaplasia (p = 0.001, p = 0.016, p = 0.025; respectively, Table 2) than current smokers. PAS/AB$^+$ area also tended to be lower in ex-smokers, but this was not statistically significant (p = 0.081, Table 2). When adjusting for the presence of squamous metaplasia (which by definition does not contain goblet cells), the difference in PAS/AB$^+$ area became significant (p = 0.014). When differences in sex, age, and FEV_1/IVC were taken into account in multivariate linear and logistic regression

Table 2. Bronchial epithelial features of smokers and ex-smokers with COPD.

	COPD Smokers	COPD Ex-smokers			p-value §
		combined group	quit <3.5 years	quit ≥3.5 years	
SCM (% of epithelium)	0 (0-27.5)	0 (0-0)*	0 (0-10.4)	0 (0-0)	0.076
SCM (% of patients)	45.7	22.5*	26.3	19.0*	0.049
PAS/AB$^+$ area (%)	14.4 (5.2-20.7)	8.1 (3.7-17.2)†	13.5 (6.6-19.6)	7.9 (2.2-16.2)*‡	0.011
Ki-67$^+$ cells (/mm BSM)	18.6 (5.3-38.8)	6.2 (1.5-15.6)*	6.9 (4.4-27.6)	2.8 (0.23-13.1)*	<0.001
EGFR$^+$ area (%)	11.4 (3.2-17.6)	8.2 (3.2-20.4)	6.7 (2.4-20.4)	8.6 (3.9-21.5)	0.95
Intensity EGFR$^+$ area (grey value)	697 (175-1182)	513 (203-1479)	372 (125-1479)	517 (215-1424)	0.95

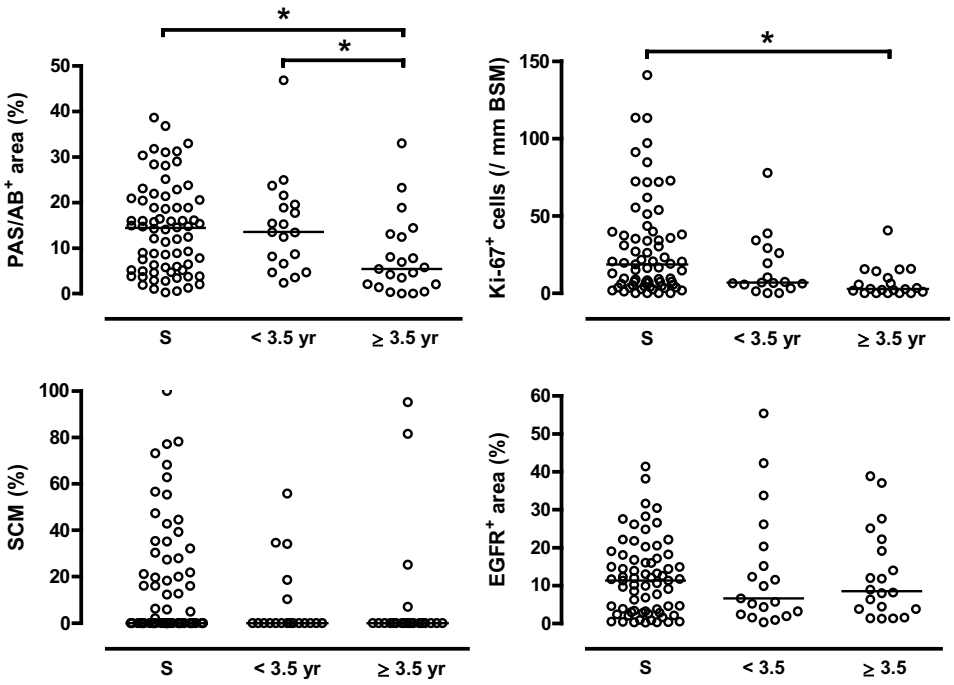
Data represent median (IQR: 25th - 75th percentile). PAS/AB = Periodic acid-Schiff/Alcian blue, SCM = squamous cell metaplasia, EGFR = Epidermal growth factor receptor.
* p<0.05: compared to COPD smokers.
† p<0.05: compared to COPD smokers adjusted for differences in squamous cell metaplasia (linear regression).
‡ p<0.05: compared to COPD ex-smokers who quit <3.5 yrs.
§ p-value from Kruskal-Wallis test between current smokers, < and ≥3.5 yrs ex-smokers with COPD.

analyses, all epithelial differences remained significant, except for PAS/AB$^+$ area. Epithelial EGFR$^+$ areas and intensities of positive areas showed no differences between smokers and ex-smokers with COPD (Table 2).

Duration of smoking cessation and epithelial features in COPD

Ki-67$^+$ cell numbers, the presence of squamous cell metaplasia, and the % PAS/AB$^+$ area, were different between current smokers, ex-smokers who quit <3.5 years ago, and ex-smokers who quit ≥3.5 years ago (Kruskal Wallis: $p = 0.011$, $p < 0.001$, $p = 0.049$; respectively, Table 2, Figure 1). Percentage squamous cell metaplasia and EGFR$^+$ areas and intensities were not significantly different between these three groups (Table 2, Figure 1). There were no differences between current smokers and those who quit <3.5 years ago for any of the epithelial features. In contrast, those who quit ≥3.5 years ago had significantly less Ki-67$^+$ cell numbers, presence of squamous cell metaplasia, and % PAS/AB$^+$ area than current smokers ($p < 0.001$, $p = 0.029$, $p = 0.005$, respectively; Table 2, Figure 1). The differences in PAS/AB and Ki-67 remained significant when adjusting for age and pack-year differences between both groups. Moreover, the % PAS/AB$^+$ area was also lower in long-term quitters than those who quit <3.5 years ago ($p = 0.008$), and tended

Figure 1. Epithelial phenotype in smokers (S) and ex-smokers with COPD.



Individual values of: (A) % PAS/AB$^+$ area of intact epithelium, (B) Ki-67$^+$ cells (/mm basement membrane = BSM) in intact epithelium, (C) % squamous cell metaplasia (SCM) of intact epithelium (note: in a large % of patients no SCM was observed), (D) % EGFR$^+$ area of intact epithelium. Data are grouped by COPD smokers (S), COPD ex-smokers who quit < 3.5 years ago (<3.5 yr), and who quit ≥ 3.5 years ago (≥3.5 yr). Horizontal bars represent median values, * $p < 0.05$.

to be lower for Ki-67$^+$ cell numbers ($p = 0.050$). When adjusting for differences in age, PAS/AB significance was lost ($p = 0.061$).

There was a significant inverse relationship between the duration of smoking cessation (including current smokers as 0 years stopped) and Ki-67$^+$ cell numbers ($r_s = -0.354$, $p < 0.001$), % squamous cell metaplasia ($r_s = -0.212$, $p = 0.004$), and % PAS/AB$^+$ area ($r_s = -0.235$; $p = 0.013$), but not with EGFR expression.

Relation of epithelial features with smoking, symptoms and lung function

The duration of smoking tended to be associated with the number of Ki-67$^+$ cells ($r_s = 0.180$, $p = 0.065$), whereas the number of pack-years smoked was not associated with epithelial features. 46.5% of all patients reported symptoms of chronic bronchitis, and although ex-smokers had significantly less symptoms of chronic

bronchitis (Table 1) and % of PAS/AB$^+$ area, there was no relation between the presence of these symptoms and the % of PAS/AB$^+$ area (p = 0.78). Epithelial features were not associated with the degree of airflow limitation.

Relation between epithelial cell proliferation and differentiation

Ki-67$^+$ cell numbers and the % squamous cell metaplasia were positively associated with one another (r_s = 0.586, p < 0.001). Finally, Ki-67$^+$ cell numbers were also positively associated with % EGFR$^+$ area (r_s = 0.210, p = 0.031).

Discussion

In the present study, we demonstrated that long-term ex-smokers with COPD had less bronchial epithelial mucin stores, proliferating cells, and squamous cell metaplasia than current smokers with COPD. Moreover, these epithelial differences in ex-smokers were only significant after a long-term period of smoking cessation (>3.5 years). In contrast, epithelial EGFR expression was not different between current and ex-smokers with COPD. These results may indicate that smoking cessation reverses smoking-induced bronchial epithelial cell proliferation and differentiation in patients with COPD, and the magnitude of this effect increases with longer duration of smoking cessation. In addition, our results suggest that these smoking cessation-induced epithelial changes in COPD are not accompanied by reduced EGFR expression.

Our observation of lower bronchial epithelial mucin stores, proliferating cells, and squamous cell metaplasia in large airways of ex-smokers as compared to current smokers with COPD, and the association with duration of cessation, is novel. These results are in contrast to other, smaller studies showing no differences in epithelial features in ex-smokers compared to smokers both with and without COPD (21;23). However, the finding that ex-smokers with chronic bronchitis (with or without airflow limitation), had less goblet cell metaplasia in small airways than current smokers (24) is in line with our findings in COPD patients. The effect of smoking cessation and duration of cessation on squamous cell metaplasia and proliferation has been examined previously in bronchial biopsies (25). Although it was not mentioned whether these patients had respiratory symptoms and/or COPD, the latter study also reported less squamous cell metaplasia and epithelial proliferation in ex-smokers. Our result of similar EGFR expression in ex-smokers compared to current smokers with COPD, is also novel and in contrast with observations in smokers without COPD, where lower bronchial EGFR expression was observed in ex-smokers (12). Taken together, it can now be inferred that

smoking cessation results in decreased epithelial mucin stores, proliferation, and squamous cell metaplasia, in large airways of patients with COPD, but that it does not affect EGFR expression.

There are a few important considerations when interpreting our results. We included a large (n = 114) group of well-characterised patients with stable COPD, not inhaling steroids for at least six months or oral steroids for at least three months, and without a clinical diagnosis of asthma. First, it needs to be emphasised that this was a cross-sectional study, and it cannot be ruled out that our ex-smoking group is a selected group of patients who quit smoking because they suffered more from smoking related symptoms, and may already have had a different epithelial morphology before quitting. Yet, in the present study ex-smokers had significantly less respiratory symptoms than current smokers, whilst having similar pack-years and duration of smoking. In addition, we also reported analyses adjusted for clinical differences between the groups (sex, age, FEV_1/IVC, pack-years). Second, comparable to previously published cross-sectional studies we did not confirm smoking status by laboratory tests, and therefore cannot rule out that some ex-smokers were still smoking. Third, our definitions of intact epithelium and squamous cell metaplasia were very strict, which could have led to an underestimation. Fourth, we cannot exclude the possibility that mechanical injury induced during bronchoscopy may have interfered with our analyses of epithelial damage. Fifth, we applied fully automated image analysis for quantification of cell numbers, positively stained areas, and densitometry analyses, and therefore minimised potential observer biases. Finally, we did not include a control group of smokers without COPD and therefore cannot conclude whether the observed effects of smoking cessation are specific to COPD. Taken together, it seems unlikely that our results are strongly affected by methodological errors.

How can we interpret these data? The (partial) reversibility of mucin stores, squamous cell metaplasia, and proliferation after smoking cessation, supports a causal relationship between cigarette smoke exposure and these epithelial features in COPD. *In vivo,* the proliferative response in smokers may be due to a direct mitogenic effect of cigarette smoke (26), but may also result from chronic inflammation, tissue damage and wound healing (26). An inadequate repair response to smoke-induced injury may lead to a sustained increase in epithelial proliferation and/or altered differentiation. Increased proliferation may accompany squamous cell metaplasia (21;25), which is in line with our observation that squamous cell metaplasia is related to proliferating cell numbers. This squamous metaplasia may serve to protect the underlying tissue against the injurious effects of cigarette smoke. According to our results, smoking cessation may reverse this process in COPD, at least partially.

The observed decrease in mucin stores in long-term ex-smokers with COPD, and its relation with duration of smoking cessation, can be explained by decreased goblet cell numbers, decreased mucin production, and/or increased mucin secretion. The few previous studies examining goblet cell hyperplasia in relation to smoking cessation in humans used semi-quantitative scoring systems, but not cell numbers (23;24). Animal studies suggest that a reduction in secretory cell numbers occurs after smoking cessation (27). The observed decrease in mucin stores in ex-smokers in our study probably reflects a decrease in the major mucin in surface epithelium, MUC5AC (28). MUC5AC and total mucin in bronchial epithelium are correlated, and both are increased in smokers compared to non-smokers (11). In addition, small airway epithelial MUC5AC expression is lower in ex-smokers compared to current smokers with and without COPD (29), which is also in line with our conclusion. Although ex-smokers exhibited fewer symptoms of chronic bronchitis and a smaller amount of mucin stores, chronic bronchitis was not associated with mucin stores. This can be explained by the fact that mucin is produced by both goblet cells in the surface epithelium and by submucosal glands (30), whereas these latter were not included in the present analysis. This conclusion is supported by the results of a recent study that also failed to reveal differences in epithelial mucin expression in peripheral airways of COPD patients with or without chronic bronchitis (29). Finally, it needs to be noted that our study focused on mucin expression and not on secretion, whereas the latter is the main feature of chronic bronchitis.

The changes in epithelial mucin stores, proliferating cells, and squamous cell metaplasia were most pronounced in COPD patients who had stopped smoking more than 3.5 years ago. Correspondingly, inflammation initially persists after smoking cessation (17;31). This suggests a long-term effect of smoking on bronchial regulatory networks, which is not restored immediately after removing the initial stimulus, i.e. cigarette smoke. In contrast, the greatest improvements in respiratory symptoms and lung function decline occur within the first year after cessation (14;15). Therefore, other pathological mechanisms that reverse more rapidly after cessation should be involved in these clinical beneficial effects of smoking cessation.

Cigarette smoke causes both mucus hypersecretion and increases the number of goblet cells through activation of the EGFR system (32). In the present study, there were no differences between current and ex-smokers with COPD in EGFR expression, suggesting that differences in EGFR activation, rather than expression, are present in ex- versus current smokers with COPD. We did not pursue this possibility further, since recent attempts in our laboratory to demonstrate *in situ* EGFR phosphorylation in lung tissue by immunohistochemical methods using

phosphospecific antibodies were not successful (13). Since pro-inflammatory cytokines, such as TNF-α, increase EGFR expression (33), our observation that there is no difference in EGFR expression between ex- and current smokers may be related to the persistence of bronchial inflammation (16;17) in ex-smokers with COPD. The large inter-individual differences in EGFR expression could also explain why there were no differences between the groups in this cross-sectional analysis. Alternatively, EGFR independent pathways may contribute to epithelial mucin production.

What could be the clinical implications of our findings? There is good evidence that smoking cessation results in less respiratory symptoms (14), less decline in FEV_1 (15), and less severe airway hyperresponsiveness (34;35), whereas inflammation persists (16;17). Our data suggest that smoking-induced bronchial epithelial goblet cell hyperplasia, proliferation, and squamous cell metaplasia are reduced with long-term smoking cessation. These epithelial features might contribute to COPD by facilitating colonization of the airways by respiratory pathogens, secondary to loss of cilia, increased mucus secretion, and epithelial injury (36). The chronic colonization of the airways may enhance airway inflammation and further epithelial injury. In addition, mucus hypersecretion may cause airways obstruction in peripheral airways (37). Reversal of epithelial remodelling may therefore contribute to reduced progression of COPD attributable to restored mucociliary clearance, resulting in reduced respiratory colonization (38) and exacerbations, and less small airways obstruction. In addition, reduced epithelial proliferation and squamous cell metaplasia in ex-smokers with COPD may decrease the risk of squamous cell carcinoma development. Whether the observed (partial) reversibility of epithelial remodelling is associated with the clinical benefits of smoking cessation in patients with COPD, remains to be established in longitudinal studies.

Conclusions

The present study has shown that ex-smokers with COPD have less bronchial epithelial mucin stores, proliferating cells, and squamous cell metaplasia than current smokers with COPD, whereas epithelial EGFR expression was not different between both groups. These epithelial changes in ex-smokers were more pronounced with longer duration of smoking cessation, and significant after 3.5 years smoking cessation. This suggests that the clinical benefits of smoking cessation in COPD patients may be in part attributable to a restoration of epithelial homeostasis.

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Chapter 6

Effect of fluticasone with and without salmeterol on pulmonary outcomes in chronic obstructive pulmonary disease

A randomized trial

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Abstract

Background: Inhaled corticosteroids (ICS) and long-acting beta$_2$-agonists (LABAs) are used to treat moderate to severe chronic obstructive pulmonary disease (COPD).

Objective: To determine whether long-term ICS therapy, with or without LABAs, reduces inflammation and improves pulmonary function in COPD.

Design: Randomized, placebo-controlled trial. (ClinicalTrials.gov registration number: NCT00158847)

Setting: 2 university medical centers in The Netherlands.

Patients: 114 steroid-naïve current or former smokers with moderate to severe COPD.

Measurements: Cell counts in bronchial biopsies and sputum (primary outcome); methacholine responsiveness at baseline, 6 and 30 months; and clinical outcomes every 3 months.

Intervention: Random assignment by minimization method to receive fluticasone propionate, 500 µg twice daily, for 6 months (n=31) or 30 months (n=26); fluticasone , 500 µg twice daily, and salmeterol, 50 µg twice daily, for 30 months (single inhaler; n=28); or placebo twice daily (n=29).

Results: 101 Patients were greater than 70% adherent to therapy. Fluticasone therapy decreased counts of mucosal $CD3^+$ cells (-55% [95% CI, -74% to -22%]; p = 0.004), $CD4^+$ cells (-78% [CI, -88 to -60%]; p < 0.001), $CD8^+$ cells (-57% [CI, -77% to -18%]; p = 0.010), and mast cells (-38% [CI, -60% to -2%]; p = 0.039] and reduced hyperresponsiveness (p = 0.036) versus placebo at 6 months, with effects maintained after 30 months. Fluticasone therapy for 30 months reduced mast cell count and increased eosinophil count and percentage of intact epithelium, with accompanying reductions in sputum neutrophil, macrophage, and lymphocyte counts and improvements in FEV_1 decline, dyspnea, and quality of life. Reductions in inflammatory cells correlated with clinical improvements. Discontinuing fluticasone therapy at 6 months increased counts of $CD3^+$ cells (120% [CI, 24% to 289%]; p = 0.007), mast cells (218% [CI, 99% to 407%]; p < 0.001), and plasma cells (118% [CI, 9% to 336%)]; p = 0.028) and worsened clinical outcome. Adding salmeterol improved FEV_1 level.

Limitations: The study was not designed to evaluate clinical outcomes. Measurement of primary outcome was not available for 24% of patients at 30 months.

Conclusions: ICS therapy decreases inflammation and can attenuate decline in lung function in steroid-naïve patients with moderate to severe COPD. Adding LABAs does not enhance these effects.

Introduction

Chronic obstructive pulmonary disease (COPD) is characterized by a progressive decrease in lung function, accompanied by worsening respiratory symptoms and health status (1). These clinical features are associated with airway inflammation (such as that resulting from neutrophils, macrophages, lymphocytes, and mast cells) (2-5) and alterations of the bronchial epithelium (such as that resulting from squamous cell metaplasia or goblet and basal cell hyperplasia) (6).

Current guidelines (1) recommend treating patients who have severe COPD and frequent exacerbations with inhaled corticosteroids (ICSs) and adding long-acting β_2-agonists (LABAs) for patients with moderate to severe COPD. Regular ICS treatment leads to clinical benefits in terms of symptoms, exacerbation rates, and initial improvements in FEV_1 (7-10). However, withdrawal of ICS treatment results in deterioration of clinical outcome (11;12). Combining a LABA with an ICS provides additional clinical improvements (13;14). A recent analysis of the TORCH (Towards a Revolution in COPD Health) study suggests that prolonged therapy with ICS and LABA attenuates FEV_1 decline in COPD (15), in contrast to previous studies (13;14;16-19).

The clinical benefits of ICS therapy for COPD, with or without a LABA, may be at least partially mediated its anti-inflammatory efficacy. Short-term treatment of COPD (2-3 months) with ICS reduced the number of bronchial mast cells but not $CD8^+$ cells, neutrophils, or macrophages (20;21). Combination therapy with ICS and LABAs for 3 months provided more anti-inflammatory effects than ICS monotherapy by reducing bronchial $CD8^+$ cells and macrophages (22). No long-term anti-inflammatory effects have been reported for these interventions. Our goal was to link pathological and clinical efficacy during 30-month treatment.

We hypothesized that:

1. long-term maintenance therapy with ICS provides anti-inflammatory effects (primary outcome) in the airways of patients with COPD;
2. such effects are associated with clinical improvements;
3. discontinuing ICS therapy induces a flare-up of inflammation and clinical deterioration;
4. adding a LABA to ICS therapy provides no further anti-inflammatory effects.

Methods

Our study is investigator-initiated, with a double-blind, parallel, 4-group, placebo-controlled, randomized design.

Setting and participants

The GLUCOLD (Groningen Leiden Universities Chronic Obstructive Lung Disease) project (23) enrolled patients with COPD who were aged 45 to 75 years, were current or former smokers, had smoked for 10 or more pack-years, and had lung function levels compatible with Global Initiative for Chronic Obstructive Lung Disease (GOLD) stages II and III (1). Exclusion criteria were asthma and receipt of ICS within 6 months before random assignment. We determined the presence of asthma on the basis of a physician 's diagnosis or selfreported history, symptoms, treatment, or diagnosis of asthma. Patients were clinically stable and were allowed to continue taking short-acting bronchodilators. We determinec smoking status on the basis of self-reports and gave standard clinical advice to quit smoking in accordance with Dutch national guidelines. We recruited almost all patients from family practices by electronically selecting patients aged 45 to 75 years who did not have an International Classification of Primary Care code for asthma (R96). Their general practitioner sent them a letter asking for participation in research. A telephone interview revealed 4617 potentially eligible patients, who received spirometry. In addition, were recruited patients by advertising in local newspapers. We performed chest radiography and electrocardiography to rule out important comorbid conditions. Recruitment and follow-up was between 2000 and 2007. Both centers' ethics committees approved the study, and all patients provided written informed consent.

Random assignment and interventions

We randomly assigned patients to receive 1 of 4 regimens: fluticasone propionate, 500 µg twice daily, for the first 6 months followed by placebo, twice daily, for 24 months; fluticasone, 500 µg twice daily, for 30 months; fluticasone, 500 µg twice daily, and salmeterol, 50 µg twice daily, in a single inhaler for 30 months; or placebo, twice daily, for 30 months. Study medications were individually numbered, and we used Diskus dry-powder inhalers (GlaxoSmithKline, Zeist, The Netherlands) with 60 doses per inhaler; all active treatment medication and placebo were identical in appearance. The placebo consisted of lactose monohydrate (also included in other treatment groups). At entry, an independent randomization center provided patient and medication numbers by using a minimization procedure that balanced treatment groups for center, sex, smoking status, FEV_1/IVC (<60%

or ≥ 60%), and methacholine PC_{20} (the provocative concentration of methacholine that causes a 20% decrease in FEV_1) (<2 mg/mL or ≥ 2 mg/mL).

Outcomes and Measurements

Our predefined primary outcome was inflammatory cell counts in bronchial biopsies and induced sputum. We performed fiberoptic bronchoscopy, biopsy processing, and quantification as described elsewhere (24). We stained paraffin-embedded biopsy sections with Periodic acid-Schiff/Alcian blue to identify goblet cells, epithelial intactness, and squamous metaplasia as described elsewhere (25). We performed immunohistochemistry by using specific antibodies against T lymphocytes (CD3, CD4, and CD8), macrophages (CD68), neutrophil elastase, mast cell tryptase (AA1), eosinophils (EG2), plasma cells (CD138), and proliferating cells (Ki-67). We expressed subepithelial cells as number of cells per 10^{-7} m^2 by fully automated image analysis (26). We used the full sample method (23) to perform sputum induction.

Secondary outcomes included postbronchodilator spirometry and hyperresponsiveness to methacholine PC_{20}, assessed by using standardized procedures (23), dyspnea score by the modified Medical Research Council (MRC) dyspnea scale (range, 1 to 5); and health status by the St. George's Respiratory Questionnaire (SGRQ) (range, 0 to 100; 100 = maximum disability) (27) and Clinical COPD Questionnaire (CCQ) (range, 0 to 6; 6 = worst) (28).

Follow-up Procedures

We measured symptoms, health status, self-reported smoking status, medication adherence, and spirometry every 3 months. We checked adherence by counting the doses on the inhalers. We performed bronchoscopy, sputum induction, and methacholine challenge at baseline and at 6 and 30 months.

Statistical analysis

We based our sample size on the latest data released in 2002 (29) regarding the standard deviation (0.77) of the fluticasone-induced short-term change in submucosal CD8 cell count in patients with COPD. A 2-fold difference in change from baseline to 6 months and from 6 to 30 months in the fluticasone group versus placebo should be detectable with 80% power with 20 patients per treatment group. Because this was an efficacy trial, per-protocol analysis included all available data from randomly assigned patients who adhered to their therapy regimen (using ≥70% of the prescribed dose), including data from patients who did not complete follow-up.

We used linear mixed-effects models with a random intercept at the patient level to analyze the data and assumed that data were missing at random. We used STATA, version 9.0 (StataCorp, College Station, Texas) for analysis. The linear mixed models included the main effect of treatment (3 indicators), the main effect of time (2 indicators), and the interaction of treatment and time. For outcomes with 3-month measurements, we replaced the time effect with terms that allowed a shift or linear change in the average outcome during the first 6 months and a subsequent linear change in the average outcome after 6 months. Because of the considerable number of model parameters and the sample size, we did not include center, age, or sex as covariates in the baseline model. We performed a post hoc analysis to adjust for smoking status at baseline and during the study. We present the effects as adjusted means in the figures and as percentage of change in estimates, CIs, and *P* values in the text.

We analyzed correlations between statistically significant treatment effects on inflammatory outcomes and lung function by using the Spearman correlation coefficient (Rs). Data are presented as means (SDs) or medians (interquartile ranges). We considered 2-sided *P* values less than 0.05 to be statistically significant.

Role of the funding source

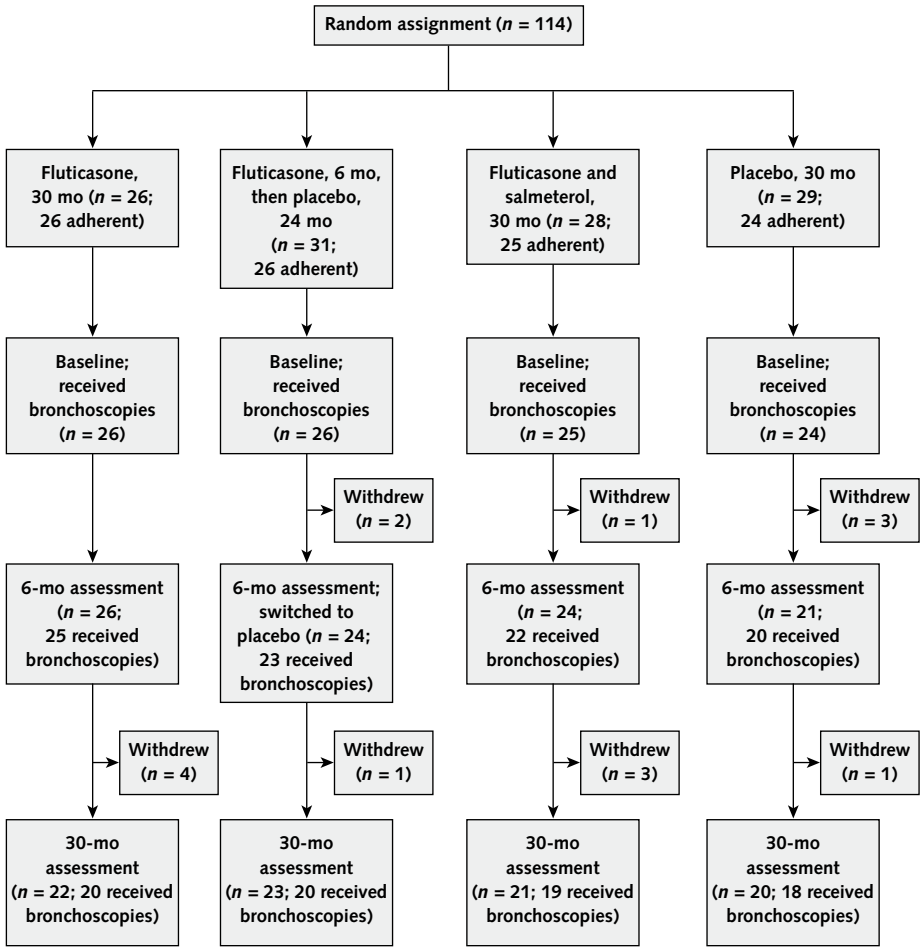
This was an investigator-initiated trial. The study was funded by the Netherlands Organization for Scientific Research, Netherlands Asthma Foundation, GlaxoSmithKline of The Netherlands, University Medical Center Groningen, and Leiden University Medical Center. The funding sources had no role in the design, conduct, and analysis of the study or in the decision to submit the manuscript for publication.

Results

Of the 114 randomly assigned patients, we analyzed 101 adherent patients (Figure 1). Mean postbronchodilator FEV_1 was 63% predicted (SD, 9%) (91 patients were GOLD stage II and 10 were GOLD stage III) and geometric mean methacholine PC_{20} was 0.6 mg/mL (SD, 2.6 doubling dose). Seven patients had ever received a short course of corticosteroids and only 5 had ever received ICS maintenance therapy.

Baseline patient characteristics were similar among the 4 treatment groups (Table 1). Sputum and biopsy inflammatory cells counts did not differ. The amount of missing data, including missing data due to dropouts, for each study

Figure 1. Study flow diagram.



Total number of patients randomized and compliant (>70% medication use) per treatment group. At each stage of the study (0, 6 and 30 months) the numbers are listed of those who underwent bronchoscopy amongst the number of patients remaining in the study.
Definition of abbreviations: n = number.

measure were 12% for FEV_1, 13.9% for methacholine PC_{20}, 12.5% for MRC score, 13.9% for SGRQ score, 14.7% for CCQ score, 12.5% for bronchial inflammatory cells, 14.2% for epithelial features, and 14.2% sputum cells.

Short-term therapy with ICS

Fluticasone therapy decreased counts of bronchial $CD3^+$ cells (-55% [CI, -74% to

Table 1. Patient characteristics at baseline *.

Characteristics	Placebo, 30 mo	Fluticasone 6 mo, Then Placebo, 24 mo	Fluticasone, 30 mo	Fluticasone plus salmeterol, 30 mo	P value †
Patients, n	24	26	26	25	
Clinical					
Men/women, n/n	20/4	22/4	23/3	22/3	0.94
Age, y	59 (8)	64 (7)	62 (8)	62 (8)	0.31
Current smoker/ not current smoker, n/n	17/7	14/12	16/10	17/8	0.61
Median smoking history (range), pack-years	42 (34-54)	41 (29-57)	44 (31-55)	47 (31-56)	0.62
Lung Function					
Prebronchodilator FEV_1, % predicted	54.1 (8.3)	56.8 (11)	56.6 (9.9)	55.0 (11)	0.742
Postbronchodilator FEV_1, % predicted	61 (8.3)	65 (8.6)	64 (9.1)	61 (9.4)	0.41
Change in FEV_1, % predicted ‡	7.1 (4.5)	7.3 (5.3)	7.1 (4.0)	6.2 (6.3)	0.87
Postbronchodilator FEV_1/IVC, %	47 (9.0)	51 (8.3)	49 (9.0)	46 (8.4)	0.157
Geometric mean methacholine PC_{20}, mg/mL §	0.7 (2.0)	0.7 (3.2)	0.4 (2.4)	0.7 (2.7)	0.64
K_{CO}, % predicted	65 (19)	79 (29)	77 (22)	74 (27)	0.188
Symptoms and health status					
MRC dyspnea score ||	2.7 (0.8)	2.5 (0.6)	2.6 (0.6)	2.9 (1.0)	0.53
SGRQ total score ¶	33.5 (18.5)	25.7 (15.2)	32.9 (10.9)	28.1 (13.2)	0.27
CCQ total score **	1.77 (1.3)	1.16 (0.6)	1.26 (0.6)	1.43 (0.7)	0.35

CCQ = Clinical COPD [chronic obstructive pulmonary disease] Questionnaire; IVC = inspiratory vital capacity; K_{CO} = transfer factor for carbon-monoxide; methacholine PC_{20} = provocative concentration of methacholine that causes a 20% decrease in FEV_1; MRC = Medical Research Council; SGRQ = St. George's Respiratory Questionnaire.

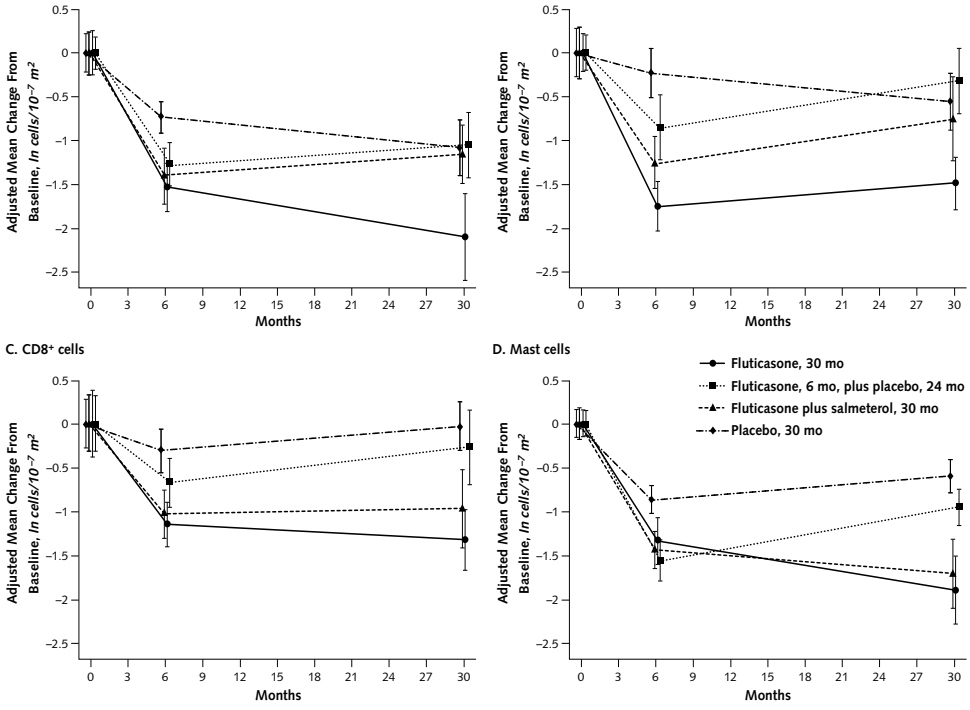
* Values are means (SDs) unless otherwise indicated. † By analysis of variance or Kruskall-Wallis tests between groups. ‡ Reversibility in FEV_1 by 400-µg inhaled salbutamol. § Methacholine PC_{20} values are expressed as mean doubling doses. || Range of 1 to 5 (a higher score indicates more dyspnea). ¶ Range of 0 (best) to 100 (worst). ** Range of 0 (best) to 6 (worst)

-22%]; p = 0.004), $CD4^+$ cells (-78% [CI, -88% to -60%]; p < 0.001), $CD8^+$ cells (-57% [CI, -77% to -18%]; p = 0.010), and mast cells (-38% [CI, -60% to -2%]; p = 0.039) at 6 months compared with placebo (Figure 2 and Table 2]. This was accompanied by an increase in methacholine PC_{20} (1.5 doubling dose [CI, 0.1 to 3.0]; p = 0.036) (Figure 3B) and CCQ mental score (0.2 point [CI, 0.01 to 0.4 points]; p = 0.037) compared with placebo. We found no other statistically significant effects of 6 months of fluticasone therapy. The change in FEV_1 after 6 months did not significantly differ between patients who were randomly assigned to continue fluticasone therapy and those assigned to switch to placebo.

Long-term continuation of ICS therapy

Continuing fluticasone therapy from 6 to 30 months maintained the reduction in

Figure 2. Pathological outcomes.



Adjusted mean change in log-transformed bronchial cell numbers (/10^{-7} m^2 lamina propria) over time during treatment with fluticasone (500 µg bid) for 30 months (FP30), fluticasone (500 µg bid) for 6 months (FP6), the combination of fluticasone/salmeterol (500/50 µg bid) for 30 months (FP/S) and placebo for 30 months in patients with COPD. Error bars represent 95% confidence intervals (CI). Data of bronchial $CD3^+$ cells (2A), $CD4^+$ cells (2B), $CD8^+$ cells (2C), and mast cells (2D) are presented.

Table 2. Bronchial inflammatory cell counts at baseline and after 6 and 30 months*.

	Placebo, 30 mo			Fluticasone, 6 mo, then Placebo, 24 mo			Fluticasone, 30 mo			Fluticasone Plus Salmeterol, 30 mo		
	Baseline	6 mo	30 mo	Baseline	6 mo	30 mo	Baseline	6 mo	30 mo	Baseline	6 mo	30 mo
Patients, n	24	20	18	26	23	20	25	24	19	25	22	19
$CD3^+$ cells	135 (76-197)	57 (37-84)	50 (24-79)	111 (69-180)	28 (21-45)	38 (19-89)	124 (63-192)	21 (16-33)	12 (5.5-38)	118 (74-191)	35 (17-54)	36 (15-53)
$CD4^+$ cells	44 (21-66)	33 (18-67)	24 (11-42)	34 (24-67)	11 (6.5-19)	27 (12-57)	68 (43-100)	10 (6.0-19)	22 (6.5-26)	48 (26-82)	11 (6.9-25)	15 (11-57)
$CD8^+$ cells	19 (10-33)	14 (9.0-23)	22 (12-33)	17 (6.9-29)	5.5 (3.0-9.0)	11 (4.3-19)	23 (11-41)	6.8 (3.3-9.5)	4.0 (2.0-9.5)	23 (16-52)	8.8 (6.3-19)	7.5 (4.0-24)
Neutrophils	4.0 (2.1-8.0)	3.0 (1.5-10)	5.3 (2.9-15)	5.0 (1.5-9.0)	7.0 (3.0-11)	9.8 (5.6-22)	2.5 (1.5-5.0)	5.5 (2.6-12)	13 (8.5-24)	5.0 (3.0-8.0)	6.3 (3.0-18)	7.5 (4.0-24)
Eosinophils	1.0 (0.5-5.8)	0.5 (0-2.2)	1.0 (0.4-3.4)	2.0 (0.5-7.5)	0.5 (0-1.0)	5.5 (1.1-11)	1.5 (0.5-3.3)	0.5 (0-1.4)	2.5 (1.0-8.5)	1.5 (0.5-2.5)	0.3 (0-3.3)	1.0 (0-5.0)
Plasma cells	7.8 (3.5-17)	2.0 (1.5-11)	5.5 (2.0-12)	11 (7.4-14)	2.0 (1.0-3.0)	6.3 (1.6-13)	8.0 (2.8-15)	2.0 (0.6-3.4)	1.0 (1.0-3.0)	6.5 (4.0-18)	1.3 (0.4-2.5)	4.0 (1.0-7.5)
Macrophages	8.3 (4.1-10)	5.3 (2.6-12)	4.0 (2.9-15)	9.3 (4.5-12)	3.5 (2.0-7.5)	5.3 (2.3-14)	10 (5.0-23)	4.0 (2.5-7.9)	3.0 (0.5-8.5)	9.5 (5.5-12)	4.8 (1.9-12)	4.0 (0.5-21)
Mast cells	24 (17-32)	11 (8.5-13)	14 (9.6-18)	31 (23-41)	6.0 (3.0-9.0)	12 (7.5-16)	22 (16-34)	8.0 (3.0-10)	2.5 (0.5-4.5)	26 (17-32)	7.0 (4.4-8.8)	5.0 (1.5-10)

* Cell counts are expressed as medians (25^{th}-75^{th} percentiles) count/10^{-7} m^2 of subepithelium.

$CD3^+$, $CD4^+$ and $CD8^+$ cell counts (Figure 2 and Table 2) after 30 months, compared with placebo. This was accompanied by a further -56% change in mast cell count (CI, -73% to -29%; p = 0.001), a 125% increase in eosinophil count (CI, 2% to 399%; p = 0.046), and a 101% increase in the percentage of intact epithelium in bronchial biopsies (CI, 10% to 268%; p = 0.024) after 30 months (Figure 2 and Tables 2 and 4). In addition, the 30-month fluticasone group had lower counts of sputum neutrophils (-58% [CI, -82% to -1%); p = 0.047), macrophages (-57% [CI, -81% to -3%]; p = 0.041), and lymphocytes (-52% [CI, -76% to -5%]; p = 0.035) at 30 months than did the placebo group (Table 3).

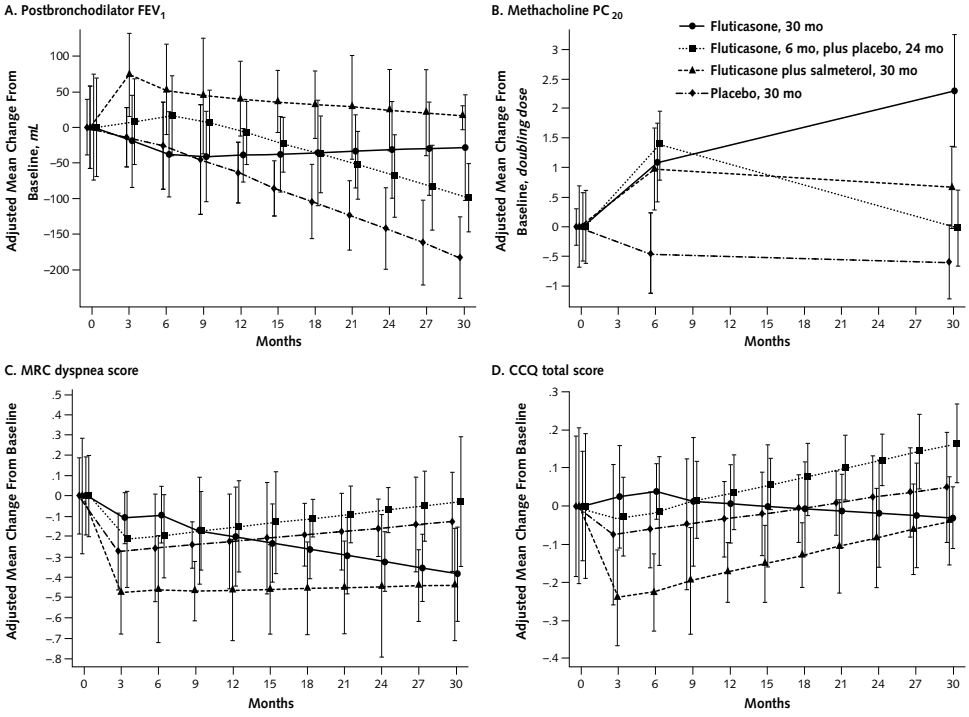
The rates of FEV_1-decline from 6 to 30 months were -79 mL/y (CI, -112 to -46 mL/y) for the placebo group, -62 mL/y (CI, -93 to -31 mL/y) for the 6-month fluticasone group, 7.3 mL/y (CI, -21 to 35 mL/y) for the 30-month fluticasone group, and -16 mL/y (CI, -46 to 15 mL/y) for 30-month fluticasone and salmeterol group. Fluticasone significantly diminished annual FEV_1 decline over the last 2 years of the study compared with placebo (difference, 86 mL/y [CI, 43 to 129 mL/y]; p < 0.001) (Figure 3A). The improvement in methacholine PC_{20} by fluticasone compared with placebo that we observed during the first 6 months was maintained during the following 2 years (Figure 3B). In addition, maintaining fluticasone therapy reduced dyspnea scores more than placebo over the last 2 years of the study (-0.2 point/y [CI, -0.3 to -0.06 point/y]; p = 0.003) (Figure 3C), and significantly improved SGRQ activity score (-3.1 points/y [CI, -5.5 to -0.7 points/y]; p = 0.012) and CCQ total score (-0.1 point/y [CI, -0.2 to -0.01 points/y]; p = 0.036), symptom score (-0.1 points/y [CI, -0.3 to -0.02 points/y]; p = 0.026), and functional score (-0.1 points/y [CI, -0.2 to -0.01]; p = 0.027) (Figure 3D).

Discontinuation of ICS therapy

Discontinuing fluticasone therapy after 6 months increased $CD3^+$ cell count by 120% (CI, 24% to 289%; p = 0.007), mast cell count by 218% (CI, 99% to 407%; p < 0.001), and plasma cell count by 118% (CI, 9% to 336%; p = 0.028) at 30 months versus continuing therapy (Figure 2 and Table 2). Bronchial epithelial parameters and sputum inflammatory cells did not change significantly (Tables 3 and 4).

Discontinuing fluticasone therapy after 6 months worsened subsequent FEV_1 decline compared with continuing therapy during the last 2 years of follow-up (difference in slope, -70 mL/y [CI, -111 to -28 mL/y]; p = 0.001) (Figure 3A), with an accompanying deterioration in methacholine PC_{20} (-2.6 doubling dose [CI, -4.1 to -1.2 doubling dose]; p < 0.001) (Figure 3B). Stopping fluticasone therapy also worsened dyspnea scores by 0.2 points/y (CI, 0.08 to 0.3 points/y; p = 0.001) (Figure 3C), SGRQ total score by 1.7 points/y (CI, 0.19 to 3.2 points/y; p = 0.028) and activity score by 2.9 points/y (CI, 0.6 to 5.3 points/y; p = 0.015),

Figure 3. Clinical outcomes.



Adjusted mean change ± 95% CI over time during treatment with fluticasone (500 µg bid) for 30 months (FP 30), fluticasone (500 µg bid) for 6 months (FP 6), followed by placebo (as indicated by the vertical line), the combination of fluticasone/salmeterol (500/50 µg bid) for 30 months (FP/S 30) and placebo (bid), in patients with moderately severe COPD. Changes in PC_{20} are expressed as mean doubling doses. Data are presented for forced expiratory volume in one second (FEV_1) (3A), log-transformed provocation concentration of methacholine causing a 20% fall in FEV_1 (PC_{20}) (3B), Medical Research Council dyspnea score (3C) and Clinical COPD Questionnaire (CCQ) (3D).

and CCQ total score by 0.1 point/y (CI, 0.04 to 0.2 points/y; p = 0.003) and symptom score by 0.2 points/y (CI, 0.1 to 0.3 points/y; p < 0.001), compared with continuing therapy (data not shown).

Addition of LABAs to ICS therapy

At 6 months, combination treatment provided no additional anti-inflammatory effects compared with fluticasone alone; however, at 30 months, $CD3^+$ cell count had increased by 126% (CI, 27% to 303%; p = 0.006) and plasma cell count by 144% (CI, 21% to 393%; p = 0.013) (Figure 2 and Table 2), and eosinophils in bronchial biopsies had changed by -55% (CI, -79% to -1%; p = 0.047). Salmeterol had no additional effect on bronchial epithelial parameters or sputum inflamma-

Table 3. Sputum inflammatory cell counts at baseline and after 6 and 30 months *.

	Placebo, 30 mo			Fluticasone, 6 mo, Then Placebo, 24 mo			Fluticasone, 30 mo			Fluticasone Plus Salmeterol, 30 mo		
	Baseline	6 mo	30 mo	Baseline	6 mo	30 mo	Baseline	6 mo	30 mo	Baseline	6 mo	30 mo
Total cell count, x10^4 cells/mL)	168 (77-235)	62 (41-212)	107 (18-268)	117 (53-380)	101 (80-320)	95 (57-164)	175 (101-316)	95 (53-178)	58 (23-74)	136 (78-247)	114 (60-201)	55 (17-160)
Neutrophils, %	72 (54-80)	74 (54-81)	70 (50-85)	73 (63-82)	67 (56-79)	75 (61-79)	66 (50-77)	68 (47-78)	71 (45-79)	72 (61-81)	74 (64-81)	75 (65-81)
Eosinophils, %	0.9 (0.3-2.2)	0.8 (0.2-1.3)	1.0 (0.2-1.8)	1.3 (0.5-2.6)	1.0 (0.5-1.6)	1.0 (0.5-1.9)	1.2 (0.3-2.2)	0.8 (0.2-1.9)	0.8 (0.5-1.8)	1.3 (0.2-2.3)	0.8 (0.4-1.3)	0.8 (0.3-2.0)
Macrophages, %	22 (16-36)	23 (16-39)	22 (11-31)	22 (13-27)	20 (14-34)	20 (15-28)	29 (19-37)	25 (17-37)	19 (14-38)	23 (17-32)	19 (14-31)	19 (16-29)
Lymphocytes, %	1.8 (1.3-3.0)	1.7 (1.0-3.2)	1.8 (1.2-3.7)	1.8 (1.5-2.2)	1.5 (1.2-2.3)	2.0 (1.2-3.3)	2.2 (1.2-3.1)	2.0 (1.2-2.9)	1.9 (1.1-2.3)	1.3 (0.8-2.4)	2.0 (0.7-2.8)	1.7 (1.2-2.5)

*Data are expressed as medians (25^{th} - 75^{th} percentiles).

Table 4. Bronchial epithelial features at baseline and after 6 and 30 months *.

	Placebo, 30 mo			Fluticasone, 6 mo, Then Placebo, 24 mo			Fluticasone, 30 mo			Fluticasone Plus Salmeterol, 30 mo		
	baseline	6 mo	30 mo	baseline	6 mo	30 mo	baseline	6 mo	30 mo	baseline	6 mo	30 mo
Intact epithelium, %	23 (18-35)	22 (10-30)	12 (3-20)	30 (18-42)	21 (10-30)	15 (4-27)	20 (14-33)	20 (14-28)	16 (10-31)	29 (16-47)	29 (24-38)	25 (20-50)
Squamous-cell metaplasia, % of epithelium	0 (0-30)	0 (0-0)	0 (0-0)	0 (0-8.4)	0 (0-0)	0 (0-8.4)	1.1 (0-21)	0 (0-0)	0 (0-0)	0 (0-24)	0 (0-0)	0 (0-0)
Squamous-cell metaplasia, % of patients	24	20	24	26	26	26	25	27	25	25	26	25
PAS/AB-positive area, %	15 (5.9-20)	17 (8.2-26)	9.9 (4.3-27)	8.4 (3.3-20)	14 (6.1-20)	5.0 (1.0-12)	9.2 (3.9-15)	20 (7.9-32)	9.1 (4.6-23)	10 (4.7-16)	17 (8.0-24)	13 (4.9-23)
Ki-67^+ cells, per mm of basement membrane	15 (3.6-23)	6 (0.5-28)	3.4 (1.6-8.6)	9.9 (3.7-34)	5.4 (1.2-30)	33 (9.3-67)	12 (0.2-39)	4.1 (0.4-12)	5.4 (1.3-13)	9.2 (5.6-33)	7.3 (1.5-25)	11 (6.2-36)

PAS/AB = periodic acid-Schiff/Alcian blue. *Data are expressed as medians (25^{th} - 75^{th} percentiles).

tory cells (Table 3 and 4).
At 6 months, combination therapy increased postbronchodilator FEV_1 (96 mL [CI, 16 to 176 mL]; p = 0.018) (Figure 3A) and improved dyspnea scores (-0.4 points [CI, -0.7 to -0.04 points]; p = 0.027) (Figure 3C) more than fluticasone alone. Improved FEV_1 was maintained during prolonged combination therapy without further alteration of FEV_1 decline, compared with fluticasone alone, but the dyspnea score increased after 30 months (0.1 points/y [CI, 0.01 to 0.3 points/y]; p = 0.029). During the first 6 months, combination therapy resulted in a change of -0.3 points (CI, -0.5 to -0.07 points; p = 0.007) in CCQ total score, -0.3 points (CI, -0.6 to -0.04; p = 0.028) in symptom score, and -0.3 points (CI, -0.6 to -0.08 points; p = 0.008) in functional score (Figure 3D). The minimal clinically important difference of 0.4 was not reached (30). During the subsequent 24 months, combination therapy did significantly worse than fluticasone alone on these outcomes, with a change of 0.1 point/y (CI, 0.04 to 0.2 points/y; p = 0.003) in total score, 0.1 point/y (CI, 0.03 to 0.3 points/y; p = 0.013) in symptom score, and 0.1 point/y (CI, 0.03 to 0.2 points/y; p = 0.012) in functional score.
We analyzed the data by using a model that also included individual variances of the slopes and obtained similar results.

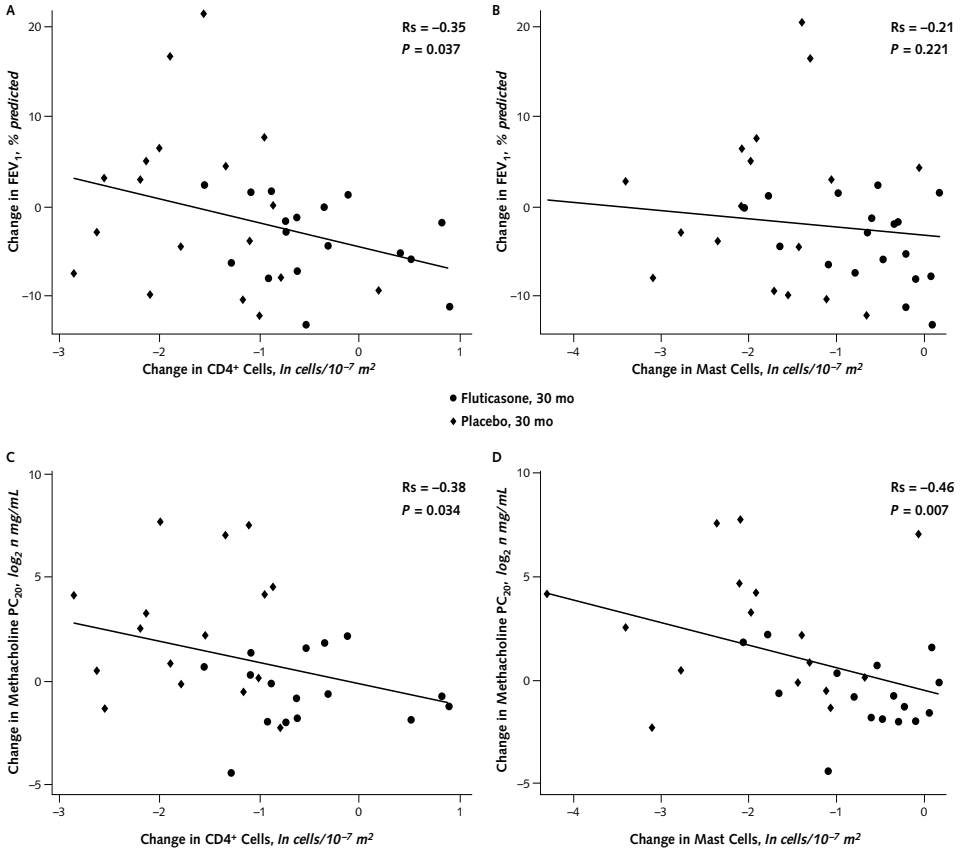
Smoking and treatment effects
During the study, 3 patients started smoking and 13 patients stopped smoking (balanced among groups). All above results remained statistically significant when adjusted for smoking status throughout the study, except for the reduction in sputum lymphocyte numbers by long-term fluticasone therapy.

Relation of treatment effects with pathology and lung function
Analyses of patients that received either fluticasone or placebo for 30 month showed that decreases in $CD4^+$ cells were associated with improvements in predicted postbronchodilator FEV_1 (Rs, -0.35; p = 0.037) (Figure 4). Improvements in methacholine PC_{20} were associated with reductions in $CD3^+$ cells (Rs, -0.36; p = 0.041), $CD4^+$ cells (Rs, -0.38; p = 0.034), and mast cells (Rs, -0.46; p = 0.007), and increases in percentage intact epithelium (Rs, 0.40; p = 0.024) (Figure 4).

Discussion

Our study shows that 2.5-year maintenance therapy with ICS in COPD reduces bronchial T-lymphocyte and mast cell numbers and increases eosinophils and the integrity of bronchial epithelium, with an accompanying reduction in sputum

Figure 4. Correlation between pathological and clinical outcomes.



Upper panel. Correlation of changes (30 months minus baseline) in postbronchodilator forced expiratory volume in one second (FEV_1, % predicted) with changes in log-transformed $CD4^+$ cell numbers ($/10^{-7}$ m^2) (4A) and changes in log-transformed mast cell numbers ($/10^{-7}$ m^2) (4B) in the lamina propria of bronchial biopsies in COPD patients treated with fluticasone propionate 30 months or placebo.
Lower panel. Correlation of changes (30 months minus baseline) in log-transformed provocative concentration of methacholine causing a 20% fall in FEV_1 (PC_{20}) with changes in log-transformed $CD4^+$ cell numbers ($/10^{-7}$ m^2) (4C) and changes in log-transformed mast cell numbers ($/10^{-7}$ m^2) (4D) in the lamina propria of bronchial biopsies in COPD patients treated with fluticasone propionate 30 months or placebo.

cell counts. These effects are associated with a reduced rate of FEV_1 decline and improvements in airway hyperresponsiveness, dyspnea, and health status. Stopping ICS therapy at 6 months leads to relapse of bronchial inflammation and hyperresponsiveness, dyspnea, and poorer health status, with acceleration of FEV1 decline. Combination therapy with ICS and a long-acting β_2-agonist does not provide further anti-inflammatory effects compared with fluticasone alone

but improves the level of FEV_1 without further influencing FEV_1 decline. Our findings indicate that a subphenotype of patients with COPD who are steroid-naïve and have moderate airway obstruction and airway hyperresponsiveness are sensitive to long-term ICS therapy. These prolonged effects on inflammation and lung function do not imply causality but suggest that disease modification can be achieved in particular phenotypes of patients with COPD.

We observed differential effects of ICS on inflammatory cell counts. Although smoking may reduce corticosteroid responsiveness (31), our data show that at least part of the inflammation in COPD remains sensitive to this treatment. The contribution of $CD8^+$ cells to inflammation and the relevant antigen-specific triggers in COPD are still unknown. $CD4^+$ cells may contribute to activation and memory formation of $CD8^+$ cells, as well as provide help for B cells (32). Mast cells and their secreted enzymes can drive various processes relevant to inflammation and remodeling (33). Although in vitro studies suggest that corticosteroids are less effective in inhibiting activation of mast cells than activation of T-cells (34), our data indicate corticosteroids can have selective anti-inflammatory effects in COPD. The observed increase in intact epithelium by ICS has also been found in persons with asthma (35). Corticosteroid-induced changes in epithelial integrity and inflammation correlated with improvements in methacholine PC_{20}, which supports the notion that airway hyperresponsiveness in COPD can be a marker of disease activity (36;37).

The clinical novelty of our findings is that anti-inflammatory effects observed with long-term ICS treatment associate with reduced FEV_1 decline in COPD. Previous short-tem studies that investigated patients with COPD and similar degrees of airway obstruction (20;21;38) have shown anti-inflammatory effects of ICS in COPD. We show that these beneficial effects are maintained during long-term treatment up to 30 months. The detrimental effects of discontinuing ICS therapy on bronchial inflammation are also novel. Previous short-term studies of the combination of a LABA and ICS demonstrated anti-inflammatory effects versus placebo (39) or additional reductions of bronchial $CD8^+$ cells and macrophages versus ICS alone (22). Our data suggest that this is not a long-lasting additional effect; we observed a slight increase in $CD3^+$ and plasma cells. The attenuated FEV_1 decline in our patients with COPD contrasts with large COPD trials from the 1990s (7-9). The more recent TORCH study (15) did show reductions of FEV_1 decline in patients with COPD who received therapy ICSs, LABAs, or both. Our results suggest that the improvement in the level of FEV_1 in the combination group might be due to a residual bronchodilator effect of salmeterol and not further disease modification. Discrepancies between the previous trials and our study may be due to differences in study samples, which may provide a clinical message.

Our study comprised a common subset of patients with COPD. First, by choosing steroid-naïve patients, we aimed to exclude patients with unknown previous benefits from ICS at baseline and avoid the problem of selective dropouts in the placebo group. Second, our patients had predominantly moderate degrees of airway obstruction and most demonstrated airway hyperresponsiveness or modest reversibility of FEV_1. Recent studies (10;40) show that these characteristics, previously attributed to asthma alone, can also be components of COPD. This COPD phenotype may be particularly sensitive to ICS, similar to the documented beneficial effects of smoking cessation (37). Of note, the decrease in postbronchodilator FEV_1 in the placebo group was quite similar to that observed in previous studies (8;10;15). We were particularly careful to exclude patients with a previous or concurrent diagnosis of asthma by carefully taking histories, checking family practice medical records, and obtaining clinical judgments from chest physicians. Furthermore, most patients had low numbers of eosinophils in sputum and biopsies (similar to those reported by Bourbeau and colleagues (22)), had smoked for many years, and had a mean reversibility of FEV_1 to salbutamol of only 7% of predicted value, and most (83%) were nonreversible according to European Respiratory Society criteria - yet all adhered to the GOLD criteria. This is consistent with the patient characteristics of short-term COPD studies that show benefits with ICS therapy (22;39). Airway hyperresponsiveness was similar to that in the Lung Health COPD study (10), which measured long-term changes in airway hyperresponsiveness. Finally, our post hoc analysis showed that actual smoking throughout the study was unlikely to be a major confounder. Taken together, our findings suggest that ICS therapy, when given for the first time and for longer duration to steroid-naïve patients with relatively moderate disease, has the potential to change the clinical course of COPD.

Our study has limitations. First, only 77 of 101 analyzed patients had biopsies at 30 months because patients dropped out or were unwilling to have another bronchoscopy. This might have resulted in selection bias; however, lost-to-biopsy rates were similar among treatment groups. Second, our study was not powered to examine clinical outcomes. Nevertheless, the primary and secondary outcome parameters were all pre-specified. According to international standards on clinical investigations (41), the secondary outcomes point toward a clinically relevant treatment benefit, given our positive findings in the primary outcome. In addition, the positive findings on FEV_1 decline are consistent with the symptomatic benefit we observed (42). Third, because this was an efficacy trial, we used data from adherent patients. As expected, the placebo group had more nonadherent patients, which may have led to underestimate the treatment effect. Fourth, the pathologic changes in COPD are not uniformly distributed among central and

peripheral airways (43;44). We inevitably focused on the central airways. Fifth, despite its beneficial effects, long-term ICS treatment has potentially meaningful adverse effects, such as increased frequency of pneumonia (14). Our sample size was too small to draw conclusions on this.

Our study should lead to subsequent analyses of the benefits of inhaled steroids in COPD. Histological outcomes need to include inflammatory and epithelial cell activity and aspects of airway wall remodeling and fibrosis. Studies are also needed to determine the best inflammatory and clinical predictors of steroid efficacy in COPD. Finally, our results indicate a need to study the cost benefit of changing disease progression by using maintenance ICS therapy.

In conclusion, long-term maintenance therapy with ICS can reduce inflammation in bronchial biopsies and sputum in COPD. This is mirrored by attenuated lung function decline, airway hyperresponsiveness, dyspnea, and improved quality of life. Adding a LABA provided supplementary benefit for lung function but did not further alter the course of FEV_1 decline. Clinicians who are treating patients recognize that COPD is a heterogeneous disease that includes various phenotypes (45). Our observations indicate that progressive decline in lung function can be attenuated in steroid-naïve patients with moderate COPD, a long history of smoking, and airway hyperresponsiveness. The observed treatment response by this particular subphenotype of COPD underscores the potential of tailored therapy in COPD to achieve clinical benefit.

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Chapter 7

Conclusions and General discussion

Introduction

Chronic obstructive pulmonary disease (COPD) is characterized by progressive airflow limitation and airway inflammation mainly caused by tobacco smoking. COPD is a heterogeneous disease in terms of clinical, physiological, and pathological presentation. Hence, COPD has multiple disease domains that each can be highly relevant for disease monitoring, progression and treatment. This thesis has focused on: the potential independent roles of airway inflammation and functional impairment in COPD, the monitoring of airway function and inflammation in patients with COPD, the effects of smoking cessation on inflammation and epithelial remodeling in COPD, and the integrative effects of anti-inflammatory treatment on each of the disease domains in COPD.
This thesis comprises cross-sectional and follow-up analyses of data from the GLUCOLD study (Groningen Leiden Universities and Corticosteroids in Obstructive Lung Disease). The GLUCOLD Study is a prospective, four-arm, placebo-controlled, and double-blind study, comparing the effects of 30 months treatment with inhaled steroids, inhaled steroids and a long-acting β_2-agonist combination therapy, placebo, and 6 months inhaled steroids followed by 24 months placebo on clinical and pathological outcome parameters. First, the conclusions of the studies in this thesis will be summed up, followed by a general discussion and directions for future research.

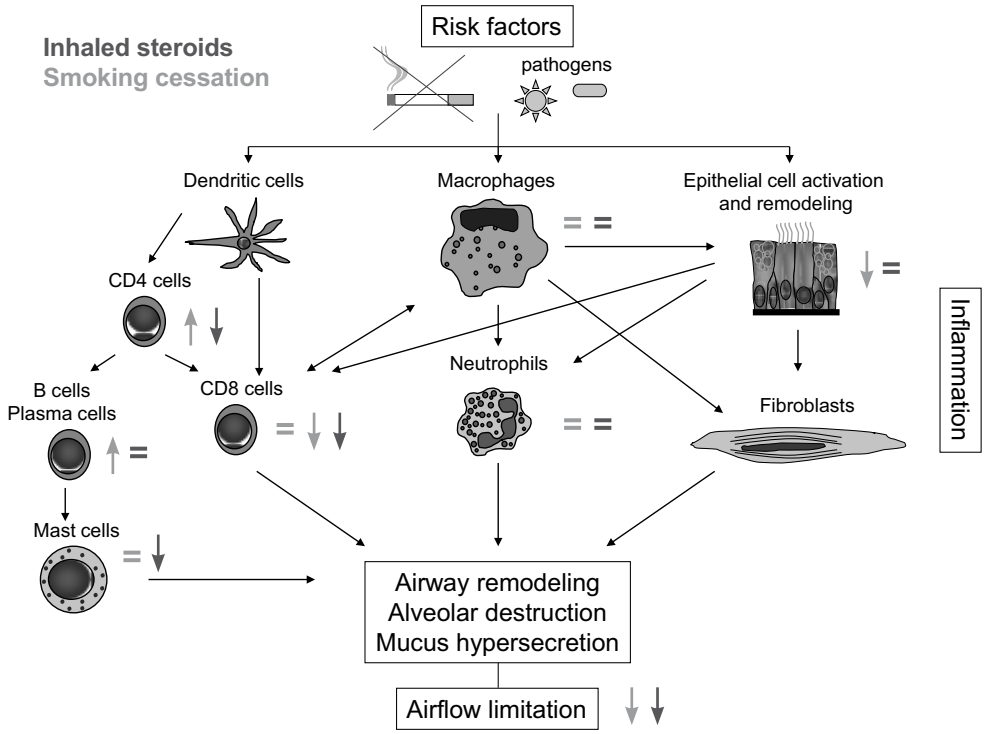
Conclusions of the thesis

Relation between airway inflammation and pathophysiology in COPD

- Airflow limitation, asthma-like components (i.e. airway responsiveness and IgE), exhaled nitric oxide and sputum inflammatory cell counts (neutrophils and eosinophils) offer separate and additive information about the pathophysiological condition of COPD patients (*Chapter 2*).
- Uneven distribution of ventilation and airway closure in patients with stable COPD are associated with neutrophil numbers in bronchial biopsies and bronchoalveolar lavage (BAL) fluid, and with neutrophil elastase and its local inhibitor secretory leukocyte proteinase inhibitor (SLPI) in BAL (*Chapter 3*).

Effect of smoking cessation on airway pathology in COPD

- Ex-smokers with COPD have higher numbers of bronchial $CD4^+$ and plasma cells than current smokers, whereas numbers of neutrophils, macrophages, and $CD8^+$ cells are not different between both groups. T-lymphocytes are higher in short-



term quitters, whereas longer duration of smoking cessation is associated with lower CD8/CD3 ratios, and higher numbers of plasma cells (*Chapter 4*).

- Ex-smokers with COPD have less bronchial epithelial mucin stores, proliferating cells, and squamous cell metaplasia than current smokers with COPD, whereas epithelial epidermal growth factor receptor (EGFR) expression is not different between both groups. These epithelial changes in ex-smokers were more pronounced with longer duration of smoking cessation, and only significant after 3.5 years smoking cessation (*Chapter 5*).

Treatment effects on airway pathology in COPD

Long-term treatment with fluticasone propionate in COPD patients reduces the number of bronchial T-lymphocytes and mast cells, whilst increasing the integrity of bronchial epithelium and number of bronchial eosinophils, which was accompanied by a reduction in sputum cell counts. This is accompanied by a reduced rate of decline in FEV_1 and improvements in airway hyperresponsiveness, dyspnea and quality of life (*Chapter 6*).

- Fluticasone propionate-induced improvement in FEV_1 after long-term treatment in patients with COPD is associated with a reduction in bronchial $CD4^+$ cells, whilst the improvement in airway hyperresponsiveness is associated with a reduction in $CD3^+$, $CD4^+$, and mast cells and with an increase in intact epithelium (*Chapter 6*).
- Discontinuation of fluticasone at 6 months leads to a relapse of bronchial inflammation and airway hyperresponsiveness, dyspnea and quality of life, with acceleration of FEV_1-decline (*Chapter 6*).
- Adding salmeterol to fluticasone propionate does not provide further anti-inflammatory effects compared to fluticasone alone, but improves FEV_1-level without further influencing FEV_1-decline (*Chapter 6*).

General discussion

Airway inflammation and airflow limitation in COPD are not simply related

A crucial pathologic feature of COPD is airway inflammation and remodeling. The chronic inflammation in COPD is characterized by an accumulation of neutrophils, macrophages, B-cells, lymphoid aggregates and $CD8^+$ T-cells, particularly in and near the small airways (1). Multiple cross-sectional studies have demonstrated relationships between this inflammatory profile and airflow limitation in COPD, suggesting a role for inflammation in the pathogenesis of the disease. Increased airflow obstruction is associated with a higher degree of airway inflammation (2), and more specifically with increased numbers of $CD8^+$ cells (3-5), B-lymphocytes (2), neutrophils (6-10), macrophages (10), dendritic cells (11), and in some COPD patients even with eosinophils (12). In addition, one prospective study has shown that patients with an accelerated decline in lung function have an increased baseline sputum neutrophil differential count (13). However, prospective data examining the natural course of airway inflammation in relation to FEV_1 decline within COPD patients are still lacking. Also, cross-sectional correlations do not differentiate properly between cause and effect. So is chronic airway inflammation involved in COPD progression?

To clarify relationships between various parameters, without reference to a specific criterion, we performed a factor analysis using sputum inflammatory and functional parameters in patients with COPD. Interestingly, our data suggest that airflow limitation is greatly independent of neutrophilic and eosinophilic inflammation in the larger airways (chapter 2). Neutrophils are involved in the induction of mucous metaplasia and tissue damage through the release of serine proteases and oxidants (14). However, emphysema is not a prominent feature of other

pulmonary diseases where chronic airway neutrophilia is even more prominent, such as cystic fibrosis and bronchiectasis (15). In addition, increased neutrophil numbers are found in the airway lumen, but they are not a prominent feature of the inflammation in the airway wall or parenchyma in patients with COPD (16). This suggests that other factors are involved in the generation of emphysema. Obviously, sputum does not cover all the inflammatory and structural changes of the airways in COPD. It may well be that selection of other inflammatory cell types characteristic for COPD, such as $CD8^+$ T-lymphocytes and macrophages, or selection of cells in other compartments of the lung, would have given different results of the factor analysis. This is also supported by our observation that small airways dysfunction (uneven distribution of ventilation and early airway closure), as measured with the sbN_2-test, is associated with neutrophil numbers in bronchoalveolar lavage fluid and bronchial biopsies, and with NE and NE/neutrophil ratio, but not with sputum neutrophils or other inflammatory cell types (chapter 3). In patients with established airflow limitation the sbN_2-test contributes to prediction of the decline in FEV_1 (17). Therefore, we may speculate that neutrophilic inflammation is indirectly involved in the progression of COPD by contributing to small airways and/or alveolar pathology.

Dissociation of inflammation and lung function is partly supported by the results of intervention studies in COPD. For instance, although smoking cessation reduces lung function decline in COPD (18), inflammation persists (chapter 4) (19-21). On the other hand, intervention with inhaled steroids is able to slow down FEV_1 decline in a subgroup of COPD patients which is correlated with suppressive effects on the number of bronchial T-lymphocytes, but not with other inflammatory cells (chapter 6). This latter result suggests a role for at least T-lymphocytes in COPD progression.

Apparently airflow limitation requires more than the presence of inflammatory cells per se. Activity of these cells will obviously be of importance, and would therefore be of interest to investigate in more detail in future studies. Additionally, the degree of airflow limitation is also correlated with airway wall thickness (2), and in a recent study the annual changes in airway thickening assessed by CT measurements correlated with annual decline in air flow limitation (22). These data provide indirect evidence for a role for airway wall remodeling in airflow obstruction in COPD. This remodeling includes epithelial remodeling, including squamous metaplasia and mucous metaplasia, increased smooth muscle mass, and peribronchial fibrosis. In addition, breakdown of extracellular matrix occurs in parenchymal tissues, also resulting in airflow limitation.

COPD has been recognized as a heterogeneous disorder in terms of clinical presentation, physiological and pathological characteristics (23;24). This suggests

that distinct pathophysiological pathways contribute to COPD development. In agreement with this, we observed that multiple functional and inflammatory characteristics were categorized into 4 independent dimensions using factor analysis. Factor 1 included airflow limitation and hyperinflation; factor 2 features commonly associated with asthma (β_2-response, total serum IgE, airway hyperresponsiveness); factor 3 exhaled nitric oxide; and factor 4 sputum % neutrophils and eosinophils (chapter 2). Our data suggest that these four dimensions offer separate and additive information about the pathophysiological condition of COPD patients. Therefore, prospective long-term studies monitoring different aspects of pathological changes in the airways and different functional parameters may further explore the mechanisms leading to progressive airflow limitation in various phenotypic subgroups of COPD patients.

Ongoing airway inflammation and reversal of epithelial remodeling after smoking cessation in COPD

Smoking cessation is able to reduce COPD progression (18), respiratory symptoms and hyperresponsiveness as compared to continued smoking (25;26), and improves survival (27). What are the exact changes that occur in the airways and lung parenchyma after smoking cessation? Since airway inflammation is a key characteristic of COPD, it was hypothesized that this inflammation might be reduced after cessation. However, we have shown that ex-smokers with COPD have higher numbers of bronchial $CD4^+$ and plasma cells than current smokers, whereas numbers of neutrophils, macrophages, $CD8^+$ and mast cells are not different between both groups (chapter 4). The persistence of airway inflammation in ex-smokers is in line with other recent studies (19-21), although decreased numbers of macrophages in ex-smokers with COPD have been reported (19). In contrast, in subjects without COPD inflammatory changes are at least partially reversible with cessation (28). These results suggest that inflammation becomes or remains self-perpetuating after stopping smoking in COPD patients, which could be related to latent adenovirus or bacterial colonization, an auto-immune response (29), persistent apoptosis (30), or persistent and/or aberrant repair processes. The discrepancy between the improvement in FEV_1 decline and the ongoing airway inflammation suggests that inflammation does not necessarily contribute substantially to disease progression.

Interestingly, we observed increased bronchial $CD4^+$ and plasma cells in ex-smokers compared to current smokers (chapter 4). In addition, a more recent study demonstrated that current smokers with COPD have lower numbers of bronchial dendritic cells which reversed upon smoking cessation (31). These results might be explained by reversal of immunosuppressive effects induced by tobacco smoke,

and thereby ameliorating lung defence mechanisms. Alternatively, smoking cessation may contribute to restoration of epithelial characteristics in the large airways of COPD patients, which are directly and continuously exposed to the noxious substances present in cigarette smoke, thereby contributing to the clinical benefits observed after smoking cessation. Consistent with this, we demonstrated that long-term ex-smokers with COPD had less bronchial epithelial mucin stores, proliferating cells, and squamous cell metaplasia than current smokers with COPD (chapter 5). These epithelial features might contribute to COPD by facilitating colonization of the airways by respiratory pathogens, secondary to loss of cilia, increased mucus secretion, and epithelial injury (32). The chronic colonization of the airways may enhance airway inflammation and further epithelial injury. In addition, mucus hypersecretion may cause airways obstruction in peripheral airways (2). Reversal of epithelial remodeling may therefore contribute to reduced progression of COPD attributable to restored mucociliary clearance, resulting in reduced respiratory tract colonization (33) and exacerbations, and less small airways obstruction.

Finally, our results demonstrate that longer duration of smoking cessation (>3.5 years) in COPD patients is associated with higher plasma cell numbers, lower CD8/CD3 ratios, and more pronounced reductions in epithelial mucin stores, proliferating cells, and squamous cell metaplasia (chapter 3 and 4). This suggests a long-term effect of smoking on bronchial regulatory networks, which is not restored immediately after removing the initial stimulus, i.e. cigarette smoke. In contrast, the greatest improvements in respiratory symptoms and lung function decline occur within the first year after cessation (18;25). Therefore, other pathological mechanisms that reverse more rapidly after cessation should be involved in the mechanism leading to the clinical beneficial effects of smoking cessation. It could therefore be interesting to study effects of smoking cessation on for instance cell activity, oedema in de airways, vascular changes and airway smooth muscle. In addition, these results indicate that when studying the effects of smoking cessation the duration of cessation should be taken into account when interpreting the results.

Anti-inflammatory properties of inhaled steroids in COPD

Systemic and local inflammation has been implicated in the pathogenesis of COPD, and consequently the use of systemic and inhaled corticosteroids (ICS) has been considered critical to COPD treatment. As a result, ICS treatment has been clinical practice for decades in stable COPD patients; however, their precise role remains controversial. Previous studies have shown clinical benefits of ICS in patients in terms of symptoms, exacerbation rate, and initial improvement in

FEV_1 (34;35). The attenuated decline in FEV_1 in COPD patients in the present thesis (chapter 6) contrasts with large COPD trials from the 90's showing initial improvement in FEV_1 without benefits on the subsequent progressive FEV_1-decline (34-36). Interestingly, the more recent TORCH study did show slight reductions of FEV_1-decline in COPD patients by inhaled fluticasone, but also by LABA (37). Discrepancies between the negative trials and the present study may be due to differences in study populations, which may provide a clinical message. We studied steroid-naïve patients, with predominantly moderate degrees of airway obstruction and the majority demonstrated airway hyperresponsiveness and/or modest reversibility of FEV_1, although patients with a previous or concurrent diagnosis of asthma were excluded. Recent studies showed that these characteristics, previously attributed to asthma, can also be components of COPD (38;39). It may well be that this COPD phenotype is particularly sensitive to inhaled corticosteroids, similar to beneficial effects of smoking cessation (40). Therefore, our findings raise the option that inhaled corticosteroids, when given for the first time and for longer duration to steroid-naïve patients as recruited from general practices having relatively moderate disease, have a realistic potential to change the clinical course of COPD. Moreover, there seems to be a great deal of patient-to-patient variability regarding efficacy of inhaled steroids. As a result, it would be highly valuable to identify phenotypic disease markers (clinical or pathophysiological) that can distinguish which patients with COPD experience the greatest benefit by inhaled corticosteroids. Previous studies have suggested that smoking cessation (34;41;42), larger bronchodilator response (34;35;43), airway hyperresponsiveness (43-45), and eosinophilic airway inflammation (46;47) may be able to predict a beneficial response of steroid treatment in patients with established COPD. However, larger long-term prospective studies should confirm whether these markers can predict the effects of inhaled steroids on FEV_1 decline.

Clinical benefits of ICS in COPD may be mediated, at least partially, by their anti-inflammatory efficacy. Short-term (2-3 months) ICS treatment in COPD was previously shown to reduce numbers of bronchial mast cells, but not $CD8^+$ cells, neutrophils or macrophages (48;49), cells that are characteristic for COPD. We observed differential effects of ICS on inflammatory cell numbers with long-term treatment. Bronchial T-cells, mast cells and sputum neutrophils, macrophages, and lymphocytes were reduced, whereas bronchial neutrophils and macrophages were not (chapter 6). Although smoking may reduce corticosteroid responsiveness (50), our data show that at least part of the inflammation in COPD is not insensitive to this treatment. The contribution of $CD8^+$ cells to inflammation and the relevant antigen-specific triggers (e.g. microbial or autoantigens) in COPD are still unknown. $CD4^+$ cells may contribute to activation and memory formation of $CD8^+$ cells as well as

providing help for B cells (51). Mast cells and their secreted enzymes can drive a variety of processes relevant to inflammation and remodeling, including fibrosis, extracellular matrix turn-over, angiogenesis, smooth muscle and epithelial hyperplasia, and hypersecretion (52). This aspect requires follow-up studies including inflammatory cell activity in addition to cell numbers as outcome parameter. Notably, the observed increase in intact epithelium by long-term corticosteroid treatment has also been found in asthmatic patients (53). This might be due to alterations in inflammation and/or in fragility of the epithelium secondary to changes in epithelial integrity, apoptosis or proliferation status. We can exclude the latter since we did not find such treatment effects within the intact epithelium, or effects on mucin stores, squamous cell metaplasia or EGFR expression. Interestingly, corticosteroid-induced changes in epithelial integrity (and inflammation) correlated with improvements in PC_{20}, supporting the notion that airway hyperresponsiveness in COPD can be a marker of disease activity (40;54).

The reduction in $CD3^+$ cells and mast cells and the functional benefits are reversed after stopping inhaled steroids (chapter 6), indicating that the anti-inflammatory effects are not persistent and that those processes inducing ongoing inflammation in COPD are not affected permanently by steroids. This suggests a role for continuous and long-term treatment with inhaled steroids in COPD as opposed to intermittent treatment. We cannot infer from our study how fast inflammation increases after stopping steroids or whether duration of treatment influences this process. Previous biopsy studies included patients with COPD who withdrew for at least 4-8 weeks from inhaled steroid treatment before entry, and therefore may have interfered with the effects of stopping steroids on inflammation (44;48;55). The next step required when examining effects of corticosteroid intervention in the airways of patients with COPD, is to focus on inflammatory and epithelial cell activity and on airway wall remodeling and fibrosis.

Although both smoking cessation and treatment with inhaled steroids are able to reduce FEV_1 decline in COPD, airway inflammation is partly reduced with steroid treatment whereas it persists after smoking cessation. In addition, whereas smoking cessation reverses epithelial remodeling, treatment with inhaled steroids does not. These apparent discrepancies indicate that other factors, next to inflammatory cell numbers and epithelial remodeling, play a role in lung function decline in COPD. These may include for instance activity of inflammatory cells, remodeling of the airway wall (matrix remodelling and airway smooth muscle hyperplasia/hypertrophy), or alveolar destruction. It should be noted however that the observed discrepancies may also be a consequence of methodological differences between the studies on smoking cessation and inhaled steroid treatment in the present thesis (i.e. retrospective versus prospective, time effect).

Limitations of the studies and methodological considerations

The patients recruited for the GLUCOLD study had to meet the in- and exclusion criteria as discussed in the introduction. These comprised: GOLD stage II and III, no maintenance treatment with inhaled steroids in the past 6 months, no asthma, willing to undergo three bronchoscopies, and participate in a long-term follow-up study with 3-monthly outpatient clinic visits. It turned out to be difficult to find these patients, because the majority of these patients diagnosed with COPD already were treated with inhaled steroids. Therefore, we had to change our recruitment strategy to find those patients who were not yet diagnosed with the disease. It took 2.5 years of intensive recruitment in outpatient clinics and general practices, including approximately 40,000 letters send to potential candidates and 3600 lung function screenings in Leiden only. Finally, we included 114 patients in the study, which was less than the aimed number, but still one of the largest studies including bronchial biopsies world-wide. Steroid withdrawal has shown to result in detoriation of clinical as well as inflammatory parameters in COPD (55-57) (chapter 6). Therefore, we believe that the fact that we included mainly steroid-naïve patients is one of the strengths of the study. It should be taken into account that COPD is a heterogeneous disease in terms of clinical, physiological and pathological presentation. As a result of our inclusion criteria we selected a specific subgroup of the total COPD population, and therefore our results may not be representative for COPD in general.

It needs to be emphasised that the studies on smoking cessation (chapter 4 and 5) were cross-sectional studies, and it cannot be ruled out that our ex-smoking group is a selected group of patients who quit smoking because they suffered more from smoking related symptoms, and may already have had different cell numbers before quitting. Nevertheless, ex-smokers had significantly less respiratory symptoms than current smokers, whilst having similar pack-years and duration of smoking. In addition, in the analysis we did adjust for clinical differences between the groups.

Bronchoscopy with biopsy and induced sputum access the proximal bronchi only, and we have therefore not been able to assess the effects of therapy on small airways and lung parenchyma, the site considered to contribute most to reduced lung function in COPD (2). However, the predominance of $CD8^+$ cells is seen in both proximal and small airways and the correlation between this cell type and impaired lung function is similar in both large and small airways and lung parenchyma (2;3). This and other data (58) suggest that similar processes of inflammation and airway wall thickening appear to be taking place in both large and small airways. Thus, biopsy samples of large airways may be a reasonable surrogate for assessing the potential effects of treatment on small airway inflammation and

remodeling. Additionally, most studies examining peripheral airways pathology have used resection material from patients with lung cancer. It may well be that airway inflammation is influenced by these malignant processes in the airways. We attempted to investigate treatment effects on peripheral airways inflammation assessed in bronchoalveolar lavage fluid. Unfortunately, because of ethical considerations, the BAL procedure was discontinued (i.e. a few patients reported side effects in relation to the BAL) and therefore it was performed only in the first half of patients. An alternative approach of studying treatment effect on smaller airways pathology could be using ultra thin bronchoscopes.

A fully automated image analysis system was applied for cell counting in airway area sections (59). We are aware that counting cells in a 2 dimensional manner has limitations, since it does not take into account the volume of the cell in a given sample – the smallest cells have the least chance to be counted in a single biopsy. Nevertheless, we were able to demonstrate differences in the smallest cells (lymphocytes) in our studies. There is still debate in the literature whether the theoretic basis of stereology fits well with the limitations of endobronchial biopsies (60). Still, most of the present data in the literature is based on counting profiles/area, which allows, although somehow limited by other methodological factors, comparison among studies.

Directions for future research

The studies described in this thesis have provided more insight into the role of airway inflammation in the pathogenesis and treatment of patients with COPD. However, many issues remain to be explored. Interesting questions for future studies may include:

- Are airflow limitation, airway responsiveness, and airway inflammation in bronchial biopsies separate entities underlying the pathophysiology of COPD by using factor analysis?
- What is the natural course of airway pathology in relation to FEV_1 decline within COPD patients in a prospective long-term study? (this is currently ongoing)
- Can the long-term clinical and pathological course of COPD in patients with usual medical care, in a long-term follow-up study of patients from the GLUCOLD study, be predicted by phenotypic disease markers of cellular, physiological and clinical origin? (this is currently ongoing)
- What is the effect of long-term smoking cessation on inflammatory cell activity and airway remodeling in COPD?
- What is the effect of long-term treatment with inhaled corticosteroids on inflammatory cell activity and regulation of matrix remodelling and airway smooth muscle hyperplasia/hypertrophy in airways of COPD patients?
- Is it possible to predict which COPD patients benefit from treatment with inhaled corticosteroids? (this currently ongoing)
- Which non-invasive markers are useful for monitoring COPD patients and treatment effects?
- Can development of novel treatments for COPD result in reversal of disease progression and reduction in mortality?
- How can the apparent discrepancy between beneficial effects of smoking cessation and ICS on lung function decline in COPD, but differential effects on airway inflammation and epithelial features be explained?

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Chapter 8

Nederlandse samenvatting

Achtergrond

Wat is COPD?
COPD is een Engelse afkorting van chronic obstructive pulmonary disease, in het Nederlands chronisch obstructieve longziekten. COPD omvat chronische bronchitis en longemfyseem. Chronische bronchitis wordt gekenmerkt door klachten van chronisch hoesten en sputum opgeven. Bij longemfyseem is er verlies van longweefsel door destructie van longblaasjes, ook wel "rek uit de longen" genoemd. Patiënten met COPD hebben klachten van kortademigheid, vooral bij inspanning, productieve hoest, piepende ademhaling en/of prikkelbaarheid van de luchtwegen voor bepaalde prikkels als rook, mist en kou (hyperreactiviteit). COPD wordt gekenmerkt door een versnelde achteruitgang van de longfunctie. Een deel van de patiënten maakt periodes door met toename van klachten, welke "exacerbaties" worden genoemd. Deze exacerbaties worden vaak veroorzaakt door luchtweginfecties met virussen en/of bacteriën en leiden frequent tot ziekenhuis opname.
In 2003 hadden 316.400 mensen in Nederland COPD: 176.500 mannen en 139.900 vrouwen. Het aantal mensen met COPD stijgt met de leeftijd. COPD komt voornamelijk voor bij mensen van 55 jaar en ouder. In 2004 stierven 3.381 mannen en 2.281 vrouwen ten gevolge van COPD: 4,1% van de totale sterfte in Nederland wordt veroorzaakt door COPD. Hiermee behoort COPD tot de ziekten met de hoogste sterfte. Internationaal gezien is de sterfte aan COPD in Nederland relatief hoog.

Risicofactoren voor het krijgen van COPD
De belangrijkste risicofactor voor COPD is roken. Hoe meer en hoe langer men heeft gerookt, des te groter is de kans op COPD. De trends in COPD volgen de trends in het rookgedrag. 90% van alle COPD patiënten heeft gerookt of rookt nog steeds. Naar schatting ontwikkelt 15-20% van alle rokers in de loop van het leven deze aandoening. Er is dus een zekere mate van gevoeligheid voor sigarettenrook nodig voor het ontstaan van COPD. Waardoor deze gevoeligheid wordt bepaald is nog onbekend, maar er zijn aanwijzingen dat erfelijkheid een rol speelt. Tevens dragen luchtverontreiniging (onder andere passief roken) en luchtweginfecties mogelijk ook bij aan COPD ontwikkeling.

Longfunctie en COPD
Bij patiënten met COPD is de longfunctie verminderd. Daarom is het noodzakelijk om deze longfunctie te meten voordat de diagnose gesteld kan worden. Ten gevolge van luchtwegvernauwing, ook wel obstructie genoemd, ontstaat bij de uitademing een toegenomen weerstand tegen de luchtstroom. De uitademing

kost hierdoor meer moeite, terwijl dit bij gezonde mensen een passief proces is. De ernst van de luchtwegobstructie kan gemeten worden door het volume lucht te meten dat een patiënt in 1 seconde kan uitademen, de zogenaamde 1-secondewaarde (FEV_1). Hoe lager deze waarde is, hoe ernstiger de ziekte is. Als de luchtwegobstructie ernstig is, lukt het de patiënt niet meer om alle lucht uit te blazen voordat een nieuwe inademing begint. Hierdoor wordt de hoeveelheid lucht, die in de longen achterblijft, steeds groter. Dit noemt men hyperinflatie en kan bepaald worden met een bodybox meting.
Bij toenemend verlies van longweefsel en daardoor destructie van longblaasjes neemt de capaciteit van gastransport (zuurstof en koolstofdioxide) af. Een diffusiecapaciteit meting geeft weer in welke mate gastransport kan plaatsvinden en zegt daarmee indirect iets over de mate van weefselverlies.
Daarnaast kan de aanwezigheid van hyperreactiviteit gemeten worden door een provocatietest. Hierbij wordt een prikkelende stof ingeademd die luchtwegvernauwing kan veroorzaken bij gevoelige luchtwegen.
Ook de functie van de kleine luchtwegen kan gemeten worden met longfunctie tests. De stikstof uitwascurve (single breath nitrogen test) is een dergelijke test, waarbij men het "closing volume" en de "stikstof helling" meet. Het "closing volume" is het uitademingsvolume waarbij kleine luchtwegen dichtklappen. De stikstof helling geeft de mate van ongelijkmatige distributie van luchtstroom in de long weer. Bij toegenomen luchtwegontsteking en bij emfyseem klappen kleine luchtwegen eerder dicht en is er meer ongelijkmatige distributie van luchtstroom.

Luchtwegontsteking en veranderde anatomie bij COPD
Het langdurig inademen van sigarettenrook, wat duizenden schadelijke stoffen bevat, heeft een irriterend effect op de luchtwegen. De cellaag die normaal de luchtwegen bekleed (trilhaardragend epitheel) is slecht bestand tegen de schadelijke stoffen en wordt vervangen door een epitheel bestaande uit meerdere cellagen plaveiselcelepitheel, vergelijkbaar met de bekleding van wang of slokdarm (squameuze metaplasie). Echter dit epitheel heeft geen trilharen waardoor slijm niet meer naar de mond wordt getransporteerd. Daarbij is er een toename van slijmproducerende cellen (goblet cellen) en klieren, waardoor er meer sputum productie is. Dit stilstaande slijm in de luchtwegen vormt een risico voor het ontstaan van infecties. De tabaksrook veroorzaakt een chronische ontsteking van het slijmvlies in de luchtwegen. Deze ontsteking bestaat voornamelijk uit neutrofiele granulocyten, macrofagen en $CD8^+$ T-lymfocyten. De ontstekingscellen worden geactiveerd en scheiden een scala aan eiwitten uit, die deels schadelijk zijn voor het longweefsel. Hierdoor verandert de structuur van de luchtwegen met als gevolg een verdikte luchtwegwand, terwijl de longblaasjes worden afgebroken. Deze pro-

cessen worden voornamelijk in de kleine luchtwegen gezien en spelen een rol bij het ontstaan van de verminderde longfunctie in COPD.

Behandeling van COPD

Stoppen met roken is het uitgangspunt van de behandeling en het voorkomen van COPD. Stoppen met roken heeft een gunstig effect op klachten, de achteruitgang in longfunctie en de overleving van patiënten met COPD. Tot nu toe is er geen behandeling met medicijnen die de ziekte kan genezen. Wel zijn er medicijnen die ingeademd worden (inhalatie medicatie) die gunstige effecten hebben: luchtwegverwijders en inhalatiecorticosteroïden (ontstekingsremmers). Luchtwegverwijders bewerkstelligen ontspanning van de kleine spiertjes rondom de luchtwegen en dragen zo bij aan een verbetering van klachten en kwaliteit van leven. Echter dit gaat niet gepaard met een verbetering in achteruitgang van longfunctie. Inhalatiecorticosteroïden lijken vooral een gunstig effect te hebben op het aantal exacerbaties dat een patiënt doormaakt; echter het effect op de versnelde achteruitgang in longfunctie is niet geheel duidelijk. Enkele grote studies uit de jaren 90 laten zien dat inhalatiecorticosteroïden wel een initiële verbetering van longfunctie kunnen geven, maar de versnelde achteruitgang in longfunctie op lange termijn niet kunnen voorkomen; een recente grote studie laat dat echter wel zien. COPD exacerbaties worden behandeld met prednison tabletten of per infuus, met of zonder antibiotica. Indien er tevens sprake is van zuurstoftekort kan zuurstof toegediend worden. Het is nog onduidelijk wat de invloed is van stoppen met roken en de langdurige behandeling met luchtwegverwijders en inhalatiecorticosteroïden op de veranderingen in de luchtwegen en de chronische ontsteking.

Heterogeniteit

COPD is een heterogene ziekte met betrekking tot klinische presentatie, longfunctie afwijkingen, pathologische veranderingen die in de longen worden gezien en reactie op behandeling. Daaruit blijkt dat waarschijnlijk verschillende mechanismen een rol spelen in de ontwikkeling, klinische presentatie en het beloop van COPD. Deze heterogeniteit biedt mogelijkheden voor gerichter behandeling van subgroepen van COPD patiënten.

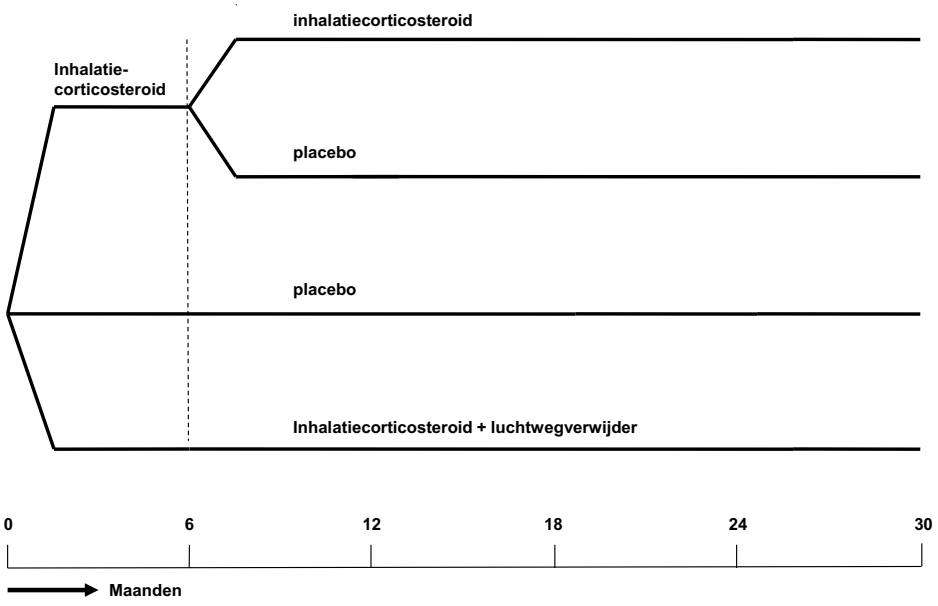
Dit proefschrift

Dit proefschrift gaat over de luchtwegontsteking bij patiënten met COPD. Hierbij is op verschillende manieren naar ontsteking gekeken.
- Rol van luchtwegontsteking bij verminderde longfunctie
- Stoppen met roken en luchtwegontsteking
- Behandeling en luchtwegontsteking

De GLUCOLD studie
De GLUCOLD studie (GLUCOLD = Groningen Leiden Universities Corticosteroids in Obstructive Lung disease) is een gezamenlijk project van de universitair medische centra van Leiden en Groningen. Alle onderzoeken beschreven in dit proefschrift betreffen data die afkomstig zijn van deze studie. De belangrijkste vraagstellingen van de GLUCOLD studie zijn:
- Wat is het effect van langdurig behandelen met inhalatiecorticosteroïden op de longfunctie en luchtwegontsteking?
- Maakt het uit of COPD patiënten kortdurend (6 maanden) of langdurig (2,5 jaar) behandeld worden met inhalatiecorticosteroïden?
- Heeft het stoppen van behandeling met inhalatiecorticosteroïden nadelige effecten op klachten, longfunctie en luchtwegontsteking?
- Heeft het toevoegen van lang werkende luchtwegverwijders aan inhalatiecorticosteroïden (combinatietherapie) additionele gunstige effecten?

Voor de GLUCOLD studie werd een groot aantal vrijwilligers (114 in totaal) gerekruteerd met matig tot ernstig COPD. Deelnemers werden bij aanvang van de studie uitgebreid onderzocht, waarbij gegevens verzameld werden met betrekking tot: luchtwegklachten, kwaliteit van leven, longfunctie, hyperreactiviteit en luchtwegontsteking. Dit laatste werd gemeten door de verschillende ontstekingscellen te tellen in opgegeven sputum, in longspoeling (lavage) en in kleine stukjes weefsel afkomstig uit de luchtwegwand (biopt). Lavage en biopt kunnen uitsluitend worden verkregen door het verrichten van een kijkonderzoek van de luchtwegen (bronchoscopie), wat een veilig maar belastend onderzoek is voor de patiënt. Vervolgens werden de deelnemers door loting ingedeeld in een van de vier behandelgroepen (figuur 1). Zowel de deelnemers als de onderzoekers waren niet op de hoogte van de behandelgroep waartoe deelnemers behoorden (dubbelblind). Patiënten werden geïnstrueerd de studiemedicatie tweemaal per dag te gebruiken gedurende 2,5 jaar en bezochten elke drie maanden de polikliniek longziekten. Tijdens deze driemaandelijkse bezoeken werd de longfunctie gemeten, vragenlijsten ingevuld over klachten en kwaliteit van leven. Na 6 maanden behandeling en aan

Figuur 1. De vier behandelgroepen.



het einde van de studie werden alle metingen die voor aanvang van de behandeling verricht werden herhaald.
Door het grote aantal patiënten met COPD waarbij zeer veel informatie werd verzameld, uiteenlopend van kwaliteit van leven, longfunctie tot ontsteking in biopten, kon ook veel geleerd worden over de ziekte op zich.

Rol van luchtwegontsteking bij verminderde longfunctie

Hoofdstuk 2: Dissociatie tussen luchtwegobstructie en luchtwegontsteking in COPD
COPD wordt gekenmerkt door verminderde longfunctie en luchtwegontsteking. COPD is een heterogeen ziektebeeld met betrekking tot klinische presentatie, longfunctie afwijkingen, pathologische veranderingen die in de longen worden gezien en reactie op behandeling. Daaruit blijkt dat waarschijnlijk verschillende mechanismen een rol spelen in de ontwikkeling, presentatie en het beloop van COPD. Eerdere onderzoeken lieten zien dat COPD patiënten met een slechtere longfunctie ook meer ontstekingscellen in de luchtwegen hebben. Dat suggereert dat ontsteking een rol speelt in de ontwikkeling van COPD. In dit hoofdstuk hebben we onderzocht of luchtwegobstructie, hyperinflatie, diffusiecapaciteit, hyper-

reactiviteit, de mate van reversibiliteit van luchtwegobstructie en verschillende onderdelen van luchtwegontsteking, overlappende of onafhankelijke dimensies zijn in het ontstaan van COPD. Dit werd onderzocht door middel van een factor analyse op deze verschillende parameters. Factor analyse is een statistische analyse die verschillende ziektekenmerken groepeert in enkele onafhankelijke groepen (factoren genoemd) van onderling geassocieerde ziektekenmerken. Een dergelijke factor analyse resulteerde in 4 verschillende factoren (groepen geassocieerde ziektekenmerken).
- Factor 1: luchtwegobstructie en hyperinflatie
- Factor 2: reversibiliteit, hyperreactiviteit, diffusie capaciteit en IgE in bloed
- Factor 3: uitgeademd NO (ontstekingskenmerk in uitgeademde lucht)
- Factor 4: ontstekingscellen in sputum

De parameters in de eerste factor passen bij luchtwegobstructie. De tweede factor omvat parameters die vaak bij astma patiënten gezien worden. De derde en vierde factor omvatten ontstekingskenmerken. Hieruit werd geconcludeerd dat luchtwegobstructie en luchtwegontsteking en kenmerken passend bij astma verschillende onafhankelijke kenmerken van COPD zijn en daarmee aanvullende informatie geven. Dit suggereert dat deze verschillende factoren meegenomen dienen te worden in de evaluatie van COPD patiënten en in de beoordeling van behandeleffecten.

Hoofdstuk 3: Kleine luchtweg functie en luchtwegontsteking in COPD
De luchtwegveranderingen die optreden bij COPD patiënten en tot verminderde longfunctie leiden, spelen zich vooral af in de kleine luchtwegen en in de longblaasjes. Deze veranderingen kunnen alleen bestudeerd worden in longweefsel wat doormiddel van een operatie verwijderd is. De stikstof uitwascurve is een longfunctie meting die de functie van de kleine luchtwegen kan meten. Eerder onderzoek heeft laten zien dat hoe meer veranderingen van de kleine luchtwegen en longblaasjes in operatiemateriaal van COPD patiënten gezien worden, hoe slechter de functie van kleine luchtwegen gemeten met de stikstof uitwascurve. Deze longfunctie test zegt dus iets over wat er in de periferie van de long gebeurd. In dit hoofdstuk hebben we onderzocht in welke mate de ontstekingscellen gemeten in sputum, lavage en biopten uit grote luchtwegen gerelateerd is aan de kleine luchtwegfunctie gemeten met de stikstof uitwascurve. De belangrijkste uitkomsten van dit onderzoek zijn:
- COPD patiënten met meer ongelijkmatige distributie van luchtstroom (stikstof helling) hadden meer ontsteking met neutrofiele granulocyten in biopten en meer geactiveerde neutrofiele granulocyten in lavage.
- Hoe groter het uitademingsvolume waarbij kleine luchtwegen samenvallen (clo-

sing volume) in COPD patiënten, hoe meer ontsteking met neutrofiele granulocyten in lavage.
- Kleine luchtwegfunctie was niet gerelateerd aan andere ontstekingscellen in sputum, biopten en lavage.

Deze resultaten suggereren dat de neutrofiele ontsteking in de luchtwegwand en in de longblaasjes een rol speelt bij de ontwikkeling van slechtere kleine luchtwegfunctie bij patiënten met COPD. Deze longfunctie test is een eenvoudige en niet belastende manier om de ontsteking en veranderingen in kleine luchtwegen en longblaasjes indirect te evalueren bij COPD patiënten.

Stoppen met roken en luchtwegontsteking

Hoofdstuk 4: Duur van stoppen met roken beïnvloedt luchtwegontsteking in COPD
Stoppen met roken heeft gunstige effecten op symptomen en vermindert de achteruitgang in longfunctie bij COPD patiënten. Deze gunstige effecten treden al binnen 1 jaar na stoppen op. Het is nog onduidelijk wat het mechanisme hierachter is, en het is mogelijk dat een vermindering in luchtwegontsteking na stoppen een rol speelt. Recente onderzoeken hebben laten zien dat ex-rokers evenveel luchtwegontsteking hebben als rokers. Echter hierbij is niet onderzocht of de duur van stoppen met roken een rol speelt en hoe eventueel de aard van de ontsteking verandert. In dit hoofdstuk hebben we het aantal ontstekingscellen in sputum en biopten onderzocht van rokers en ex-rokers met COPD (ex-rokers waren minstens 1 maand gestopt). Daarbij hebben we onderzocht of er verschillen in ontsteking waren tussen patiënten die kort (<3.5 jaar) of lang (≥3.5 jaar) gestopt waren met roken. De belangrijkste resultaten van dit onderzoek zijn:
- Patiënten die langer gestopt zijn met roken hebben lagere aantallen $CD8^+$ T-lymfocyten in biopten dan zowel rokers als patiënten die kort gestopt zijn. Deze groep heeft ook meer plasma cellen dan rokers.
- Ex-rokers hebben meer T-lymfocyten en plasma cellen in luchtweg biopten dan rokers met COPD.
- Andere ontstekingscellen en aantal ontstekingscellen in sputum zijn niet verschillend tussen de 3 groepen.

Uit deze resultaten blijkt dat de duur van stoppen met roken het ontstekingsprofiel van T-lymfocyten en plasma cellen beïnvloedt in COPD patiënten. Daarnaast blijkt dat alle andere ontstekingscellen verhoogd aanwezig blijven na stoppen. Dit geeft aan dat de gunstige effecten van stoppen met roken op symptomen en longfunctie niet simpelweg verklaard kunnen worden door een vermindering in

luchtwegontsteking. Tevens lijkt er een andere prikkel dan roken te zijn die het ontstekingsproces in stand houdt; gedacht kan worden aan virussen of bacteriën, auto-immuun prikkels of een afwijkend proces van weefselherstel na longschade.

Hoofdstuk 5: Herstel van veranderingen in epitheelcellen na stoppen met roken in COPD
Bij patiënten met COPD worden veranderingen in de bekledende cellaag van de luchtwegen (epitheelcellen) gezien. Deze veranderingen zijn: toename van slijmproducerende cellen, verandering van de epitheellaag in plaveiselcelepitheel (metaplasie) en toegenomen proliferatie van epitheelcellen. In navolging van het vorige hoofdstuk, werd onderzocht of de veranderingen aan de epitheellaag herstellen na stoppen met roken en of dit afhankelijk is van de duur van stoppen. De bevindingen van dit onderzoek zijn:
- Ex-rokers met COPD hebben minder metaplasie en proliferatie van epitheelcellen dan rokers. Er was tevens een trend voor minder slijmproducerende cellen in ex-rokers, maar deze was niet statistisch significant.
- Er is geen verschil in epitheel kenmerken tussen rokers en COPD patiënten die kort gestopt zijn met roken.
- Bij patiënten met COPD die lang gestopt zijn worden minder slijmproducerende cellen gezien dan bij zowel rokers als patiënten die kort gestopt zijn, en minder proliferatie van epitheelcellen dan rokers.

Deze resultaten laten zien dat de veranderingen van de epitheelcellaag reversibel zijn na stoppen met roken. Echter de afname in proliferatie, metaplasie en slijmproducerende cellen is pas na langdurig stoppen zichtbaar (>3.5 jaar). Dit herstel van de epitheelcellaag zou kunnen bijdragen aan de gunstige effecten van stoppen met roken op symptomen en longfunctie door minder slijm productie en stase van slijm in de luchtwegen. Dat zou mogelijk kunnen leiden tot minder luchtweginfecties en exacerbaties.

Behandeling en luchtwegontsteking

Hoofdstuk 6: Klinische en ontstekingsremmende effecten van inhalatiecorticosteroïden met of zonder langwerkende luchtwegverwijders bij COPD
Eerder onderzoek heeft aangetoond dat inhalatiecorticosteroïden een gunstig effect hebben op klachten en het aantal exacerbaties bij COPD patiënten. Daarnaast is er een verbetering in longfunctie in de eerste maanden na starten van de behandeling, maar de meeste studies laten geen effect op longfunctie achteruitgang zien op lange termijn. In dit hoofdstuk hebben we onderzocht wat het effect is van langdurig (2,5 jaar) versus kortdurend (6 maanden) behandelen met inha-

latiecorticosteroïden op de longfunctie en luchtwegontsteking. Daarnaast hebben we onderzocht wat de additionele effecten van een langwerkende luchtwegverwijder zijn. De belangrijkste resultaten van dit onderzoek zijn:

- Langdurig behandelen met inhalatiecorticosteroïden vermindert de achteruitgang in longfunctie, hyperreactiviteit en kortademigheidklachten van COPD patiënten.
- Deze gunstige klinische effecten gaan gepaard met een vermindering in ontstekingscellen in de biopten (lymfocyten en mestcellen) en in sputum (lymfocyten, neutrofielen, macrofagen) en een toename in intact epitheel in biopten.
- Stoppen met inhalaticorticosteroiden na 6 maanden behandeling doet de achteruitgang in longfunctie, hyperreactiviteit en kortademigheidklachten weer toenemen. Daarnaast treedt er een toename van lymfocyten, plasmacellen en mestcellen op in de biopten na stoppen.
- Toevoegen van een langwerkende luchtwegverwijder aan inhalatiecorticosteroïden geeft een initiële verbetering in longfunctie en kortademigheidklachten, maar heeft geen additioneel effect op achteruitgang in longfunctie op lange termijn. Zo'n combinatie behandeling heeft geen additionele ontstekingsremmende werking in de luchtwegen.

Deze resultaten suggereren dat progressie van luchtwegobstructie in COPD vertraagd kan worden door behandeling met inhalatiecorticosteroïden. Daarnaast hebben inhalatiecorticosteroïden een ontstekingsremmende werking in de luchtwegen. Echter, het verschil in respons op behandeling en de karakteristieken van de onderzochte patiënten met andere onderzoeken suggereert dat binnen de heterogene groep van COPD patiënten er een subgroep bestaat die meer baat heeft bij inhalatiecorticosteroïden.

Perspectief voor toekomstig onderzoek

De onderzoeken in dit proefschrift hebben bijgedragen aan het inzicht in de rol van luchtwegontsteking in ontstaan en behandeling van COPD. Uiteraard roepen deze nieuwe bevindingen nieuwe vragen op.
Wat is het natuurlijk beloop van luchtwegontsteking in biopten op lange termijn in COPD patiënten?
Wat is het effect van stoppen met roken en van behandeling met inhalatiecorticosteroïden op andere pathologische kenmerken (zoals activiteit van ontstekingscellen, bindweefselvorming en spiercellen) in de luchtwegen van COPD patiënten?
Zijn er kenmerken te identificeren in patiënten die voorspellend zijn voor een gunstig effect van behandeling met inhalatiecorticosteroïden?
Zijn er nieuwe behandelingen voor COPD te ontwikkelen die progressie van de ziekte kunnen stoppen en mortaliteit aan COPD kunnen verminderen?

Curriculum vitae

The author of this thesis was born on August 20th, 1974 in Wilrijk, Belgium. In 1992, she graduated from secondary school at the Koninklijk Atheneum Tervuren, Belgium, and started medical school at the University of Leiden in the same year. In 1996 she started a research project of nine months at the Department of Pulmonology of the Leiden University Medical Center supervised by Dr. T.J.N. Hiltermann, Dr. J. Stolk and Prof. Dr. P.S. Hiemstra. Subsequently, in 1997 she performed a three months research project at the Respiratory Medicine Department, Rayne Laboratory, of the University of Edinburgh, supervised by Dr. I. Rahman and Prof. Dr. W. MacNee. In August 1999, following her medical training period, she obtained her medical degree and began working as a research fellow at the Department of Pulmonology of the Leiden University Medical Center. Her research project 'Modification of disease outcome in COPD. Intermittent versus continuous treatment with inhaled corticosteroids, either or not combined with a long-acting β_2-agonist', based on grants from the Netherlands Organization for Scientific Research (NWO), Netherlands Asthma Foundation (NAF), GlaxoSmithKline (GSK, NL), Leiden University Medical Center (LUMC), Groningen University (RUG), was supervised by Prof. Dr. P.J. Sterk, Prof. Dr. D.S. Postma, Prof. Dr. P.S. Hiemstra and Prof. Dr. W. Timens. In February 2005 she started her clinical training at the department of Internal Medicine of the Kennemer Gasthuis hospital in Haarlem (head: Prof. Dr. R. ten Kate). In August 2007 she continued her clinical training to become a respiratory physician at the Department of Pulmonology of the Leiden University Medical Center (head: Prof. Dr. K.F. Rabe).

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