# Effects of inhaled corticosteroids on clinical and pathological outcomes in COPD - Insights from the GLUCOLD study

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# Effects of inhaled corticosteroids on clinical and pathological outcomes in COPD

Insights from the GLUCOLD study

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**Als we wisten wat we deden, heette het geen onderzoek**

*Albert Einstein (1879-1955)*

Voor mijn ouders

# Contents

# CHAPTER 1

# General introduction

# Introduction

Chronic Obstructive Pulmonary Disease (COPD) is a chronic disease that causes major morbidity and mortality around the world [1]. It is the currently the third leading cause of death worldwide and the fifth leading cause of morbidity [2, 3]. COPD is a preventable disease that is usually characterized by a progressive decrease in lung function associated with chronic lung inflammation as a consequence of exposure to inhaled noxious gases and particles, such as cigarette smoke. Patients with COPD suffer from chronic and progressive dyspnea on exercise and in advanced stages also dyspnea at rest, cough and sputum production. Currently, bronchodilators can relieve symptoms of dyspnea in many patients, but there is a continuing debate about the efficacy of anti-inflammatory medication. This thesis describes the results of studies on the clinical and pathological changes in the airways in COPD during use and after withdrawal of anti-inflammatory intervention.

## Classification of COPD

COPD is classified in severity stages by the Global Initiative for Chronic Obstructive Lung Disease (GOLD) [1]. From 2007, the severity COPD was based on the degree of airflow limitation obtained by spirometry (GOLD stage 1 to 4, Figure 1). A ratio of the forced expiratory volume in one second ($FEV_1$) and forced vital capacity (FVC) $\leq$ 0.70 after bronchodilator use and the level of $FEV_1$ are the main parameters used for the GOLD classification. Since 2011, the assessment of COPD is based on the combination of post-bronchodilator spirometric values, the annual number of exacerbations and the impact of the disease of the patient's health status, as measured by the mMRC (Modified Medical Research Council) and CAT (COPD Assessment Tool) [1], as presented in Figure 1. In the GLUCOLD study presented in this thesis, we also used measurements of health status not included in the assessment tool, such as the CCQ (Clinical COPD Questionnaire) and the St George Respiratory Questionaire (SGRQ). The combined COPD assessment tool was developed to predict future exacerbations and mortality. Some COPD patients do not fit perfect in one category and severity of the disease should be determined by the method with the highest score, thereby shifting the COPD severity distribution towards more severe categories [4]. In addition, both COPD GOLD classifications cannot adequately predict total mortality for an individual COPD patient [4]. This suggest that there is still room for improvement to optimize the COPD assessment tool.

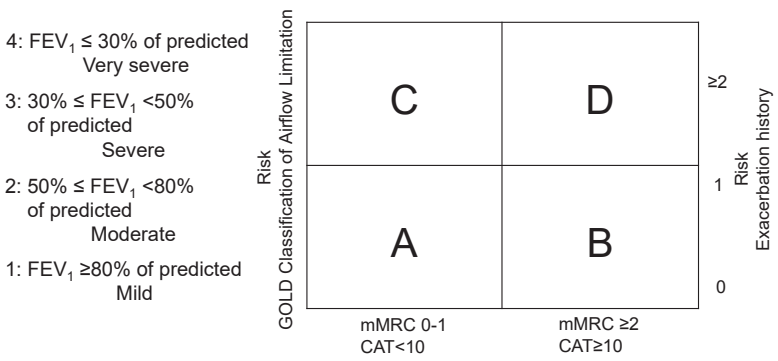


**Figure 1:** COPD assessment of severity. Adapted from Vestbo, et al. [1].

## Comorbidities

Comorbidity can be defined as two or more diseases which exist simultaneously in an individual, but have a different pathogenetic mechanisms; there is however no agreement on this definition [5]. Multimorbidity implicates that two or more diseases are simultaneously present which do not have a mutual connection [5]. When these definitions are applied to patients with all stages of COPD, they have one or more comorbidities (or multimorbidities) which have a major impact on quality of life and survival [6]. Smoking is not only a risk factor for COPD, but also for numerous other diseases, such as cardiovascular disorders [7], including ischemic heart disease, coronary artery disease and cardiac failure. Other comorbidities of COPD are metabolic syndrome, osteoporosis [8], skeletal muscle weakness, gastroesophageal reflux, obstructive sleep apnea, normocytic anemia and depression. However, for the majority of these diseases it is unclear whether these are a comorbidity or multimorbidity of COPD. Severe COPD patients have a 3-4 times increased risk to develop lung cancer compared to smokers with a normal lung function, fitting it to the definition of a comorbidity [9]. A recent study showed that after correction for smoking, no elevated risk was found between COPD and ischemic and hemorrhagic stroke, implicating that COPD and stroke are multimorbidities [10].

## Exacerbations

Exacerbations of respiratory symptoms are common in patients with COPD and can be triggered by bacterial and/or viral infections, air pollution and/or unknown factors [1].

During an exacerbation, COPD patients suffer from increased dyspnea, which in stable state already shows a day-to-day variation, coughing and sputum production. These features are accompanied by increased airway inflammation, severe hyperinflation of the (already hyperinflated) thorax and decreased expiratory flow [11]. Exacerbations are associated with a steeper decline in $FEV_1$ and an increased mortality, especially in those who are admitted to the hospital [12]. In-hospital mortality of hypercapnic patients is approximately 3-8%, and all-cause mortality during 3 years after hospital admission is 31-49% [13-15]. This shows that long-term mortality after admission for an exacerbation is higher than that following admission for myocardial infarction (30 day mortality 15%, 1-3 year mortality 9-24%) [16-18]. Therefore, it is highly relevant to early detect and treat exacerbations of COPD. Timely treatment is associated with faster recovery of symptoms, reduced number of future hospital admittances and improved quality of life [19]. Assessment and treatment of acute exacerbations of COPD is beyond the scope of this thesis.

## Epidemiology

The prevalence of COPD varies across countries and even within different populations [1], but is directly related to the cumulative exposure to cigarette smoke. For decades, COPD has been an underrecognized and underdiagnosed disease, especially in low income countries [20, 21]. However, even in industrialized countries, COPD is often underdiagnosed. This is evident from several large epidemiologic population-based and general practice-based studies, in which participants were screened for COPD. Among those who had COPD, 70% did not have a previous physician-recorded diagnosis of COPD that was confirmed by spirometry [22-24]. Approximately 4-10% of the general adult population worldwide will develop COPD [25]. However, among long-term smokers the prevalence of COPD can be as high as 47% [26]. The World Health Organization (WHO) estimates that worldwide 64 million people suffer from COPD, thereby it is the third leading cause of death worldwide [3]. Currently, COPD is already the third cause of death in the United States [2]. Moreover, hospitalizations contribute to the costs of and the burden to COPD patients. In the Netherlands over 350.000 people suffer from COPD and each year almost 50.000 new patients are added [27] and in 2014 almost 6000 patients had COPD as a primary cause of death [28], indicating that even in our country COPD is the fifth cause of death. Therefore, COPD is a major health problem around the world with substantial morbidity and mortality.

## Risk factors for COPD

Why do some smokers develop COPD, while others do not? COPD results from a tangled interplay between genetic susceptibility and exposure to environmental stimuli, of which cigarette smoking is the major risk factor [29]. Already in 1977, Fletcher and Peto showed that $FEV_1$ decline over time is much higher in susceptible smokers compared to non-smokers (Figure 2) [30]. Smoking cessation in a group of susceptible smokers can revert the $FEV_1$ decline to 'normal physiologic' deterioration [30, 31]. Although smoking is the most important risk factor in over 1 billion people globally, it is certainly not the only one. Inhalation of secondhand smoke, smoke from biomass fuel, occupational exposure to vapor, gases, dust and fumes and outdoor pollution have been particularly associated with the development of airflow limitation and chronic respiratory symptoms especially in low income countries in 3 billion people worldwide but also in the Netherlands [32, 33]. This suggests that exposure to biomass smoke is even a bigger risk factor for COPD in these countries than cigarette smoking, as 25-45% of the patients with COPD has never smoked. In addition, birth weight, maternal smoking, childhood asthma, atopy and (childhood) respiratory infections as well as previous tuberculosis are all associated with a reduced lung function [34, 35]. α1-Antitrypsin deficiency is a well-known genetic (and familial) cause and is present in 1-2% of patients with COPD [36]. Genome-wide association studies have shown several single nucleotide polymorphisms (SNPs) that are associated with an increased susceptibility for COPD, like 'hedgehog interacting protein' (*HHIP*) and 'family with sequence similarity 13, member A' (*FAM13A*, both on chromosome 4), 'advanced glycosylation end product-specific receptor' (*AGER*, on chromosome 6), 'bicaudal homolog 1' (*BICD1*, on chromosome 12), 'α-nicotinic acetylcholine receptor 3 and 5' (*CHRNA3* and *CHRNA5*) and 'iron-responsive element binding protein 2' (*IREB2*, on chromosome 15) and 'transforming growth factor-β1' (*TGF-β1*, on chromosome 19) [37-42]. Although the role of genetic polymorphisms in most of these genes in disease pathogenesis is incompletely understood, the gene products may clearly be linked to COPD pathogenesis.

## Heterogeneity of COPD

Traditionally COPD was clinically phenotyped as chronic bronchitis and emphysema, reflecting the 'blue bloater' and the 'pink puffer', respectively. However, COPD is a far more complex and heterogeneous disease than this division and can be classified on the basis of clinical, physiologic, molecular, cellular, pathologic and radiographic variables with effects modified by varied host susceptibility [43]. Clinical parameters that differentiate one COPD patient from the other are age, smoking, number of packyears, degree of dyspnea, exercise

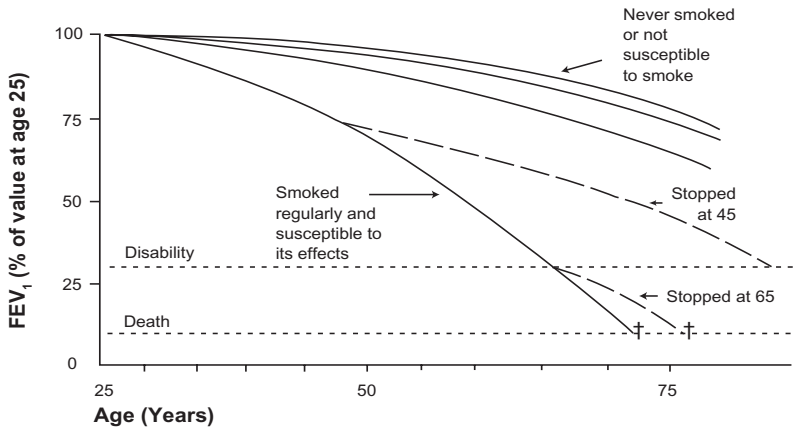


**Figure 2:** Effect of smoking cessation on lung function decline by Fletcher and Peto [30].

capacity, frequent exacerbations, poor quality of life, low body mass index (BMI) and symptoms of depression or anxiety [44, 45]. Physiological phenotypes in COPD are based on airflow limitation, rate of $FEV_1$ decline (rapid versus slow decliners), responsiveness to bronchodilators, airway hyperresponsiveness, low diffusion capacity (DLco), hyperinflation and pulmonary hypertension [46]. More extensive emphysema, increased airway wall thickness and an increased ratio of pulmonary artery and aortic diameter are associated with an increased rate of exacerbations [47, 48]. One study group found a relation between some parameters in COPD patients: one group with severe airflow limitation and many respiratory complaints; the second group had less severe airflow limitation; and the third group had mild airflow limitation, but had comorbidities such as obesity, cardiovascular diseases, diabetes and systemic inflammation [49]. These outcomes were related with more frequent hospitalizations due to COPD in the first group and a higher all-cause mortality, whereas the third group had more admissions due to cardiovascular diseases. To make the heterogeneity of COPD even more complex, some patients with COPD demonstrate typical features of asthma, including reversible airway obstruction, bronchial hyperresponsiveness, sputum eosinophilia, high IgE levels and atopy [50, 51]. This has recently been covered by the term asthma-COPD overlap syndrome (ACOS), although the exact definition of ACOS is still a matter of debate and this likely does not reflect a syndrome, but rather the heterogeneity of COPD that is also influenced by aging [52, 53]. These considerations indicate that the diagnosis 'COPD' alone is incomplete to cover the wide variety of phenotypic differences between patients, and that the adequate phenotyping as is being developed in asthma [54] is also needed for COPD and preferably should be done for chronic airways diseases collectively [55].

# Pathology of COPD

Chronic exposure to cigarette smoke induces pathological changes in the lungs of mouse models, such as an inflammatory response in the small and large airways, destruction of alveolar walls and structural changes of the airways [56, 57]. Although the pathological and functional changes in these animal models are not fully comparable to the human situation, chronic smoking does lead to similar changes in humans. First, due to smoking an inflammatory reaction occurs, as presented in Figure 3. In classically described 'chronic bronchitis', the airways, mucosa and submucosa are infiltrated with inflammatory cells (see below in the paragraph 'Inflammatory cells in innate immunity'), which contributes to airway obstruction [58]. Furthermore, enlarged bronchial mucus glands with increased mucus production in the bronchi and an increase in mucus-producing goblet cells in the surface epithelium can be found, as well as increased proliferative activity of the epithelial cells to form squamous cell metaplasia [59]. Both in the large and small airways peribronchiolar fibrosis can be found, contributing to irreversible airflow obstruction [58]. In emphysematous lungs, destruction of the alveolar walls results in permanent abnormal enlargement of air spaces [60] and the loss of elastic recoil additionally contributes to airflow obstruction. However, as COPD is a heterogeneous disease, often these pathological changes are (to some extent) present in every COPD patient. The structural changes in the airways, also called remodeling, are more extensively discussed below.

## Pathogenesis of COPD

Several mechanisms have been proposed to describe the pathogenesis of COPD. First, in response to tobacco smoke, an inadequate inflammatory response develops in the airways of smokers [63], of which neutrophils, $CD8^+$ T-cells and macrophages are the most predominant cells. Secondly, an imbalance between oxidants and antioxidants in the lungs of COPD patients results in oxidative stress, thereby amplifying airway inflammation and inducing cell death of e.g. epithelial and endothelial cells [64]. Thirdly, an imbalance between proteinases (e.g. elastase produced by neutrophils) and proteinases inhibitors (such as α1-antitrypsin) contributes to lung tissue changes and destruction. The combination of increased numbers and activation state of inflammatory cells in the lungs, increased production of chemokines and cytokines as well as an impaired response to infection, all are likely to contribute to the pathogenesis of COPD. The inflammatory cells of the innate and adaptive immune system implicated in COPD will be discussed more extensively below.

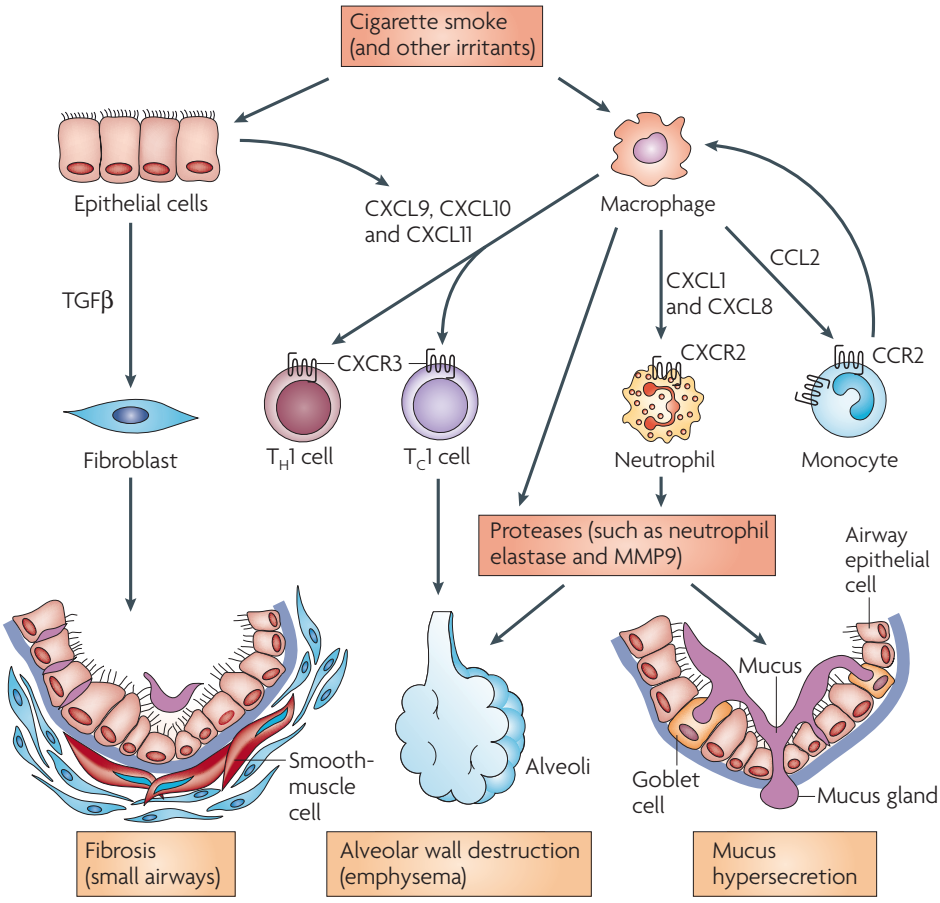


**Figure 3:** Pathological changes due to cigarette smoking in the airways in COPD. Reproduced from Barnes [61].

## Tobacco smoke

The lungs of a 60-year old person who has smoked one pack of cigarettes per day starting at age of 20 will have inhaled smoke generated from approximately 290.000 cigarettes, which equals 40 packyears [59]. The chemical composition of tobacco smoke consists of over 5000 chemicals, including alkanes, polycyclic aromatic hydrocarbons, sterols, alcohols, nitrils, acid, phenol(ic ethers), alkaloids (such as nicotine), carbohydrates, inorganics components, metals, tar, and others, some of them being radicals, oxidants and carcinogens [65], which makes it hard to define which component(s) lead(s) to smoking-related features of COPD

[66]. Tobacco smoke is an aerosol containing a mixture of both gaseous components and particles [67]. The particles have a diameter of approximately 0.1-0.8 μm, depending for instance on the puffing regimen of the smoker and presence in mainstream smoke (directly inhaled smoke) or sidestream smoke (smoke released from the burning tip of a cigarette) [68]. Each component has its own distribution between the gas and liquid phase. Exposure of the airways can take place in four different ways: 1) direct gas exposure; 2) evaporation of gas from the particle followed by exposure; 3) particle deposition, followed by evaporation from the particle; and 4) particle deposition followed by diffusion directly in the tissue [67]. As it is impossible to describe the effects of all separate components of cigarette smoke on the immune system, they are summarized by the term 'cigarette smoke' when discussed in the following paragraphs.

# Innate immunity in COPD

## The lung microbiome

The past opinion was that the lower airways are sterile [69]. However, with current techniques, such as 16S rRNA (ribosomal RNA) sequence analysis, it was discovered that micro-organisms from above the larynx can also be found in the lower respiratory tract [70]. This 'microbiome', which comprises (traces of) living and dead micro-organisms and their components, contributes to the development of the immune system, thereby creating a symbiosis. Every individual, even when healthy, has his/her own (lung) microbiome, which varies in number and diversity and may change during lifetime [71]. The microbiome composition can be altered in several ways, such as by anatomical, pathological (smoking) and physiological changes or by a defective immune system [72]. An imbalance in the microbiome may contribute to several diseases, such as COPD. A reduction in microbial diversity is found in COPD patients, with more abundance of pathogenic bacteria (e.g. *Haemophilus* species), whereas non-pathogenic *Bacteroides* and *Prevotella* species are reduced [70, 73, 74]. In addition, some micro-organisms have increased microbial pathogenicity [72]. This disruption of the local microbiome may result in an imbalance between immune regulation and immune responses in the lungs, resulting in tissue destruction, emphysema and airflow limitation in COPD [75].

## The innate immune response

To prevent invasion of microbes or noxious effects of inhaled substances, such as cigarette smoke, the innate immune system is protecting our lungs. Several mechanisms of innate immunity are present in the respiratory tract: mucociliary clearance, the epithelial barrier, humoral factors and several innate and adaptive immune and inflammatory cells [59, 63]. Cigarette smoke impairs host defense against infections by decreasing epithelial barrier function, ciliary function and antimicrobial peptide production [76].

The airways are lined by a pseudostratified epithelium that functions as a barrier, and consists of basal cells that act as progenitors, and a variety of luminal cell types, including ciliated cells and mucin producing goblet cells [77]. The cilia promote sputum containing trapped inhaled particles to be moved towards the throat, and this mucociliary clearance forms the first line of defense against inhaled microparticles. A considerable proportion of smokers with and without COPD have increased sputum production by mucus gland hypertrophy and an increase in the goblet cells of the surface epithelium, which is accompanied by a decrease in cilia. As a result of this impaired clearance, mucostasis occurs in the airway lumen.

In addition to this barrier function and role in mucociliary clearance, the airway epithelium is also involved in inflammation, immunity, host defense and tissue remodeling by producing a range of mediators. Due to long-term presence of noxious gases and particles, the epithelial cell layer of smokers with and without COPD is often characterized by squamous cell, goblet cell and basal cell hyper- or metaplasia [78]. Cigarette smoke affects epithelial cell function by various mechanisms, including direct oxidant activity, Toll-like receptor (TLR) signaling and may induce endoplasmic reticulum stress and an integrated stress response [79, 80]. In addition, the activity of adherent and tight junctions of epithelial cells, which normally form an impermeable barrier, is transiently decreased. This facilitates entering of macromolecules and microbes into the interstitium of the lung [81].

Antimicrobial peptides (AMP) are part of the innate immunity and are small peptides that have antimicrobial activity against bacteria, viruses and fungi [77]. Besides this, they are important players in e.g. inflammation, repair and regeneration. Defensins and cathelicidins (in humans there is only one cathelicidin: hCAP-18 or LL-37) are the most commonly studied AMP in humans. β-Defensins and cathelicidins are mainly expressed in airway epithelial cells, whereas α-defensins and cathelicidins are also produced by neutrophils, macrophages and other cells [82]. Although the exact role of β-defensins beyond their direct antimicrobial activity is unknown, they also attract immune cells and activate dendritic cells, thus playing a role in innate and adaptive host defense [83]. Cathelicidins act in angiogenesis, wound healing, cancer growth and regulate immune cells [84]. Smoking leads to an impaired

antimicrobial function of LL-37 and increased pro-inflammatory activity [85].

## Inflammatory response in innate immunity

When a microbe enters the airways, a cascade of innate immunity reactions starts. First, the microbe must be recognized, which is done by pathogen-associated molecular patterns (PAMPs). Several pattern recognition receptors (PRRs) contribute to microbial recognition, for instance membrane-bound Toll-like receptors (TLRs), cytosolic NOD-like receptors (NLRs) and RIG-I-like receptors (RLRs). These PRRs are expressed on various cell types, including alveolar macrophages, dendritic cells and epithelial cells [86]. PRRs are also activated by specific endogenous, often intracellular molecules, which are released after cell damage, called damage-associated molecular patterns (DAMPs). The recognition of PAMPs and DAMPs are crucial to start the inflammatory response and to help shaping the adaptive immune responses.

Cigarette smoke also leads to activation of several PRRs, both directly (effect of e.g. lipopolysaccharide [LPS] present in cigarette smoke) and indirectly (by damage on epithelial cells, which release DAMPs) [87]. Both the hydrophilic constituents as well as the tar fraction of cigarette smoke contribute to cell injury [88]. Activation of PRRs like TLRs and RAGE (receptor for advanced glycation end products) lead to increased expression of the pro-inflammatory cytokine pro-interleukin-1β [89, 90]. The contribution of this event to lung injury was demonstrated in a mice study with IL-1R knock-out mice, showing less pulmonary inflammation and protection against emphysema after exposure to cigarette smoke [91].

When the inflammatory response starts, several pro-inflammatory cytokines and chemokines such as tumor necrosis factor α (TNFα) or IL-8/CXCL8 are released from airway epithelial cells and alveolar macrophages. This leads to the recruitment of neutrophils and monocytes, [63], which explains why neutrophils and macrophages are present in higher numbers in the lungs of COPD patients compared to non-smokers. When activated, these cells release oxygen radicals and proteolytic enzymes such as neutrophil elastase and matrix metalloproteinases (MMPs) [92, 93]. Cigarette smoke and neutrophil elastase also stimulate the production and secretion of mucin, which also contribute to airway obstruction in COPD [94].

## Inflammatory cells in innate immunity

### *Macrophages*

Macrophages play an important role in the pathogenesis of COPD and are essential in innate immunity and host defense [95, 96]. The number of alveolar macrophages in lung parenchyma and bronchoalveolar lavage (BAL) of patients with COPD is increased up to 20-fold compared to healthy smokers and never-smokers [97, 98]. Increased numbers of macrophages have been found in small and large airways, lung parenchyma, BAL fluid and sputum of patients with COPD [99-103]. The number of macrophages in the airways is associated with the severity of COPD [58, 104]. After their activation by for instance cigarette smoke, macrophages release proteases, metalloproteinases (such as MMP-2, MMP-9, MMP-12) and IL-8/CXCL8, which all contribute to destruction of the alveoli and the development of emphysema (Figure 3) [61, 105, 106].

Diverse macrophage subpopulations are present throughout the lung, such as alveolar macrophages and tissue macrophages. These macrophages constitute a heterogeneous cell population with substantial plasticity both *in vivo* and *in vitro* [95]. *In vitro* cultured human monocytes exposed to e.g. granulocyte or macrophage colony-stimulating factors (GM-CSF and M-CSF, respectively) differentiate into MΦ1 and MΦ2 cells, respectively [107]. Due to environmental stimuli present in the lung, monocytes differentiate into a classical MΦ1 (pro-inflammatory) or an alternatively activated MΦ2 (anti-inflammatory) phenotype. MΦ1 cells are activated by exposure to TLR ligands and/or IFN-γ and are characterized by secretion of pro-inflammatory cytokines, such as IL-12, by promotion of Th1 response and by their antimicrobial activity. In contrast, MΦ2 cells promote tissue remodeling and phagocytic activity of apoptotic cells (efferocytosis), have a high expression of scavenger and mannose receptors, such as CD163 and produce anti-inflammatory cytokines, such as IL-10. However, the concept of this dichotomy in phenotypes is probably an oversimplification of the actual situation, which is even different between mice and men [108, 109]. It is plausible that *in vivo* macrophages with an intermediate phenotype exists [109]. A previous study showed that (small) MΦ1 macrophages, characterized by TNF-α and human leukocyte antigen (HLA)-DR were more abundant in induced sputum of COPD patients compared to large (possibly MΦ2) macrophages [110]. Another study investigating alveolar macrophage gene expression in COPD found that expression of genes related to MΦ1 macrophages in BAL is decreased and that of MΦ2 macrophages is increased in BAL of smokers with COPD compared to healthy smokers and non-smokers [111]. In this thesis, the subdivision between MΦ1 and MΦ2 is used (also called M1 and M2, respectively), which is an oversimplicifation of macrophage heterogeneity. This thesis further evaluates macrophage phenotypes and their distribution in the central and peripheral airways and the effects of smoking cessation and inhaled

corticosteroids (ICS) on these phenotypes in patients with COPD.

Macrophages play an important role in the defense against respiratory infections by bacteria such as (nontypeable) *Haemophilus influenza*, *Moraxella catarrhalis* and *Streptococcus pneumoniae*, which are all frequent pathogens in case of COPD exacerbations [112]. Although increased numbers of macrophages are present in the lung, the bacteria are not adequately phagocytosed. Studies have shown an impaired antimicrobial activity of alveolar macrophages in smoking and ex-smoking patients with COPD [113, 114]. The impaired phagocytic capacity of alveolar macrophages in smoking COPD patients is improved after smoking cessation [115] and after treatment with macrolide antibiotics, such as azithromycin [116].

Another way of improving phagocytic capacity of macrophages could be with (inhaled) glucocorticoids, which are frequently used as treatment in several airway diseases, including COPD. This has been confirmed in a study that showed that inhaled corticosteroids significantly increased efferocytosis in murine alveolar macrophages [117]. However, some studies suggest that these drugs are less effective in controlling pulmonary inflammation in COPD patients compared to other chronic inflammatory processes [118]. Steroids can reduce killing of pneumococci in *in vitro* cultured murine alveolar macrophages, partially by delaying phagolysosome acidification, without an effect on production of reactive oxygen or nitrogen species [119], supporting the observation that COPD patients treated with inhaled steroids have a slightly higher incidence of pneumonia [120]. Furthermore, there is a relative steroid resistance, which is associated with a decreased expression and activity of histone deacetylase 2 (HDAC2), a regulator of transcription during e.g. inflammation [121]. This reduced activity of HDAC2 is due to oxidative and nitrative stress, e.g. resulting from smoking, which combine to form peroxynitrite, resulting in inactivation and degradation of HDAC2, and thereby steroid insensitivity [122].

### *Neutrophils*

The number of neutrophils in sputum [123] and BAL fluid [100] of patients with COPD is increased, but with various results in the lung parenchyma and the airway wall [101, 124, 125], reflecting rapid passage of neutrophils through the lung tissue. A positive relation has been found between the number of sputum neutrophils and decline in lung function in patients with COPD [126], although other studies found that sputum neutrophils increased and bronchial neutrophils remained similar after smoking cessation in patients with COPD [127]. In addition, smoking cessation attenuated lung function decline in COPD [128].

These data suggest divergent roles for neutrophils in the development and progression of emphysema, which may be explained by the fact that neutrophils are not a homogeneous group of cells. Furthermore, it is unclear to which extent neutrophils contribute to disease development, or are mostly increased as a result of e.g. tissue injury. This argument also holds for various other cell types associated with COPD.

Although neutrophils are present in increased numbers in the airways in COPD, clearance of bacteria is compromised probably due to disordered host-pathogen interactions [129]. Neutrophils and other inflammatory cells can be recruited by several chemotactic factors, such as IL-8, leukotriene B4 (LTB4) and other chemoattractants, which can be found in the airways of COPD patients [100]. Activated neutrophils secrete several proteases, including neutrophil elastase (NE), cathepsin G, proteinase-3 and matrix metalloproteinases (MMP)-8 and 9. These proteases may contribute to the development of emphysema and stimulate mucus production [130, 131]. Cigarette smoke impairs efferocytosis of apoptotic neutrophils, which increase release of pro-inflammatory mediators, cytokines and chemokines from these neutrophils [129]. In addition, hypoxia in COPD prolongs survival of neutrophils *in vitro* [132], which impairs oxidase-dependent bacterial killing and promotes survival of bacteria [133]. It remains to be studied to what extent these described factors, as well as other cells of the immune system or remodeling of the airways in COPD contribute to the disordered function of neutrophils in COPD, compared to neutrophils of healthy smokers [129].

### *Eosinophils*

COPD patients can present with marked eosinophilia in sputum, BAL and in the airway wall [134], even after exclusion of patient with asthmatic features. Sputum eosinophilia in COPD has been associated with more severe airflow obstruction [135], bronchial hyperresponsiveness to methacholine and adenosine 5'-monophosphate (AMP) and a higher number of exacerbations [136, 137]. In COPD patients, an elevated sputum eosinophil count is predictive for a better response to ICS on post-bronchodilator lung function, but does not result in a reduction in sputum eosinophils [138]. In addition, sputum eosinophils can be used as management strategy to reduce COPD exacerbations, eosinophilic airway inflammation and symptoms [139]. Elevated blood eosinophils can be found during COPD exacerbations; those patients with higher blood eosinophils and treated with ICS have a larger reduction in the number of exacerbations compared to long-acting bronchodilators alone [140].

### *Mast cells*

Mast cells play an important role in the airways of asthmatics, especially those in the airway smooth muscle [141, 142]. Smokers have more mast cells in sputum compared to ex-smokers with COPD [143]. Mast cells can secrete TNF-α, proteases and IL-8 and could therefore contribute to the pathogenesis of COPD as well [144]. Like macrophages, mast cells also constitute a heterogeneous cell population, with tryptase- and chymase-positive cells. With progression of COPD, the number, density, morphology and distribution of mast cells change [145]. The most predominant and important mast cell location in the airways in COPD is yet unclear, as mast cells have been found in all lung compartments [146]. A positive correlation has been found between the number of tryptase- and chymase-positive mast cells and lung function in COPD [147].

## The cross-talk between innate and adaptive immunity

### *Dendritic cells*

Dendritic cells function as antigen presenting cells in the airways and alveoli that link the innate and adaptive immune system. These cells present an antigen in a major histocompatibility complex (MHC) class I and II to naive $CD8^+$ and $CD4^+$ T-cells, respectively [63]. Cigarette smoke attracts dendritic cells to the respiratory tract especially in patients with COPD compared to healthy smokers, and their local numbers in lung tissue are associated with severity of airflow limitation [148]. The role of adaptive immunity is discussed elsewhere in this introduction.

### *Innate lymphoid cells*

Other cells which are involved in lung inflammation are the innate lymphoid cells (ILC), which are lymphocytes without expression of classical T-cell surface markers. Currently three ILCs have been described, although it is unclear whether these cells are stable, heritable cell lineages or stages of activation or differentiation [149]. ILC1 include natural killer (NK) cells and secrete IFN-γ, ILC2 are activated by epithelium-derived alarmins, including IL-25 and IL-33 and release Th2 cytokines, such as IL-4, IL-5, IL-13, which are involved in allergic lung

inflammation; and ILC3 release IL-17 and IL-22 which is involved in allergic asthma, virus-induced lung disorders and airway remodeling in asthma [150, 151]. Especially ILC2s are involved in the cross-talk between innate and adaptive immune responses [151]. Recent studies have also suggested a role for ILC2s in the pathogenesis of COPD [152, 153], whereas the role of ILC1 and ILC3 is currently unclear. An accumulation of ILCs has been found in mouse lungs after a respiratory viral infection, and viral pathogens are thought to often contribute to acute exacerbations of COPD in humans [152]. Cigarette smoke decreases a specific receptor for IL-33 on ILC2s, but increased this receptor on NK cells. During infection, local IL-33 augmented type I pro-inflammatory responses via macrophages and NK cells [153].

## The adaptive immune response

### *T-Lymphocytes*

In the large and small airways as well as in the parenchyma in COPD, the cytotoxic $CD8^+$ T-lymphocyte is the most predominant type of T-cell [99, 154-157]. The number of $CD8^+$ cells is related to disease severity and airflow limitation in COPD [154, 158]. $CD8^+$ T-cells (and natural killer cells of the innate immune system) release pore-forming and proteolytic enzymes such as perforin and granzyme B after activation, which cause cell death of target cells [154, 159]. Granzyme B expression in $CD8^+$ T-cells and granulocytes is increased in small airways of patients with COPD, suggesting a possible role in the pathogenesis of COPD [160, 161].

$CD4^+$ T-cells are also important cells in the airways of smokers with COPD. Several subtypes of $CD4^+$ cells are present of which Th1, Th17 and regulatory T-cells (Treg) cells are the most well-studied [162, 163]. $CD4^+$ Th1 cells of COPD patients secrete more IFN-γ compared to healthy smokers, which promote attraction of other inflammatory cells to the lungs [164]. Th17 cells secrete IL-17A and IL-17F and mediate immunity against extracellular pathogens. They can induce epithelial cells to produce antimicrobial peptides, chemokines and growth factors (like GM-CSF), thereby regulating the inflammatory response [165]. IL-17A can also be secreted by macrophages, mast cells and epithelial cells [162]. Treg cells can regulate immune responses and suppress inflammation. Natural Treg have a main function in preventing auto-immunity, whereas adaptive or inducible Treg are mostly activated by exogenous antigens [166]. Smoking COPD patients have significantly fewer Treg cells, less

mRNA encoding the transcription factor FOXP3 (forkhead box P3) and less IL-10 secretion (both markers of Treg) in the lungs compared to healthy smokers and never smokers [167]. However, the exact role of Treg cells in COPD needs to be elucidated.

### *B-lymphocytes*

Increased numbers of B-cells, organized in lymphoid follicles, are found in both large and small airways and lung parenchyma during progression of COPD [58, 168-170]. The lymphoid follicles in COPD belong to inducible Bronchus-Associated Lymphoid Tissue (iBALT), which is an ectopic lymphoid tissue formed on infection and inflammation in mice and humans [171]. After contact with antigens from the airways, B-cell follicles initiate a local immune response and are maintained as memory cells in the lungs. One of the factors that regulate B-cells is B-cell activating factor (BAFF), which is present in healthy persons as well as in COPD and is associated with disease severity of COPD by promoting B-cell survival and lymphoid follicle expansion [172, 173]. When blocking the BAFF receptor in cigarette smoke-exposed mice, pulmonary inflammation and emphysema formation is attenuated [173]. The role of B-cells in the pathogenesis of COPD remains to be further clarified.

### *Is COPD an autoimmune disease?*

Both T-cells and most B-cells need antigen-presenting cells, such as dendritic cells, before they are primed with an antigen. An intriguing question is which antigens that drive COPD development and progression are important in the case of COPD. Possible candidate antigens could be microbial, cigarette smoke components or auto-antigens from e.g. the extracellular matrix [174-176]. Examples of microbial antigens are for example viral epitopes or peptides. By slight modifications of the epitopes, such as oxidation or citrullination, they are still presented by the MHC, but lead to ineffective response by the host cells and may trigger autoimmunity instead [177]. Second, cigarette and wood smoke can both induce carbonylation and citrullination of lung proteins, which may serve as auto-epitopes [178, 179]. In addition, an oligoclonal expansion of $CD4^+$ and $CD8^+$ T-cells has been found in the lungs after chronic cigarette exposure, which persists after smoking cessation [180]. Finally, breakdown products of elastin have been shown to induce proliferation and cytokine production by $CD4^+$ T-cells in lungs of COPD and the presence of anti-elastin antibodies [167, 181], although anti-elastin autoantibodies were not found in another study [182]. Furthermore, anti-elastin antibodies are present in diseases that are not accompanied by

COPD [183]. Therefore, the presence of an autoimmune response is insufficient to define COPD as an autoimmune disease as this autoimmune response may not be causative for the disease. In addition, also anti-nuclear autoantibodies [184], as well as antibodies against rheumatoid factor (RF) and heat shock protein 70 (HSP70), have been found in COPD [185].

## Remodeling and extracellular matrix

Remodeling takes place in the structural elements of the lungs, which are composed of several pillars, called together the extracellular matrix (ECM). This is a three dimensional structure, giving the lung its structural support and rigidity. The ECM is constantly changed by environmental stimuli, including mechanical forces, and is maintaining its functions such as cell adhesion, proliferation, cell trafficking, water balance and regulation of inflammatory mediators. Three major components of the ECM are collagens, proteoglycans and elastic fibers, which are produced by (myo)fibroblasts, epithelial cells and airway smooth muscle cells (Figure 4) [62, 186, 187]. The composition of the ECM in the lungs is different in healthy subjects versus COPD patients. Previous studies have shown that the airways of COPD patients contain less elastic fibers, reduced levels of the proteoglycan decorin but more of the proteoglycan versican and lower expression of collagen I and III is present [188-193]. This altered composition of the ECM, which is already present in mild and moderate COPD, contributes to the remodeling of the airways and lung parenchyma [59, 194].

Airway remodeling is a process that can result from repeated injury and repair and leads to structural changes in quantity, composition and organization of the airway structure. Remodeling is a process that has been linked to the presence of airflow limitation in COPD [59, 192, 195]. In COPD, it is mainly attributed to cigarette smoking, which induces structural changes due to chronic inflammation that occurs not only in the central, but also in the peripheral airways as well as the lung parenchyma. In both large and small airways of COPD patients, features of remodeling can be found. In the large airways epithelial metaplasia, goblet cell hyperplasia, mucus gland hypertrophy and thickening of the reticular basement membrane can be found [196]. In the small airways an increase in smooth muscle mass, angiogenesis, subepithelial collagen deposition and peribronchiolar fibrosis can be found, all contributing to reduced airway lumen and airflow limitation [58, 195, 197]. Emphysema is characterized by destruction of the parenchyma resulting in reduced recoil and loss of

alveolar integrity and emphysema. As a consequence gas exchange will be impaired, which can be measured as a lower diffusion capacity of carbon monoxide and ultimately hypoxemia.

The exact mechanism of airway remodeling has not been elucidated, but in addition to increased synthesis of matrix components, one of the possible mechanisms is the imbalance between an excess of matrix metalloproteinases (MMPs) and a shortage of tissue-inhibitor metalloproteinases (TIMPs), both produced by various cell types, including neutrophils, monocytes and macrophages [198]. The chronic inflammatory response in the airways causes chemotaxis of these cells. MMPs degrade collagens as well as proteoglycans [59, 199]. In addition, fragments of elastic fibers also have a chemotactic activity for monocytes and macrophages [200]. Fragments of proteoglycans such as versican, fibronectin and biglycan help to perpetuate inflammation through activation of Toll-like receptor (TLR)-2 and/or 4. Cytokines such as TNF-α, transforming growth factor-β (TGF-β) and interferon (IFN)-γ, secreted by several inflammatory cells can also up- or downregulate ECM molecules or MMPs [201], thereby influencing ECM turnover. This thesis evaluates the effect of smoking and ICS on extracellular matrix components in large airways of patients with COPD.

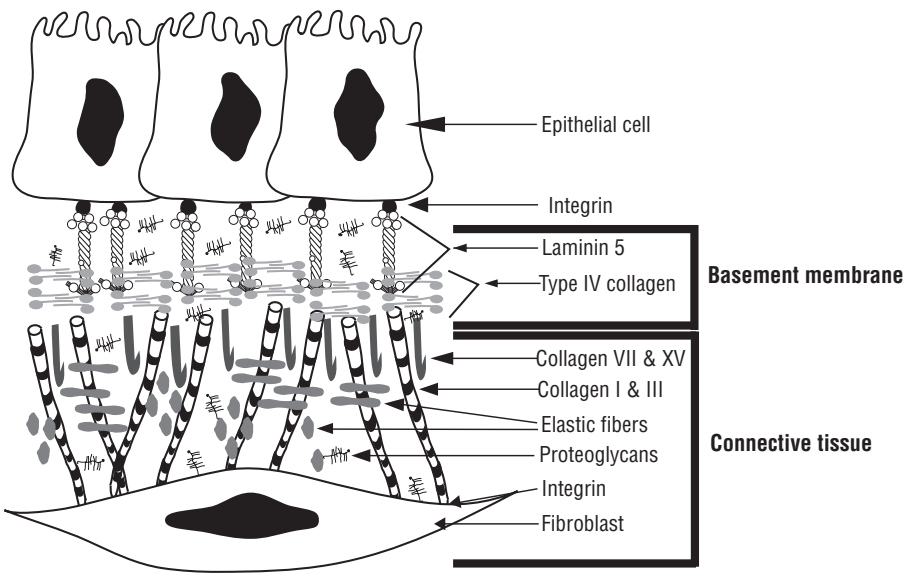


**Figure 4:** Schematic presentation of the airway wall and its extracellular matrix components. Reproduced from Dunsmore [186].

## Smoking cessation

Smoking cessation is the intervention with the greatest capacity to influence the natural course of COPD, which has already been shown in the 1970's (Figure 2) [30, 31]. Since nicotine is extremely addictive, smoking cessation should be supported by counseling combined with nicotine replacement therapy, varenicline (Champix) or bupropion (Zyban), which results in a cessation rate of roughly 50-60% after 3 months and 25-35% after 1 year [202, 203]. This clearly shows that the majority of people relapse. Therefore, active cigarette smoking should be seen as a chronic disease [204-206]. Smoking cessation has various beneficial effects, such as a slowing down of lung function decline [207], a decrease in respiratory symptoms and bronchial hyperresponsiveness [128] and increased survival [208].

In contrast, the effect of smoking cessation on airway inflammation in COPD is more difficult to interpret. Ex-smokers with COPD still have increased inflammatory cells after one-year smoking cessation, such as an increased percentage of sputum neutrophils and eosinophils, BAL percentage eosinophils and mucosal macrophages and eosinophils compared to healthy ex-smokers [209]. This may reflect a phase of repair. Some (cross-sectional) studies show a decrease in inflammation with increased levels of soluble TNF receptor-55 and -75 levels in sputum and blood in ex-smokers with mild to severe COPD [210, 211] and lower levels of neutrophil chemoattractant IL-8 in sputum [212]. These studies have been performed in small numbers of patients and some patients used steroids, which might have influenced the outcomes. Ex-smokers have lower mast cell numbers in the epithelium and lamina propria in bronchial biopsies [213]. Other studies show similar expression of pro-inflammatory markers (IL-8, MCP-1, TNFα, IL-1β, IL-2R and CCR2) and numbers of various inflammatory cells, like neutrophils, eosinophils, macrophages and lymphocytes in bronchial biopsies of current and ex-smokers with mild to severe COPD patients [214, 215]. With longer duration of smoking cessation (>3.5 years), bronchial $CD8^+$ T-cells decrease, plasma cells increase and bronchial epithelial remodeling diminishes in moderate to severe COPD patients [216, 217]. These data suggest that bronchial inflammation (at least partially) persists after smoking cessation, but may depend on the duration of smoking cessation. However, a expression of a number of genes, such as *SERPIND1*, remain altered even years after smoking cessation, although that of other genes reverses to normal [218]. Although the exact mechanism for the ongoing inflammatory response is unknown, there are suggestions for self-perpetuating innate immune responses, decreased resolution of inflammation, airway wall remodeling, impaired macrophage clearance, microbial colonization of the lung, oxidative stress, hypoxia, genetic susceptibility and epigenetic changes [92, 158]. This thesis further studies the effect of smoking on macrophage phenotypes in patients with COPD.

# Management of COPD

Currently, there is no pharmacological treatment that modifies the long-term decline in lung function in COPD [207, 219-221], although some studies suggest that ICS may have beneficial effects on lung function decline [222, 223]. The goal for treatment of stable COPD is therefore to reduce symptoms, improve exercise tolerance and health status and to prevent disease progression, exacerbations and mortality [1]. The current management of COPD can be divided into non-pharmacological and pharmacological treatment modalities, which are discussed below.

## Non-pharmacological treatment

Apart from smoking cessation, regular physical activity, pulmonary rehabilitation with attention for dietary status and influenza vaccination are also important non-pharmacological strategies [224]. Pulmonary rehabilitation followed by maintenance and regular physical activity, improves exercise tolerance as well as reduction of dyspnea and fatigue in patients with COPD [225]. Benefits of rehabilitation diminish after ending of the program, but a patients' health status remains better if exercise training is maintained [226, 227]. Yearly vaccination against influenza, but not pneumococcal vaccine, reduces the risk of hospitalization due to lower respiratory tract infections and mortality in patient with COPD [228, 229]. In case of hypoxemia ($pO_2$ <8.0kPa), long-term supplemental oxygen therapy should be considered, as this reduces mortality [230]. In a selected group of patients with severe emphysema in the apical parts of the lungs and severe impairment of exercise tolerance lung volume reduction surgery (LVRS) can be considered, which increases exercise tolerance by improving the elastic retraction of the lungs and reduction of hyperinflation. In addition, these patients have a better survival compared to patients treated with pulmonary rehabilitation [231, 232]. Newer techniques using endobronchial lung volume reduction (BLVR) with one-way valves and coils for patients with severe COPD are currently developed [233, 234].

## Pharmacological treatments

Preferentially, the pharmacological management of COPD patients should include

inhaled medications as this way of drug administration provides quick action mode in the lungs, requires a lower dose and therefore reduces the risk of (systemic) side effects [235]. The caveat of inhaled therapy in COPD is that some obstructed, peripheral regions of the tracheobronchial tree may not be accessible for inhaled aerosols. Pharmacological treatment is individualized, depending on the GOLD stage and/or combined COPD score (Table 1). The two main components of pharmacological treatment are bronchodilators and anti-inflammatory therapy: bronchodilators are given to relief complaints of dyspnea, anti-inflammatory medications decrease inflammation and aim to achieve disease modification, by affecting the underlying pathophysiology of COPD.

**Table 1**: Treatment options per COPD GOLD stage [1].*

| Patient group | 1st treatment option | Alternative option | Other options** |
|---|---|---|---|
| **A** | SABA<br>or<br>SAMA | LABA<br>or<br>LAMA<br>or<br>SABA and SAMA | Theophylline |
| **B** | LABA<br>or<br>LAMA | LABA and LAMA | SABA and/or SAMA<br>Theophylline |
| **C** | ICS with LABA<br>or<br>LAMA | LABA and LAMA | SABA and/or SAMA<br>Theophylline |
| **D** | ICS with LABA<br>And/or<br>LAMA | ICS with LABA and LAMA<br>or<br>LABA and LAMA | N-acetylcysteine<br>SABA and/or SAMA<br>Theophylline |

SABA: short-acting β2-agonists; LABA: long-acting β2-agonists; SAMA: short-acting anticholinergics; LAMA: long-acting anticholinergics; ICS: inhaled corticosteroids.

* Medication is presented in alphabetical orders and not order of preference.

** Medication in this column is only used in combination with other 1st choice options or with alternative choice options.

## *Bronchodilators*

Bronchodilators are important therapeutic options in COPD as they improve expiratory flow by widening of the airways, thereby reducing dynamic hyperinflation at rest and

during exercise, and improving exercise tolerance [236]. Several variants of bronchodilators are currently available: short-acting β2-agonists (SABA), long-acting β2-agonists (LABA), short-acting anticholinergics (SAMA) and long-acting anticholinergics (LAMA), which can be used on an as-needed basis or a regular basis to prevent or reduce symptoms. LABA (e.g. salmeterol) have a better bronchodilating effect compared to SAMA (ipratropium), although no differences were found in dyspnea-scores, number of exacerbations, quality of life and exercise tolerance [237]. LAMA (tiotropium) treatment results in a higher lung function compared to LABA (salmeterol), but no differences were found in dyspnea-scores, number of exacerbations, quality of life and exercise tolerance [238, 239]. Compared to ipratropium, tiotropium reduces the risk of an exacerbation and improves quality of life [238]. Mortality is comparable between the different bronchodilators [240-242]. Very long-acting bronchodilators are currently available (both LABA and LAMA) and are applied in one inhaler (either LABA or LAMA alone, or combination). This combination of two very long-acting bronchodilators seems safe and effective [243, 244] and improves lung function even further compared to a separate LABA and LAMA [245]. However, results of studies regarding effect on exacerbations and quality of life are not yet available.

### *Inhaled corticosteroids (ICS)*

ICS belong to one of the most prescribed medications for respiratory diseases, with approximately 30-40% use by patients with respiratory complaints in the United Kingdom and the Netherlands [246]. Of these patients, 50% use ICS for several months and 20-40% use ICS for more than 1 year [247]. The mechanisms by which corticosteroids may improve lung function in patients with COPD remain poorly understood, but 3 mechanisms of action of ICS have been described. First, bronchodilation may be enhanced by up-regulation of β2-adrenergic receptors located in the airway walls and bronchial vessels, thereby potentiating its physiological effect [248]. It is known that in asthmatics fluticasone reduces bronchial blood flow within less than 2 hours after inhalation [249]. Second, the anti-exudative effects of ICS together with vasoconstriction of the bronchial circulation may reduce airway wall edema by the anti-exudative effects of ICS. Finally, ICS may reduce the release of inflammatory mediators and induce vasoconstriction of the pulmonary vasculature.

Many studies have evaluated the effect of ICS with and without LABA in COPD. Long-term ICS treatment (1 to 3 years) improves symptoms, quality of life and lung function (measured by $FEV_1$) and decreases the number of exacerbations by about 30% in patients with COPD GOLD 2-3 [220, 221, 223, 250-253]. The GLUCOLD study group (see below in paragraph 'Aims of the present study') has previously shown that 30-month treatment with inhaled

fluticasone propionate can attenuate decline in lung function [222]. However, meta-analyses (that did not include the (relatively small) GLUCOLD study) did not confirm this attenuated lung function decline [254, 255]. This suggests that efficacy of ICS depends on the cohorts investigated and thereby on COPD phenotype and/or severity of the patients in the study.

Till now only a few trials have evaluated the anti-inflammatory effect of ICS in the airways in COPD [256]. Short term treatment with ICS with or without LABA showed no significant change in mucosal $CD8^+$ cells, macrophages and neutrophils [257-259], while another study found a reduction in $CD4^+$ and $CD8^+$ cells in bronchial biopsies after treatment with fluticasone and salmeterol [260]. Furthermore, a meta-analysis showed that ICS may reduce sputum total cell counts, neutrophils and lymphocytes [261]. The GLUCOLD study group (see below in paragraph 'Aims of the present study') has previously shown that 30 months of treatment with inhaled fluticasone decreased the number of bronchial $CD3^+$, $CD4^+$ and $CD8^+$ cells and mast cells and reduced the sputum neutrophil, macrophage and lymphocyte counts compared to placebo [222]. Furthermore, treatment with ICS in the GLUCOLD study was accompanied by a change in airway gene expression profiles [262].

As with every treatment, the benefits of ICS should be balanced against its adverse events. During the use of ICS with or without LABA, COPD patients have an increased risk of developing pneumonia, especially with use of fluticasone [120, 263-265], although they have fewer exacerbations. Other reported side effects are oropharyngeal candidiasis and hoarseness. Some studies found a decreased bone mineral density and cataract [252, 266-268]; however as the systemic bioavailability is minimal, these findings are hard to interpret. Mortality risk in COPD patients of all stages is decreased after ICS treatment [269], although this was not found in other studies [223, 254]. Therefore, it is important to select the individual patient who will probably benefit most of ICS therapy [46]. ICS with or without LABA are currently recommended in COPD patients with GOLD 3-4 with many symptoms and more than two exacerbations per year [1, 270].

### *Withdrawal of ICS*

Compliance to inhaled treatment, such as ICS, varies widely in patients with COPD and is dependent on GOLD stage [271]. Previous studies indicate that discontinuation of ICS induces a relapse in lung function decline in moderate to very severe COPD patients without an effect on the number of exacerbations [272]. However, other studies found an increased frequency of exacerbations and a lower quality of life during follow-up [273-276].

Effects of withdrawal of ICS in COPD on inflammatory parameters have been studied even more scarcely. The GLUCOLD study showed that after withdrawal following a 6-month treatment period the number of $CD3^+$ cells, mast cells and plasma cells increased in bronchial biopsies, without an effect on sputum cell counts [222]. Others showed that the percentage of sputum neutrophils increased after 6 weeks of withdrawal of ICS [277], but this has not been confirmed in another study [275]. As ICS are frequently discontinued and only a few studies are available investigating the effects of withdrawal of ICS, this warrants careful monitoring of disease outcomes after withdrawal of ICS in COPD [278]. In this thesis the long-term effects of withdrawal of ICS is studied on lung function decline and inflammatory cells in sputum and bronchial biopsies after a previous prolonged treatment with ICS in patients with moderate to severe COPD.

## Aims of the present study

This thesis describes the cellular, pathological, and clinical changes during and after treatment with ICS in patients with COPD, with respect to the heterogeneity of the disease. Data from the GLUCOLD (Groningen and Leiden Universities Corticosteroids in Obstructive Lung Disease) study were used to analyze this.

### The GLUCOLD study

The GLUCOLD study is an investigator-initiated project (ClinicalTrials.gov registration number NCT00158847) and is a placebo-controlled, double-blind, randomized trial. The trial was initiated to study the effect of short- and long-term treatment with ICS with and without long-acting bronchodilators on lung function decline, airway inflammation and quality of life in patients with moderate to severe COPD. Patients were steroid naive, which implicates that they were not allowed to have used ICS 6 months prior to start of the study, to exclude unknown previous benefits of ICS and avoid selective drop-out in the placebo group. A total of 109 out of the 114 patients had never used ICS before enrollment in the study; only seven patients had ever received a short course of oral corticosteroids. Further in- and exclusion criteria are presented in Table 2. Lapperre et al. showed in the GLUCOLD study that ICS use decreased airway inflammation, attenuated lung function decline and improved quality of life in this group of COPD patients [222]. This thesis will continue to study airway inflammation, with a focus on macrophages and their heterogeneity, airway wall remodeling after ICS treatment and clinical and inflammatory parameters after discontinuation of ICS in

COPD.

**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, sputum production, frequent exacerbations or dyspnea on exertion |
| No course of oral steroids during the last 3 months, no maintenance treatment with inhaled or oral steroids during the last 6 months |
| Post-bronchodilator $FEV_1$/IVC radio below 90% confidence interval (CI) of the predicted $FEV_1$/IVC ratio and post-bronchodilator $FEV_1$ (after 400ug salbutamol) <90% of predicted value (90% CI) [279] |
| Post-bronchodilator $FEV_1$ >1.3liter and >20% of predicted value. |
| **Exclusion criteria** |
| Prior or concomitant history of asthma |
| α1-antitrypsin deficiency (SZ, ZZ, or zero phenotype) |
| Other active lung disease except for mild bronchiectasis |
| Contra-indications for elective bronchoscopy, such as oxygen saturation <90%, abnormal coagulability, anti-coagulant therapy which cannot be temporarily withheld during 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 |

During the first part of the study (GL1) patients were randomized to one out of four treatment options (Figure 5):

- 6-month inhaled fluticasone propionate 500µg twice daily, followed by 24-month placebo (F6)
- 30-month inhaled fluticasone propionate 500µg twice daily (F30)
- 30-month inhaled fluticasone propionate with salmeterol 500/50µg twice daily in a single inhaler (FS30)
- 30-month placebo

Patients visited the outpatient clinic in Leiden University Medical Center (LUMC) or University Medical Center Groningen (UMCG) every 3 months during which lung function and quality of life were recorded. During the second part of the GLUCOLD study (follow-up study, GL2), patients visited the department every year for 5 consecutive years, and

spirometry was performed. During the follow-up study, patients were treated by their own pulmonary physician or general practitioner according to the current guidelines [1]. Airway hyperresponsiveness was recorded at baseline, after 6 and 30 months (GL1) and after 2 and 5 years of follow-up (GL2). A bronchoscopy with bronchial biopsies was performed at baseline, after 6 and 30 months (GL1) and after 7.5 years (5 years of follow-up, GL2). 114 COPD patients started with the first part of the study, 85 patients started with GL2 and 61 patients completed 7.5 years of follow-up.

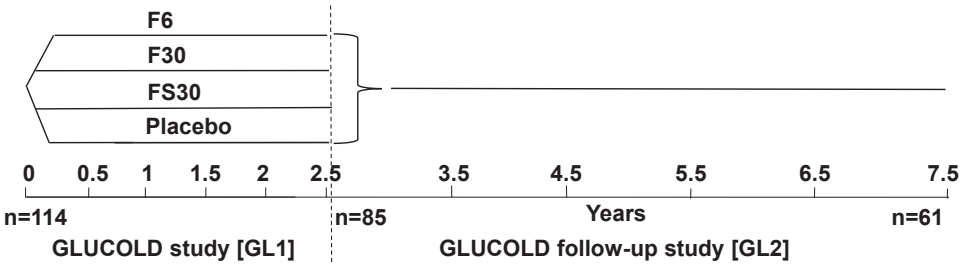


**Figure 5:** Study design during first (GL1) and second (follow-up, GL2) part of the GLUCOLD study.

# Research questions

*Relation between smoking, treatment and macrophage heterogeneity in COPD*

**Chapter 2: Does smoking cessation have an effect on macrophage heterogeneity in bronchoalveolar lavage (BAL) and sputum of patients with COPD?**

The anti-inflammatory marker CD163 for anti-inflammatory macrophages (MΦ2) was used on sputum and BAL of current and ex-smokers with COPD. In addition, we measured the pro-inflammatory mediators interleukin (IL)-6 and IL-8 and the anti-inflammatory mediators elafin, and Secretory Leukocyte Protease Inhibitor (SLPI) in BAL and sputum.

**Chapter 3: Do (inhaled) corticosteroids have an effect on macrophage polarization in *in vitro* cultured monocyte-derived macrophages as well as in serum and sputum of COPD patients?**

The novel pro-inflammatory MΦ1 marker YKL-40 was examined, and the effect of corticosteroids on secretion and expression of YKL-40 by *in vitro* cultured monocyte-derived macrophages was studied, and YKL-40 levels in serum and sputum of COPD patients were measured.

*Effect of treatment on airway remodeling in COPD*

**Chapter 4: What is the effect of smoking and long-term treatment with ICS on composition of extracellular matrix components in large airways of COPD?**

Percentage and density of stained area with collagen I and III, the proteoglycans versican and decorin and elastic fibers were quantified and compared between smokers and ex-smokers with COPD and the effect of 2.5 years of treatment with ICS on these markers was assessed.

*Effect of withdrawal of inhaled corticosteroids treatment on clinical and pathological outcomes in COPD*

**Chapter 5: What is the effect of 5-year discontinuation of ICS on lung function decline, airway hyperresponsiveness and quality of life after 2.5-year treatment with ICS in patients with moderate to severe COPD?**

Patients included in the randomized treatment during the first part of the GLUCOLD study (2.5 years, GL1) were followed for 5 consecutive years (GL2), and lung function and quality of life were measured annually. Airway hyperresponsiveness was measured after 2 and 5 years of follow-up. During the follow-up study, patients were treated by own physician according to the current guidelines.

**Chapter 6: What is the effect of 5-year withdrawal of ICS after previous 2.5-year treatment with ICS on the inflammatory cells in the large airways of patients with COPD?**

After 2 and 5 years of follow-up during GL2, a sputum induction was performed; after 5 years of follow-up bronchial biopsies were obtained. Outcomes were number of inflammatory cells in sputum and bronchial biopsies.

*General discussion*

Chapter 7: A summary is presented of the main results. Furthermore, implications of the findings presented in this thesis and suggestions for future research are discussed.

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# CHAPTER 2

# Smoking status and anti-inflammatory macrophages in bronchoalveolar lavage and induced sputum in COPD

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# ABSTRACT

**Background**: Macrophages have been implicated in the pathogenesis of COPD. M1 and M2 macrophages constitute subpopulations displaying pro- and anti-inflammatory properties. We hypothesized that smoking cessation affects macrophage heterogeneity in the lung of patients with COPD. Our aim was to study macrophage heterogeneity using the M2-marker CD163 and selected pro- and anti-inflammatory mediators in bronchoalveolar lavage (BAL) fluid and induced sputum from current smokers and ex-smokers with COPD.

**Methods**: 114 COPD patients (72 current smokers; 42 ex-smokers, median smoking cessation 3.5 years) were studied cross-sectionally and underwent sputum induction (M/F 99/15, age 62±8 [mean±SD] years, 42 (31-55) [median (range)] packyears, post-bronchodilator $FEV_1$ 63±9% predicted, no steroids past 6 months). BAL was collected from 71 patients. $CD163^+$ macrophages were quantified in BAL and sputum cytospins. Pro- and anti-inflammatory mediators were measured in BAL and sputum supernatants.

**Results**: Ex-smokers with COPD had a higher percentage, but lower number of $CD163^+$ macrophages in BAL than current smokers (83.5% and 68.0%, P=0.04; 5.6 and 20.1 $x10^4$/ml, P=0.001 respectively). The percentage $CD163^+$ M2 macrophages was higher in BAL compared to sputum (74.0% and 30.3%, P<0.001). BAL M-CSF levels were higher in smokers than ex-smokers (571pg/ml and 150pg/ml, P=0.001) and correlated with the number of $CD163^+$ BAL macrophages (Rs=0.38, P=0.003). No significant differences were found between smokers and ex-smokers in the levels of pro-inflammatory (IL-6 and IL-8), and anti-inflammatory (elafin, and Secretory Leukocyte Protease Inhibitor [SLPI]) mediators in BAL and sputum.

**Conclusions**: Our data suggest that smoking cessation partially changes the macrophage polarization *in vivo* in the periphery of the lung towards an anti-inflammatory phenotype, which is not accompanied by a decrease in inflammatory parameters.

# INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is characterized by progressive lung function decline and an abnormal inflammatory response in the airways, mainly caused by cigarette smoke [1]. The inflammation response in the small airways in COPD is characterized by the accumulation of macrophages, neutrophils, $CD8^+$-lymphocytes and B-cells and is associated with the severity of COPD [2, 3]. Smoking cessation is an effective treatment to reduce lung function decline [1]. Nevertheless, airway inflammation in bronchial biopsies, sputum and bronchoalveolar lavage (BAL) of COPD patients (predominantly) persists one year after smoking cessation [4-6]. We previously showed that the number of macrophages and neutrophils in bronchial biopsies are comparable in current and ex-smokers with COPD [7]. However, the effects of smoking on macrophage phenotypes in COPD are incompletely understood.

Macrophages play an important role in innate and adaptive immunity and form a heterogeneous population [8, 9]. Macrophages display polarized phenotypes by which they can be divided into subpopulations. Pro-inflammatory, or classically activated macrophages (M1) display pro-inflammatory and cytotoxic properties and can eradicate intracellular pathogens. In contrast, anti-inflammatory or alternatively activated macrophages (M2) display anti-inflammatory properties and are implicated in repair [8, 10]. Granulocyte-macrophage colony stimulating factor (GM-CSF) can generate M1 *in vitro* from human peripheral blood monocytes, and macrophage colony stimulating factor (M-CSF) can generate M2 [11]. M1 secrete pro-inflammatory cytokines, like IL-(interleukin)-12 and tumor necrosis factor (TNF)-α, have good antigen presenting capacity and promote Th1 immunity. In contrast, M2 secrete anti-inflammatory mediators, such as IL-10, show poor antigen presenting capacity and promote development of T-regulatory cells [11-13]. Alveolar macrophages show anti-inflammatory M2-characteristics [14-16], which can be distinguished from pro-inflammatory macrophages using M2 markers such as the scavenger receptor CD163 [17, 18]. Compared to M1 cells, M2 macrophages are highly phagocytic. The phagocytic capacity of alveolar macrophages is decreased in smoking COPD patients and improves with smoking cessation [19]. This suggests a phenotypic alteration and a role of macrophage heterogeneity in COPD, which has also been proposed in e.g. tumor progression [20], atherosclerosis [21] and renal diseases [22].

Although inflammation persists, smoking cessation shows positive clinical effects [1]. This

suggests that other mechanisms play a beneficial role, for instance regulation of macrophage polarization. We hypothesize that in moderate to severe COPD patients a) ex-smokers have more M2 and anti-inflammatory mediators in BAL and induced sputum compared to current smokers; b) M2 and anti-inflammatory mediators are relatively higher in the peripheral airways (as sampled by BAL) than in the central airways (as sampled by induced sputum).

# METHODS

## Subjects and study design

Patient characteristics and methods have been described previously [7, 23, 24]. In short, we studied 114 clinically stable moderate to severe COPD patients [GLUCOLD study (Groningen Leiden Universities Corticosteroids in Obstructive Lung Disease)] cross-sectionally. They were aged 45-75 years, smoked ≥10 packyears and were current or ex-smokers (quit ≥1 month). Patients diagnosed with asthma, α1-antitrypsin deficiency and those who used corticosteroids in the past six months were excluded; they were allowed to use short-acting bronchodilators. Approval of the medical ethics committees of both centers was obtained and all patients provided written informed consent [23]. Spirometry was performed according to international guidelines [25]. All patients underwent a bronchoscopy with BAL and a sputum induction on separate visits.

## Bronchoscopy, BAL and sputum induction

Fiberoptic bronchoscopy was performed in all patients and processed using a standardized protocol, as previously described [7, 24, 26, 27]. The BAL procedure was discontinued during the study due to ethical considerations, since four of 71 patients experienced a serious adverse event that was considered to be possibly related to the BAL procedure (pleural pain, fever, pneumonia, short-term cardiac ischemia). Sputum induction was achieved using hypertonic sodium chloride aerosols (w/v 4.5%) for a maximal duration of three times five minutes and processed according to the whole sample method.

## BAL and sputum processing

BAL was filtered through a nylon gauze and centrifuged for 10 minutes at 450*g at 4°C. If erythrocytes were macroscopically present, the cell pellet was resuspended in lysisbuffer (100 ml phosphate buffered saline (PBS) containing 0.83 gram $NH_4Cl$, 0.1 gram $KHCO_3$ and 0.004 gram Ethylenediaminetetra Acetic Acid (EDTA), pH 7.4) for 5 minutes and centrifuged (450*g, 4°C). The cell pellet was resuspended in 0.1% glucose (w/v) in PBS and centrifuged again under the same conditions. BAL processing and differential cell counts were performed analogous to the methods described for sputum processing, except that no dithiothreitol was used for homogenization. The viability of the non-squamous cells in BAL was similar in smokers and ex-smokers (82 ± 12% versus 82 ± 9%, P=0.96).

Sputum was processed according to the whole sample method and all samples were treated with dithiothreitol 0.1% (DTT, Sputolysin, Calbiochem) [28]. Cell free supernatants of both BAL and sputum were stored at -80°C.

From both BAL and sputum samples cytospins were centrifuged on apex-coated slides [28]. A sputum sample was considered adequate when the percentage squamous cells was less than 80%. After drying for 1 hour, the cytospins were wrapped in aluminum foil and stored at -80°C pending immunocytochemical staining.

## Immunocytochemical staining

Frozen cytospins of BAL and sputum were brought to room temperature in one hour. BAL cytospins were fixed in acetone at -20°C for 10 minutes, dried and endogeneous peroxidase activity was blocked by incubation in methanol and 0.3% hydrogen peroxide for 10 minutes. Sputum cytospins were fixed in 4% paraformaldehyde in PBS 0.9% (w/v) for 1 hour, rinsed with PBS and endogenous peroxidase activity was blocked with sodium azide 0.1% (w/v) and hydrogen peroxide 0.18% (w/v) in PBS for 30 minutes. Non-specific binding was blocked in PBS, 1% bovine serum albumin (BSA) and 5% normal human serum (NHS) for 45 minutes for the sputum cytospins only. Mouse-anti-human CD163 (clone GHI/61, BD Pharmingen) was used as a primary antibody to stain M2-type macrophages [17] at the dilution of 1:75 for BAL cytospins and 1:50 for sputum cytospins, and both were incubated for one hour at room temperature. The primary antibody was diluted in PBS/1% BSA for BAL cytospins and in PBS/1%BSA/1%NHS for sputum cytospins. The horseradish peroxidase conjugated anti-mouse Envision system (DAKO, Glostrup, Denmark) was used as a secondary antibody and was incubated for 30 minutes, the chromogen NovaRed (Vector, Burlingame, CA) for

7 minutes. All washing steps were with PBS. All slides were counterstained with Mayer's hematoxylin (Klinipath, Duiven, The Netherlands) and mounted afterwards with Pertex mounting medium (HistoLab, Gothenburg, Sweden).

We considered the possibility that DTT used to liquefy the induced sputum samples affects detection of CD163. To this end we generated M1 and M2 by culture of monocytes for six days in the presence of GM-CSF and M-CSF respectively [11], and treated these cells with DTT prior to FACS-based analysis of CD163 expression and preparation of cytospins followed by immunocytochemical staining for CD163.

## Analysis of cytospins

Two cytospins per sample were stained for differential cell counts with May-Grünwald Giemsa (MGG). Differential cell counts were expressed as a percentage of nucleated cells, squamous cells excluded. The median percentage squamous cells was 7.5% (2.1-13.3%). $CD163^+$ and $CD163^-$ macrophages were enumerated based on morphology by two independent, experienced researchers at 400x magnification (Figure 1). To avoid observer bias, slides were coded without knowledge of clinical data. The mean number of $CD163^+$ macrophages divided by the total counted number of macrophages was used to calculate the percentage of $CD163^+$ macrophages. The total number of $CD163^+$ macrophages per volume was calculated by the percentage of $CD163^+$ macrophages multiplied by the total number of macrophages. Repeatability between the two observers (LIK and SVW) was good, as measured by the intraclass coefficient (ICC), with the two way random model and absolute agreement. For BAL $CD163^+$ and $CD163^-$ macrophages the ICC were both 95%; for sputum $CD163^+$ and $CD163^-$ macrophages the ICC were 97% and 93% respectively.

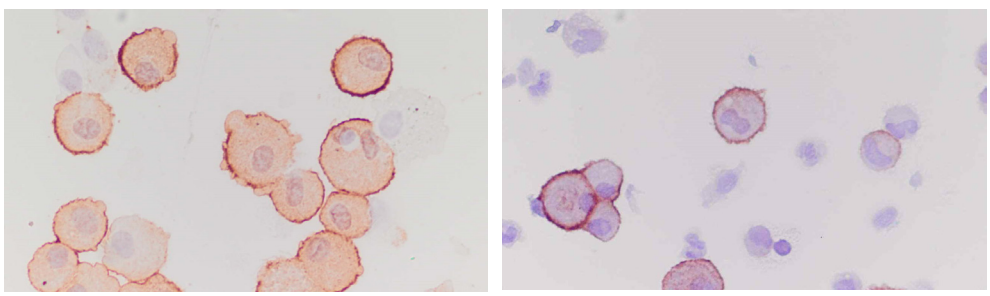

**Figure 1:** Photomicrograph of membrane-bound CD163 staining on BAL and sputum cells. A BAL cytospin is shown in the left photograph and a sputum cytospin in the right photograph. Scale bar= 20 µm.

## Enzyme-linked Immunosorbent Assay (ELISA)

Commercially available kits were used to detect GM-CSF (Bender Medsystems), M-CSF (R&D systems), IL-6, IL-8, IL-10 (Sanquin), IL-12 (IL-12/IL-23p40, R&D systems) and elafin (HBT) in sputum and BAL supernatants. SLPI ELISA was developed in our laboratory at the Leiden University Medical Center [29]. The absorbance was measured at 450nm using a Microplate reader (model 680; Bio-Rad, Hercules, CA) and Microplate Manager software (version 5.2.1, Bio-Rad). The lower limits of detection for sputum were 300 pg/ml (SLPI), 2.5 ng/ml (elafin), 38 pg/ml (IL-6) and 400 pg/ml (IL-8). The lower limits of detection for BAL were 150 pg/ml (M-CSF), 0.2 ng/ml (SLPI), 5.5 pg/ml (IL-6) and 15 pg/ml (IL-8). In BAL and sputum supernatants, IL-10, IL-12, GM-CSF levels were below the lower limit of detection. Furthermore, elafin and M-CSF were undetectable in BAL and sputum supernatants respectively. In case more than 10% of the samples were below the detection limits, the value of these samples was set at the lower limit of detection (M-CSF and IL-6 in BAL).

## Statistical analysis

Mean values and standard deviations (SD) or medians with interquartile ranges (IQR) are presented. When appropriate, variables were logarithmically transformed before statistical analysis. Differences between smokers and ex-smokers were explored using $\chi^2$-tests, two-tailed unpaired t-tests and Mann-Whitney tests. We used the Spearman (Rs) correlation coefficient to analyze correlations. Multiple linear regression was used to correct for the recovery of BAL. The statistical analysis was performed with SPSS 16.0 software (SPSS Inc., Chicago, IL). Statistical significance was inferred at $P \leq 0.05$.

# RESULTS

## Characteristics

In total, 114 COPD patients participated in the study, 72 current smokers and 42 ex-smokers, as presented in Table 1. All steroid-naive patients had moderate to severe COPD (GOLD stage 2-3) based on a mean (SD) post-bronchodilator $FEV_1$ of 63 (9)% predicted and had a median (25th and 75th percentile) smoking history of 42 (31-55) packyears. The total group of

patients and the unselected group in which BAL was performed were comparable. Of the BAL samples (first 71 patients), 62 were suitable for analysis. 106 out of 109 sputum inductions were suitable for analysis. BAL and sputum cell differentials and cell concentrations are presented in Figures 2 and 3. The percentage and number of macrophages in BAL were significantly higher in current smokers than in ex-smokers (95.8% and 74.2%, P<0.001; 34.0 and 7.6x10$^4$/ml, P=0.008 respectively). The mean recovery of BAL was 41 (18)%; the recovery in smokers was higher compared to ex-smokers (45 (16)% and 35 (19)%, P=0.039, respectively).

**Table 1**: Patient characteristics for current and ex-smokers with COPD.

| | Smokers (n=72) | Ex-smokers (n=42) |
|---|---|---|
| **Males (n (%))** | 59 (81.9) | 40 (95.2)* |
| **Age (years)** | 60.1 (7.7) | 64.1 (7.2)* |
| **Packyears** | 43.3 (32.4-55.6) | 36.8 (27.5-53.1) |
| **Smoking cessation (years)** | | 3.5 (1.0-9.8) |
| **$FEV_1$ post-bronchodilator (L)** | 2.02 (0.46) | 2.05 (0.46) |
| **$FEV_1$ post-bronchodilator (%pred)** | 63.3 (8.3) | 62.5 (9.6) |
| **$FEV_1$/IVC% post-bronchodilator** | 49.5 (8.5 | 46.0 (8.3)* |
| **KCO (%pred)** | 73.3 (25.1) | 80.4 (25.9) |

Data are presented as mean (SD) or median (IQR) unless otherwise stated. These patient characteristics have been previously described [7].

pred= predicted; $FEV_1$= forced expiratory volume in one second; IVC= inspiratory vital capacity; KCO= carbon monoxide transfer coefficient.

* P<0.05 compared with smokers with COPD ($\chi^2$ test for sex differences, two tailed unpaired t-tests for other data).

## Smoking status and CD163$^+$ macrophages in BAL and induced sputum

DTT used to liquefy the induced sputum did not affect detection of CD163 by FACS and immunocytochemical staining (data not shown). Ex-smokers with COPD had a significantly higher percentage of anti-inflammatory CD163$^+$ macrophages in BAL than current smokers (83.5% and 68.0%, P=0.04, respectively) (Figure 4), independent of BAL recovery. However, ex-smokers had a lower number of anti-inflammatory macrophages in BAL compared to current smokers (5.6 and 20.1x10$^4$/ml, P=0.001, respectively). The percentage CD163$^+$ macrophages was higher in BAL compared to sputum (74.0% and 30.3%, P<0.001,

respectively). Ex-smokers had a similar percentage and number of anti-inflammatory macrophages in induced sputum compared to current smokers with COPD (25.0% and 31.1%, P=0.89; 10.1 and 6.8 x$10^4$/ml, P=0.24 respectively).

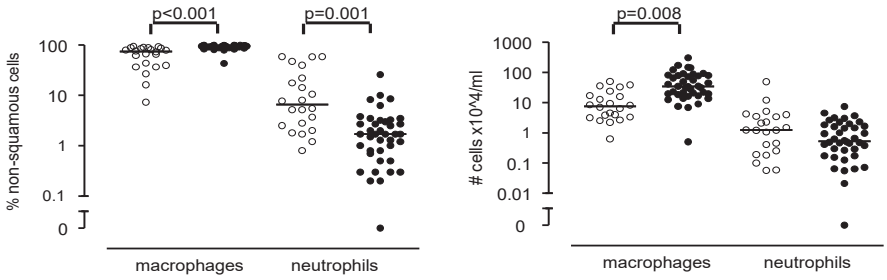


**Figure 2:** BAL differential cell counts expressed as percentage and cell concentrations of COPD patients. Percentage is shown in the left panel, cell concentrations in the right panel. Open circles represent ex-smokers, closed circles represent current smokers. Horizontal bars represent medians. P-values are corrected for recovery of BAL fluid using multiple linear regression.

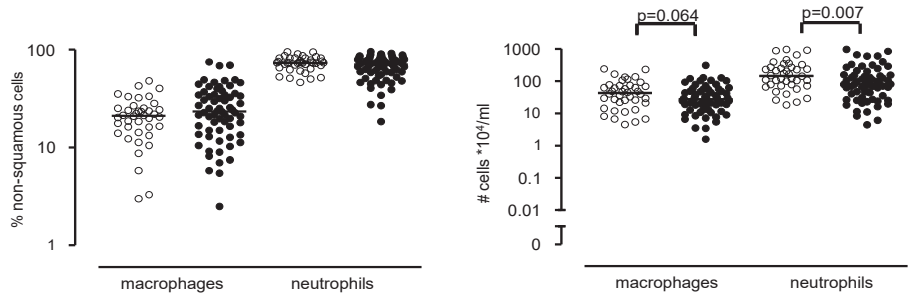


**Figure 3.** Sputum differential cell counts expressed as percentage and cell concentrations of COPD patients. Percentage is shown in the left panel, cell concentrations in the right panel. Open circles represent ex-smokers, closed circles represent current smokers. Horizontal bars represent medians.

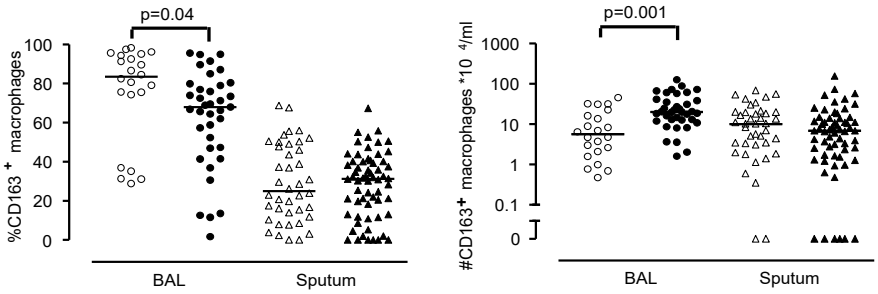


**Figure 4.** The percentage and number of CD163$^{+}$ macrophages in BAL and induced sputum in COPD patients. The percentage (left panel) and number of CD163$^{+}$ macrophages (right panel) in BAL and induced sputum between ex-smokers (open symbols) and smokers (closed symbols) with COPD. Horizontal bars represent medians. P-values are corrected for recovery of BAL fluid using multiple linear regression.

## Smoking status and soluble mediators in BAL and induced sputum supernatants

BAL M-CSF levels were lower in ex-smokers than current smokers (P=0.001) (Figure 5 and Table 2). This difference was neither explained by differences in BAL recovery between both groups, nor by the ratio of M-CSF to anti-inflammatory macrophages. No correlation was found between recovery and BAL M-CSF levels. The anti-inflammatory mediator SLPI in BAL was inversely correlated with recovery. The pro-inflammatory mediators IL-6 and IL-8 in BAL were comparable between smokers and ex-smokers and were independent of recovery. No difference was found in induced sputum for the pro-inflammatory IL-6, IL-8 levels and the anti-inflammatory mediator elafin. The levels of SLPI, IL-6 and IL-8 in sputum were higher than the levels in BAL (all P<0.001). M-CSF was below the lower limits of detection in induced sputum and elafin was undetectable in BAL.

**Table 2:** Soluble mediators measured in BAL and induced sputum supernatants of smokers and ex-smokers with COPD.

| Soluble mediator | Ex-smokers | Smokers |
|---|---|---|
| **BAL** | | |
| SLPI (ng/ml) | 156 (72-386) | 87 (48-154) |
| M-CSF (pg/ml) | 150 (150-159) | 571 (150-927) |
| IL-6 (pg/ml) | 6 (6-6) | 6 (6-6) |
| IL-8 (pg/ml) | 83 (43-193) | 64 (37-122) |
| **Sputum** | | |
| SLPI (ng/ml) | 5897 (4406-8628) | 6643 (4321-8862) |
| Elafin (ng/ml) | 44 (20-102) | 44 (12-101) |
| IL-6 (pg/ml) | 23 (10-36) | 30 (11-58) |
| IL-8 (pg/ml) | 3454 (1178-5212) | 2571 (805-5900) |

Data are presented as medians (IQR 25-75th percentile).

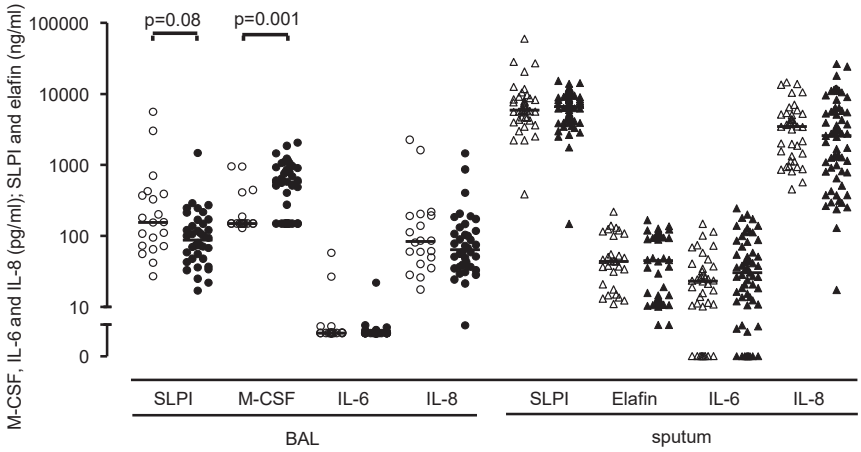


**Figure 5.** Soluble mediators measured in BAL and induced sputum supernatants of COPD patients. Ex-smokers are represented by open symbols and smokers by closed symbols. Horizontal bars represent medians. P-values are corrected for recovery of BAL fluid using multiple linear regression.

## Correlation between cells, mediators and lung function

The number of $CD163^+$ macrophages in BAL correlated with $FEV_1$ post-bronchodilator (%predicted) (Rs=0.255; P=0.05) and $FEV_1$/IVC% (Rs=0.374; P=0.004). No correlations were found between the number and percentage $CD163^+$ macrophages in BAL and sputum and the number of packyears or the duration of smoking cessation. No correlations were found between the number of packyears or duration of smoking cessation and concentrations of all soluble mediators in BAL and induced sputum.

BAL M-CSF correlated with the number of $CD163^+$ macrophages in BAL (Rs=0.379; P=0.003). BAL SLPI was negatively correlated with the number and percentage of macrophages and positively correlated with the number and percentage of neutrophils in BAL (all $P<0.05$). BAL SLPI and the number of $CD163^+$ macrophages correlated inversely (Rs=-0.353; P=0.008). Sputum SLPI correlated with the number and percentage of $CD163^+$ macrophages in sputum (Rs=0.377; $P<0.001$ and Rs=0.236; P=0.021, respectively). Both BAL and sputum IL-8 correlated inversely with percentage macrophages, but positively with the percentage and number of neutrophils (all $P<0.05$). This relation was not seen for IL-6. A trend was seen for a correlation between sputum IL-8 and the percentage of $CD163^+$ macrophages (Rs=-0.189; P=0.061). The percentage, but not the number, of $CD163^+$ macrophages in BAL showed a trend for correlation with sputum (Rs=0.267, P=0.053).

# DISCUSSION

This study is the first to show that the percentage of macrophages with anti-inflammatory, M2-type characteristics (as shown by CD163 expression) is significantly higher in BAL from ex-smokers than in current smokers with COPD. In addition, the percentage of anti-inflammatory macrophages was higher in BAL than in induced sputum, indicating a predominance of this macrophage phenotype in the periphery of the lung. BAL M-CSF correlated with the number of $CD163^+$ macrophages in BAL. The results together are in line with the hypothesis that smoking cessation causes a shift in the phenotype of luminal macrophages towards a more anti-inflammatory phenotype, which is restricted to the periphery of the lung. Although we did observe a higher percentage of M2-

type macrophages in BAL from ex-smokers, this was not accompanied by a decrease in inflammatory parameters such as neutrophils and pro-inflammatory mediators.

Our study shows that ex-smokers with COPD have a higher percentage of anti-inflammatory macrophages in BAL than current smokers. Our findings on pulmonary macrophage polarization further extend previous observations. First, we discovered that macrophages recovered from induced sputum have less anti-inflammatory features than from BAL. A previous study showed that induced sputum of COPD patients contains a majority of pro-inflammatory macrophages, based on their HLA-DR expression and capacity to produce TNFα, in contrast to control subjects [30]. However, these authors only analyzed markers of pro-inflammatory macrophages and most patients used corticosteroids which may have affected the macrophage phenotype [17]. Second, we showed that ex-smokers have more anti-inflammatory macrophages in BAL than current smokers. This is in line with a recent paper, showing that never smokers compared to current smokers had higher BAL levels of CCL18, a chemokine expressed by alternatively activated macrophages [31]. Furthermore, previous studies have shown that anti-inflammatory macrophages have a higher phagocytic capacity [8, 10]. Therefore our findings are in line with another study demonstrating that alveolar macrophages of current smokers with COPD show reduced phagocytosis compared to ex-smokers [19]. In addition, active smoking, but also the presence of COPD itself, may be associated with an impaired phagocytic capacity of alveolar macrophages (and therefore a predominance of pro-inflammatory macrophages) [32-34]. However, in contrast to these and our findings, a recent study indicated that smoking may enhance macrophage differentiation into an anti-inflammatory phenotype, since cigarette smoking polarized human alveolar macrophages of COPD patients *in vivo* towards an enhanced expression of M2-related genes and a suppression of M1 genes [35]. This study included only 12 COPD patients with predominantly GOLD stage 1. A possible explanation for this apparent difference with our observations is therefore that the direction of the effect of smoking on macrophage differentiation may be determined by disease severity.

Previously, several studies have evaluated the effect of smoking on soluble mediators. We found comparable SLPI levels in BAL between current smokers and ex-smokers with COPD, in line with results from a study of 25 smoking, ex-smoking and never smoking COPD patients with GOLD stage 2-3 [36]. We did not find a difference in BAL IL-6 and IL-8 and sputum IL-6 between current smokers and ex-smokers with COPD, in line with two previous studies [37, 38].

We believe that our study has several strengths. We studied a large cohort of well-

characterized COPD patients in which sputum (n=114) and BAL (n=71) were collected, whereas previous studies were of smaller size [30, 31, 36, 39]. In addition, we studied steroid-naive patients, excluding possible influences of inhaled corticosteroid therapy on CD163 expression. This is important, since it has been shown in previous studies that dexamethasone induces CD163 expression on monocytes and macrophages *in vitro* [17]. The BAL and sputum cytospins were counted manually by two independent researchers simultaneously (LIK and SVW). $CD163^-$ macrophages as well as $CD163^+$ macrophages were readily recognized. Repeatability between the observers was good, as measured by the intraclass correlation coefficient.

A number of limitations needs to be taken into account when interpreting our results. First, this was a cross-sectional study and it cannot be ruled out that our group of ex-smokers quit smoking because they experienced more smoking related symptoms and they may have had different macrophage phenotypes before quitting. In addition, we did not confirm smoking status by laboratory tests which is in line with other cross-sectional studies [4,5] and therefore cannot exclude the possibility that some ex-smokers were still smoking. Second, BAL samples were not available from all subjects in our study due to ethical considerations. As this was not anticipated, it is unlikely that a selection bias for the BAL results was introduced. Nevertheless, a significant difference in anti-inflammatory macrophages in BAL was found between smokers and ex-smokers. Further studies are needed to investigate whether the observed differences in CD163 staining on macrophages are also observed when comparing current or ex-smokers without COPD to non-smokers and whether CD163 expression is a specific feature of COPD. Third, we only focused on the marker CD163 for M2 macrophages, which can result in an oversimplification of our conclusions. Furthermore, it appears that the M2 macrophage population is more heterogeneous than the M1 population [9] and M2 subpopulations were not taken into account in our analysis. Obviously, it is of interest to evaluate whether the use of pro-inflammatory or other anti-inflammatory markers (like arginase or iNOS) can confirm our results and whether associated functional differences can be detected. Currently, there is no general agreement on well defined markers for M1 macrophages.

Fourth, we found that the percentage $CD163^+$ cells is higher in ex-smokers with COPD whereas the number of $CD163^+$ cells is higher in current smokers with COPD. In addition, we observed a higher percentage and number of macrophages in BAL from smokers compared to ex-smokers, which likely results from more active recruitment of monocytes from the circulation. Therefore, it is not surprising that smokers have a higher number of $CD163^+$ cells in BAL, since they have more macrophages in BAL. We hypothesize that percentages and

numbers provide different and complimentary information: percentages better reflect the environment during differentiation, whereas cell numbers result from both recruitment and differentiation. Fifth, several soluble mediators were below the lower limits of detection in sputum and BAL supernatants. Finally, analysis of cytospins using immunocytochemistry is a semi-quantitative measurement and could therefore result in incorrect interpretations. Using e.g. FACS analysis ideally combined with functional analysis of e.g. the phagocytic capacity of the macrophages, could have been more accurate to evaluate the equilibrium between pro- and anti-inflammatory macrophages in our samples. Unfortunately, fresh samples were not available at the time of this research.

How can we explain our results? Macrophages in the periphery of the lung in healthy individuals display mainly anti-inflammatory characteristics that may be involved in suppressing inflammation in this area of the lung. Our study, as well as recent data from others [19, 40], suggest that the anti-inflammatory environment may change into a pro-inflammatory environment as COPD develops in smokers. This is in line with the observation that IL-10 levels are lower and GM-CSF and Matrix Metalloproteinase (MMP)-12 levels are higher in sputum and BAL from COPD patients compared to healthy controls [39, 41, 42]. Inflammatory lung diseases, including COPD [43], are characterized by increased local production of GM-CSF which may contribute to development of a pro-inflammatory macrophage phenotype in addition to its established effect on neutrophil survival [44]. Macrophages maintain their plasticity even when differentiated into M1 or M2 cells and can switch their phenotype dependent on the presence of appropriate stimuli [45, 46]. In this study we add to the field that smoking cessation may skew alveolar macrophage heterogeneity towards a more anti-inflammatory phenotype as characterized by the M2 marker CD163. Pro-inflammatory macrophages are the predominant phenotype in the central airways, which may be explained by high exposure to pathogens and environmental stimuli compared to macrophages in the peripheral airways. The higher percentage and number of neutrophils in sputum samples are in line with this observation. The predominance of anti-inflammatory macrophages in the periphery of the lung may help to keep this area, which is central to gas exchange, free from excessive inflammation.

Our results suggest that smoking cessation can change macrophage polarization from a pro-inflammatory towards a CD163 expressing anti-inflammatory phenotype, which may decrease inflammation and enhance repair. Our findings of a positive association between a better lung function and more anti-inflammatory M2 macrophages are in line with this. We hypothesize that a shift in macrophage phenotype contributes to further clinical effects of smoking cessation. Therefore, the plasticity of the macrophage phenotype and the possibility

to modulate this phenotype may be relevant to the treatment of chronic inflammation, including COPD.

## CONCLUSION

This study shows that previous smoking cessation may contribute to the anti-inflammatory phenotype of intraluminal macrophages in BAL of ex-smoking COPD patients *in vivo*. Additional research is needed to further characterize this phenotype and to demonstrate its impact on local inflammation. Furthermore, studies are needed to investigate whether it is restricted to luminal macrophages or is also present in lung tissue. Prospective studies are required to show whether anti-inflammatory treatment contributes to the anti-inflammatory macrophage phenotype *in vivo*, and whether this contributes to treatment effects on inflammation and clinical outcomes such as lung function decline.

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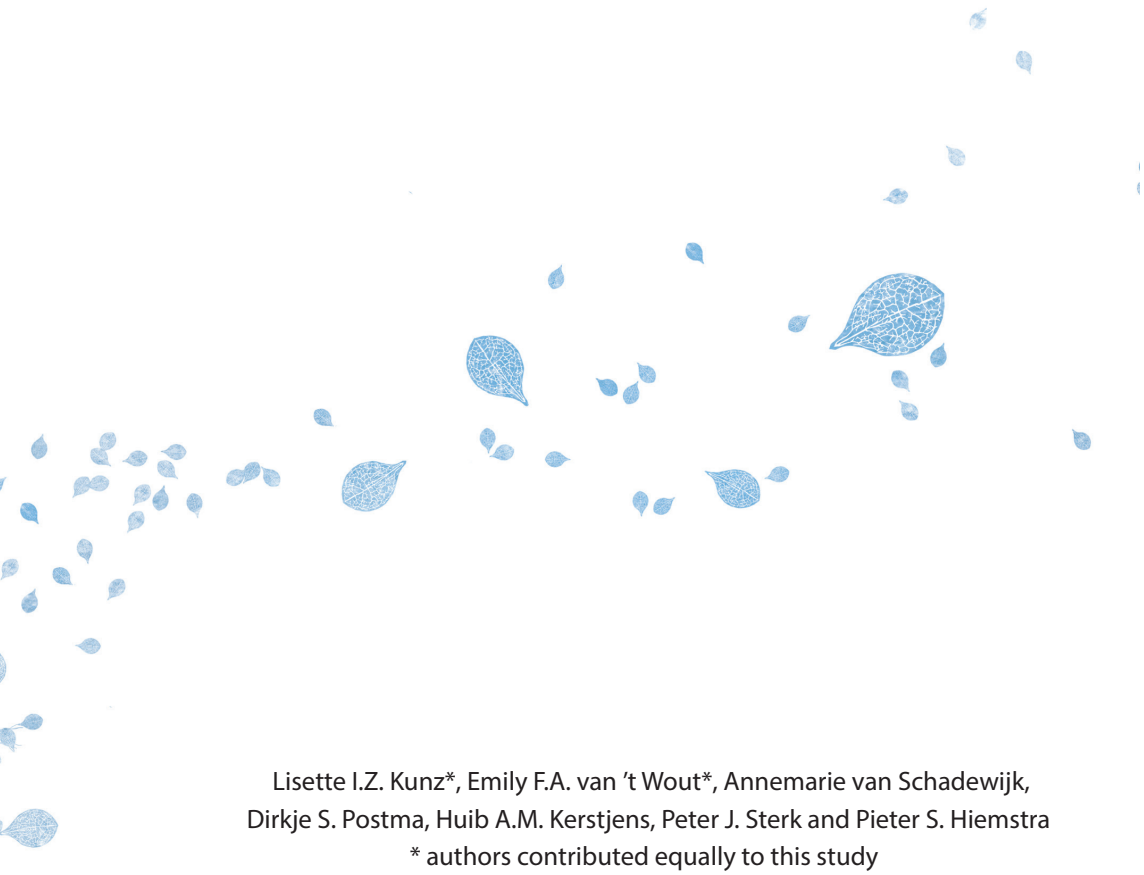
# CHAPTER 3

# Regulation of YKL-40 Expression by Corticosteroids: Effect on Pro-inflammatory Macrophages *in vitro* and its modulation in COPD *in vivo*



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# ABSTRACT

**Background:** Macrophages constitute a heterogeneous cell population with pro- (MΦ1) and anti-inflammatory (MΦ2) cells. The soluble chitinase-like-protein YKL-40 is expressed in macrophages and various other cell types, and has been linked to a variety of inflammatory diseases, including COPD. Dexamethasone strongly reduces YKL-40 expression in peripheral blood mononuclear cells (PBMC) *in vitro*. We hypothesized that: a) YKL-40 is differentially expressed by MΦ1 and MΦ2, b) is decreased by corticosteroids and c) that long-term treatment with inhaled corticosteroids (ICS) affects YKL-40 levels in serum and sputum of COPD patients.

**Methods**: Monocytes of healthy subjects were cultured *in vitro* for 7 days with either GM-CSF or M-CSF (for MΦ1 and MΦ2, respectively) and stimulated for 24h with LPS, TNFα, or oncostatin M (OSM). MΦ1 and MΦ2 differentiation was assessed by measuring secretion of IL-12p40 and IL-10, respectively. YKL-40 expression in macrophages was measured by quantitative RT-PCR (qPCR) and ELISA; serum and sputum YKL-40 levels were analyzed by ELISA.

**Results**: Pro-inflammatory MΦ1 cells secreted significantly more YKL-40 than MΦ2, which was independent of stimulation with LPS, TNFα or OSM ($P<0.001$) and confirmed by qPCR. Dexamethasone dose-dependently and significantly inhibited YKL-40 protein and mRNA levels in MΦ1. Serum YKL-40 levels of COPD patients were significantly higher than sputum YKL-40 levels but were not significantly changed by ICS treatment.

**Conclusions**: YKL-40 secretion from MΦ1 cells is higher than from MΦ2 cells and is unaffected by further stimulation with pro-inflammatory agents. Furthermore, YKL-40 release from cultured monocyte-derived macrophages is inhibited by dexamethasone especially in MΦ1, but ICS treatment did not change YKL-40 serum and sputum levels in COPD. These results indicate that YKL-40 expression could be used as a marker for MΦ1 macrophages *in vitro*, but not for monitoring the effect of ICS in COPD.

# INTRODUCTION

YKL-40 (or chitinase 3-like-1 [CHI3L1], breast regression protein [BRP]-39 or human cartilage glycoprotein-39 [HCgp-39]) is a chitinase-like protein which is found in humans [1]. It is expressed by various cell types, including neutrophils and macrophages [2-4], while macrophages have been identified as its main cellular source [2, 5]. Monocytes do not express YKL-40, and YKL-40 expression appears to be associated with later stages of macrophage differentiation [2, 6]. Although its biological function is largely unknown, YKL-40 has been suggested to play a major role in a variety of processes, including epithelial-mesenchymal transition, migration and proliferation of (malignant) cells, angiogenesis, tissue remodeling and inflammation [7-9]. Furthermore, YKL-40 has been implicated in several acute and chronic inflammatory diseases, including asthma and COPD [10, 11]. Several studies have shown that YKL-40 levels are higher in sputum and serum of COPD patients compared to asymptomatic smokers [12, 13].



These data suggest that macrophages may also be a major source of YKL-40 in inflammatory lung diseases such as COPD, a disease in which macrophages play an important role [14]. It is however unknown whether YKL-40 expression is restricted to a subset of macrophages. This is a relevant question, since macrophages constitute heterogeneous cell populations with various functions. Pro-inflammatory, or classically activated macrophages (type I, MΦ1), show pro-inflammatory properties by secreting pro-inflammatory cytokines such as interleukin-12 (IL-12), have antigen presenting capacity and promote Th1 immunity. In contrast, anti-inflammatory, or alternatively activated macrophages (type II, MΦ2), demonstrate anti-inflammatory characteristics with expression and secretion of anti-inflammatory cytokines, such as IL-10 and promote development of regulatory T cells [15]. Furthermore, Di Rosa *et al*. observed higher expression of YKL-40 in monocyte-derived MΦ1 [16].

Dexamethasone strongly suppresses YKL-40 expression in cultured monocyte-derived macrophages [17]. In line with these findings, rheumatoid arthritis patients treated with methotrexate and prednisolone had lower serum YKL-40 concentrations compared to patients treated without prednisolone [18]. However, it is unknown if YKL-40 levels are influenced by treatment with inhaled corticosteroids (ICS) in COPD and could therefore be used to monitor treatment response. Furthermore, it is not known whether modulation by steroids is a direct genomic anti-inflammatory effect or due to e.g. epigenetic mechanisms [19-22].

Based on these observations we hypothesized that YKL-40 expression is directed by macrophage polarization, with a higher expression in monocyte-derived MΦ1 compared to MΦ2. Furthermore, we hypothesized that steroids decrease YKL-40 secretion and expression in MΦ1 and that YKL-40 in serum and sputum of COPD patients is decreased by treatment with ICS.

# METHODS

## Cell culture

Monocytes were isolated from buffy coats of healthy blood donors (Sanquin Blood Bank, Leiden, The Netherlands) using anti-CD14 microbeads (Miltenyi Biotec, Auburn, CA, USA) according to the manufacturer's protocol. MΦ1 and MΦ2 were derived as described previously [23, 24]. Briefly, cells were cultured for six days in RPMI 1640 medium in 48 well plates (Invitrogen, Life Technologies, Bleiswijk, The Netherlands) containing 10% fetal calf serum (FCS, Invitrogen), 2mM L-glutamine, 100U/ml penicillin and 100µg/ml streptomycin (all Bio Whittaker, Walkersville, MD, USA) at 37°C in 5% CO2 atmosphere with either GM-CSF (5ng/ml, Invitrogen) or M-CSF (50ng/ml, R&D Systems, Minneapolis, MN, USA) to obtain MΦ1 and MΦ2, respectively (13, 14). Differentiated macrophages were stimulated with the pro-inflammatory stimuli lipopolysaccharide (LPS, from Pseudomonas aeruginosa, 100ng/ml, Sigma-Aldrich, St. Louis, MO, USA), TNF-α (10ng/ml, Peprotech, Rocky Hill, NJ, USA) or oncostatin M (OSM, 100ng/ml, R&D Systems) for 24 hours. Dexamethasone (0.1, 0.3 and 1nM, Sigma) was added during differentiation at day 0, 3 and/or day 7. The demethylating agent 5-AZA-2'-deoxycytidine (5-AZA, 0.1, 1 and 10 µM, Sigma) was added during differentiation. Every day 100 µl per well was removed and replaced by fresh medium containing growth factors and 5-AZA.

## GLUCOLD study

Serum and sputum supernatants were obtained from patients with moderate to severe COPD who participated in the GLUCOLD (Groningen and Leiden Universities Corticosteroids in Obstructive Lung Disease) study [25]. Patients were steroid-naive at baseline and were subsequently randomized to one of four inhaled treatments, all twice daily: 6- or 30-month

fluticasone propionate dry-powder inhaler (500µg, group 1 and 2, respectively), 30-month fluticasone with salmeterol (500/50µg, group 3) or 30-month placebo (group 4). In this mechanistic study, we only used data from the compliant patients (≥70% of the prescribed dose of treatment) of groups 2 and 3 combined to increase power, and group 4. At baseline and after 30 months of treatment, serum and sputum samples were collected. Sputum induction and processing were performed as previously described [26, 27]. Cell free supernatants of serum and sputum were stored at -80°C. The ethics committees of Leiden University Medical Center and University Medical Center Groningen approved the study and all patients provided written informed consent.



## Enzyme-linked Immunosorbent Assay (ELISA)

Commercially available ELISA kits were used to detect IL-12p40 (IL-12/ IL-23p40, R&D Systems; sensitivity 62.5 pg/ml) and IL-10 (Sanquin, Amsterdam, The Netherlands; sensitivity 4.1 pg/ml), to confirm that the monocytes were adequately differentiated towards MΦ1 and MΦ2, respectively (Figure 1A). YKL-40 ELISA (R&D Systems; sensitivity 16 pg/ml [10]) was performed on cell culture supernatant, serum and sputum supernatant. The absorbance was measured at 450nm using a Microplate reader (model 680; Bio-Rad, Hercules, CA, USA) and Microplate Manager software (version 5.2.1, Bio-Rad).

## Flow cytometry

Cell surface markers were assessed by standard flow cytometry using a FACS Calibur cytometer (Becton and Dickinson, La Jolla, CA, USA) and CellQuest Pro software after staining with specific APC-conjugated CD163 and CD14 (both BD Biosciences/ Pharmingen (Temse, Belgium), FITC-conjugated CD68 (eBioscience, Vienna, Austria) and/or goat-anti-human YKL-40 (R&D systems) detected by Alexa Fluor 594 F(ab')2 fragment of goat anti-mouse (Invitrogen) as secondary antibody. Cells were incubated with the antibodies for 30 minutes on ice in PBS containing 0.5% BSA (w/v) and 0.2% sodium azide (w/v) (both Sigma). After fixation with Cytofix/ Cytoperm buffer, intracellular staining was performed in Perm/ Wash buffer (both BD Biosciences). Flow cytometric analysis confirmed that the differentiated monocytes were $CD14^+$ and $CD68^+$, and that the majority of MΦ2 were $CD163^+$ (Figure 1B). Therefore, we concluded that the monocytes were properly differentiated into MΦ1 and MΦ2 cells.

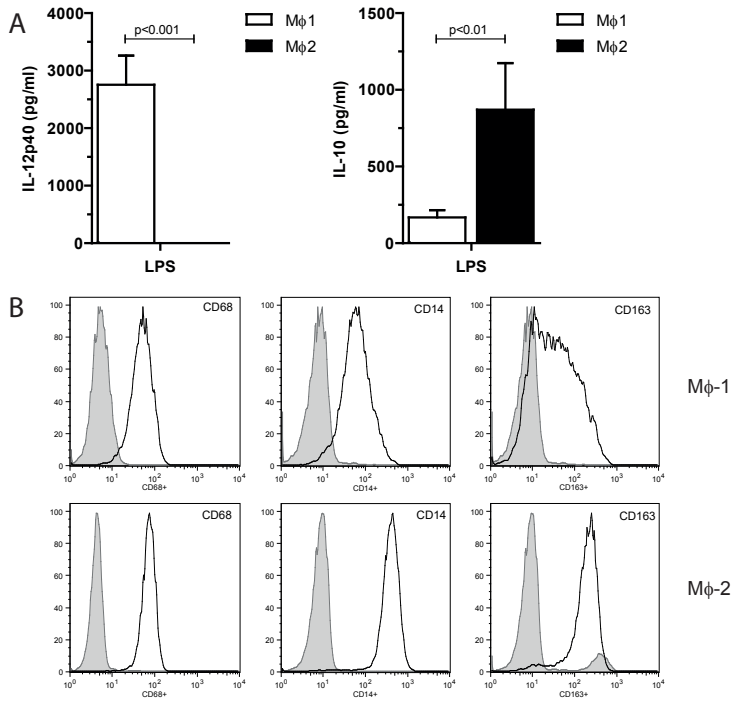


**Figure 1:** Characterization of differentiated MΦ1 and MΦ2. Figure 1A: IL-12p40 (left panel) and IL-10 (right panel) in supernatants of MΦ1 and MΦ2, respectively, stimulated with LPS for 24 hours. Data represent means with SEM of 6 donors. Figure 1B: Flow cytometry analysis for CD68, CD14 and CD163 (left, middle and right panel, respectively) for MΦ1 and MΦ2 (upper and lower panel, respectively).

## Immunofluorescence staining

YKL-40 and CD68 expression in sputum cytospins was demonstrated using immunofluorescence. Cytospins were fixed in 4% formaldehyde in PBS for 30 minutes, followed by antigen retrieval in citrate solution pH6.0 (DAKO, Glostrup, Denmark) for 30 minutes and cooled on ice. The primary antibodies goat-anti-human YKL-40 (R&D systems, dilution 1:25) and mouse-anti-human CD68 (clone PG-M1, DAKO, dilution 1:50), diluted in PBS/ 1% BSA (w/v) were incubated together overnight. Alexa Fluor568 donkey-anti-goat and Alexa Fluor 488 donkey-anti-mouse (Invitrogen, Eugene, OR, dilution of both 1:200) were incubated in a dark environment for 30 minutes. The cytospins were covered with Vectashield with DAPI (Vector Laboratories, Inc. Burlingame, CA, USA). Photographs were taken with a confocal microscope.

## Quantitative reverse-transcriptase polymerase chain reaction (qPCR)

RNA was isolated using Qiagen RNeasy mini kit (Qiagen, Venlo, The Netherlands) and cDNA was synthesized in equal amounts per experiment. Quantitative reverse-transcriptase polymerase chain reaction (qPCR) was performed with the primers for YKL-40 and LL-37 as presented in Table 1. QPCR was performed on the iCycler PCR device using iQ SYBR Green Supermix (Bio-Rad) for 40 cycles at 58°C. Relative mRNA concentrations of ACTB and ATP5B (GeNorm, PrimerDesign Ltd., Southampton, UK) were used as housekeeping genes for human genes.



**Table 1:** Primer pairs used for quantitative reverse-transcriptase polymerase chain reaction (qPCR).

| | Forward | Reverse |
|---|---|---|
| **YKL-40 (CHI3L1) (154 base pairs)** | CTG TGG GGA TAG TGA GGC AT | CTT GCC AAA ATG GTG TCC TT |
| **LL-37 (CAMP) (249 base pairs)** | TCA TTG CCC AGG TCC TCA G | TCC CCA TAC ACC GCT TCA C |

## Statistical analysis

Differences within one cell type and between cell types were analyzed by one-way and two-way ANOVA, respectively. Paired and unpaired tests were used for evaluating YKL-40 levels in serum and sputum within and between treatments, respectively, using only data from subjects with availability of samples from both baseline and 30 months. Statistical analysis was performed with SPSS 22.0 software (SPSS Inc., Chicago, IL). Data are presented as means with standard error of the mean (SEM) for *in vitro* experiments and means with standard deviations (SD) for serum and sputum samples. Differences at p-values ≤0.05 were inferred as statistically significant.

# RESULTS

## MΦ1 produce more YKL-40 compared to MΦ2

Secretion of YKL-40 by MΦ1 was markedly higher compared to MΦ2 ($P<0.001$) and not further increased by stimulation for 24 hours with 100ng/ml LPS compared to medium ($P<0.001$; Figure 2A, left panel). qPCR confirmed these results, i.e. MΦ1 express more YKL-40 than MΦ2 with and without LPS stimulation (10-fold higher in MΦ1 vs MΦ2, $P<0.001$; Figure 2A, right panel), with a trend towards more YKL-40 expression in LPS-stimulated MΦ1 ($P<0.1$). Flow cytometry analysis also showed that MΦ1 cells express more YKL-40 compared to MΦ2 (Figure 2B). In addition, MΦ1 secreted more YKL-40 compared to MΦ2 which was irrespective of further stimulation with TNF-α, and OSM for 24 or 48 hours, respectively (all $P<0.001$, Figure 2C). Immunofluorescence staining showed that only a minority of macrophages in sputum of COPD patients was positive for YKL-40 as shown by co-staining with CD68 (Figure 2D).

## YKL-40 expression is inhibited by dexamethasone

To investigate the effect of steroids on macrophage YKL-40 expression, we first assessed its effect on expression of the MΦ1 and MΦ2 markers IL-12p40 and IL-10. Whereas dexamethasone did not affect IL-10 secretion, it inhibited IL-12p40 secretion, especially when added at day 0 (Figure 3A and B, respectively). In line with these findings, dexamethasone also dose-dependently inhibited YKL-40 expression and secretion mainly in MΦ1 (Figure 4A and B, respectively). Again this effect was most pronounced when dexamethasone was added from the start of the differentiation at day 0 (Figures 4A and B, left panel). Adding dexamethasone at later time points (day 3 or day 7) showed a trend for lower YKL-40 expression and secretion (Figures 4A and B, middle and right panel, respectively), but failed to reach significance.

We have previously demonstrated that the human cathelicidin antimicrobial peptide hCAP/LL-37 directs macrophage differentiation towards MΦ1 cells [28]. In the course of the latter studies we also noted that hCAP18/LL-37 is preferentially expressed in MΦ1 cells (unpublished results). In the present study, we therefore used LL-37 expression for a comparison with YKL-40 expression. In line with the findings for IL-12p40 and YKL-40, the expression of LL-37 was also dose-dependently reduced by increasing doses of dexamethasone when added on day 0, but not on day 3 or day 7 (Figure 4C).

Collectively these data indicate that dexamethasone inhibits YKL-40 expression, but that this effect is most likely explained by a more generic effect on MΦ1 development and is not selective for YKL-40 expression.

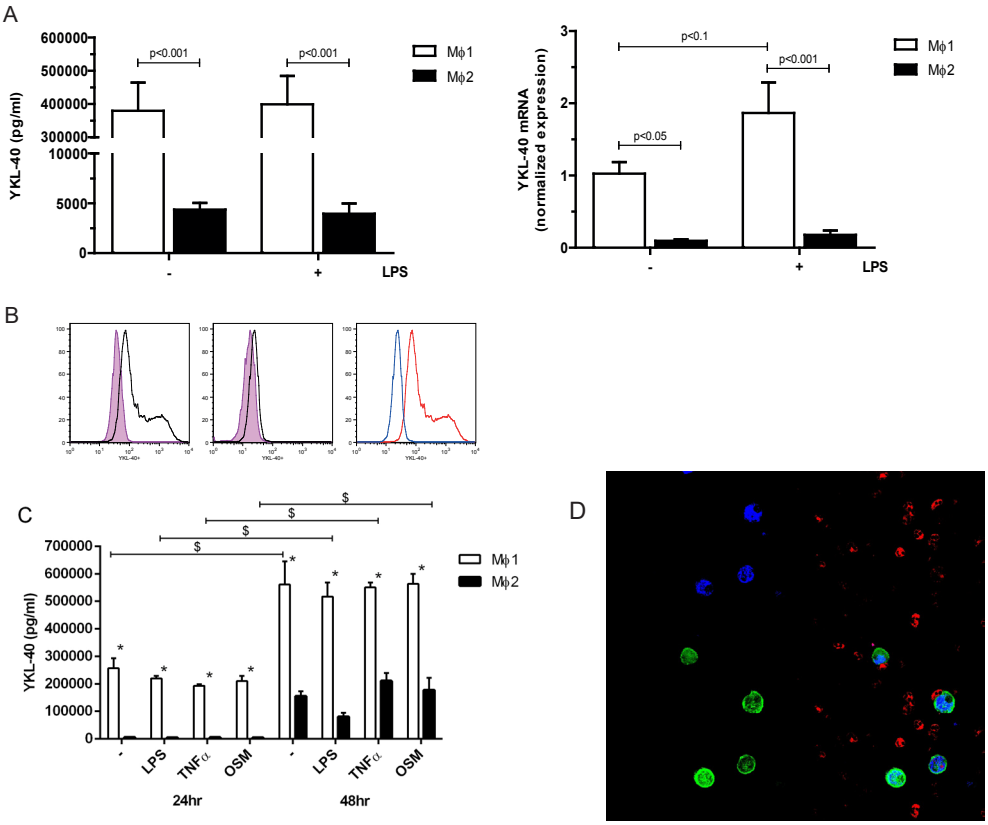


**Figure 2:** MΦ1 produce more YKL-40 compared to MΦ2. Figure 2A: YKL-40 secretion measured by ELISA (left panel, 6 donors) and mRNA levels (normalized expression, right panel, 5 donors) in MΦ1 and MΦ2 after medium and LPS stimulation for 24 hours. Figure 2B: Flow cytometry analysis of MΦ1 and MΦ2 with and without anti-YKL-40 and secondary antibody. Left panel: MΦ1 with secondary antibody (pink) and MΦ1 with anti-YKL-40 and secondary antibody (black); middle panel: MΦ2 with secondary antibody alone (pink) and MΦ2 with anti-YKL-40 and secondary antibody (black); right panel: MΦ1 and anti-YKL-40 with secondary antibody (red) and MΦ2 and anti-YKL-40 with secondary antibody (blue). Figure 2C: YKL-40 secretion in MΦ1 and MΦ2 after 24 and 48 hours of stimulation with medium, LPS, TNF-α and oncostatin M (OSM). Data represent mean and SEM of 4 and 3 donors (24 and 48 hours, respectively). *: P<0.001 between MΦ1 and MΦ2 for corresponding stimulus and time. $: P<0.001 between corresponding stimulus at different time points. Figure 2D: Immunofluorescence staining on sputum cytospin of a COPD patient (blue: DAPI; red: YKL-40; green: CD68 and overlay).

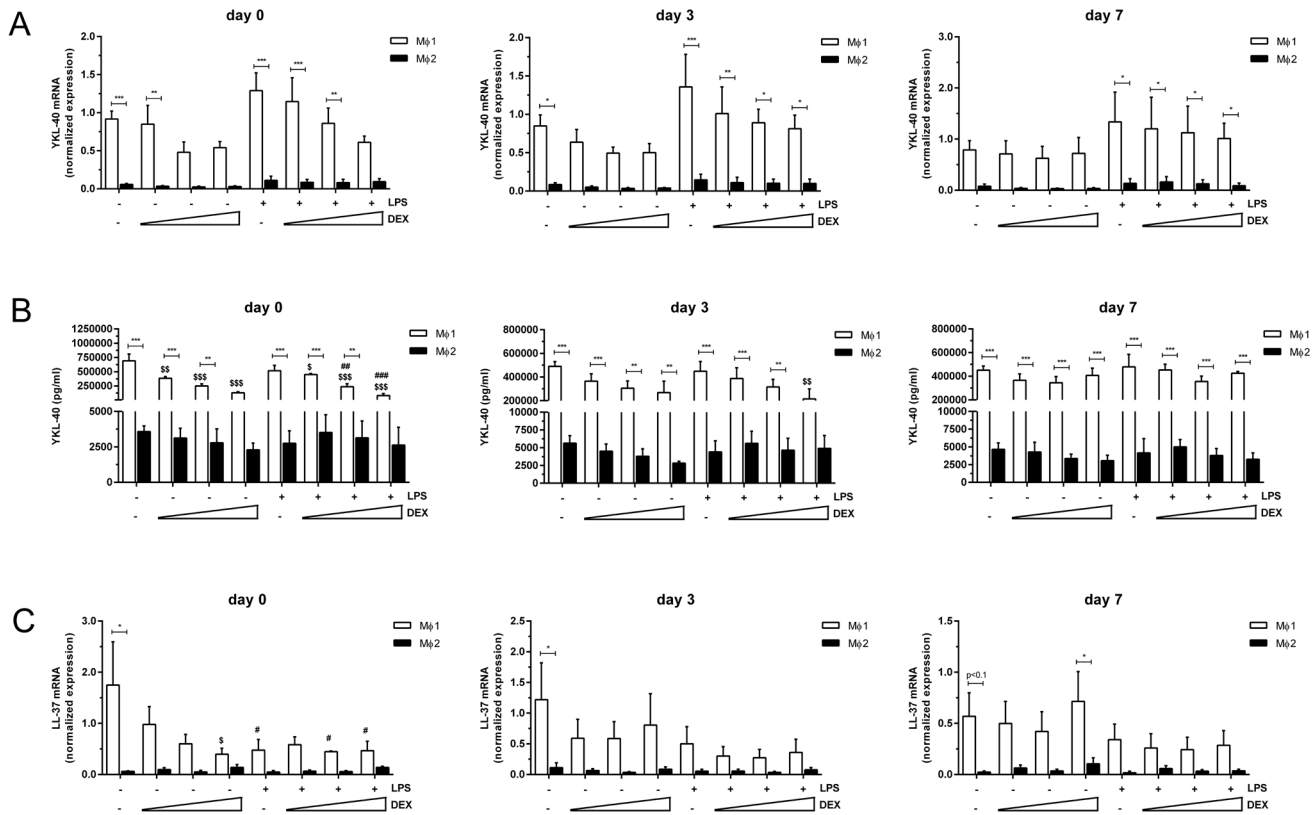


**Figure 3:** IL-12p40 secretion is dose-dependently inhibited by dexamethasone when added on day 0.

IL-10 and IL-12p40 secretion with addition of dexamethasone at day 0, 3 and 7 of differentiation (0.1, 0.3 and 1nM) (Figure 3A and B, left, middle and right panel, respectively). Data represent means and SEM (n= 3 donors). * p≤0.05, ** P<0.01 or *** P<0.001: between MΦ1 to MΦ2 with corresponding stimulus. $ p≤0.05 or $ P<0.001 between MΦ1 at different concentrations of dexamethasone.

## YKL-40 expression is not affected by the demethylating agent 5-AZA

Epigenetic mechanisms may contribute to CHI3L1 expression. This is supported by the finding of a single-nucleotide polymorphism (SNP) localized near the CpG island in the promoter region of the CHI3L1 gene, which is associated with YKL-40 expression [19, 20]. To evaluate whether YKL-40 expression in monocyte-derived macrophages is influenced by the methylation status of the CHI3L1 gene, macrophages were generated in the presence of the demethylating agent 5-AZA. We first observed that 5-AZA inhibited IL-12p40 secretion, and to a smaller extent also IL-10 secretion (Figures 5A and B, respectively). In contrast, YKL-40 protein secretion and expression was not significantly affected by 5-AZA, although it needs to be noted that the highest concentration resulted in cell toxicity (Figures 5C and D, respectively).



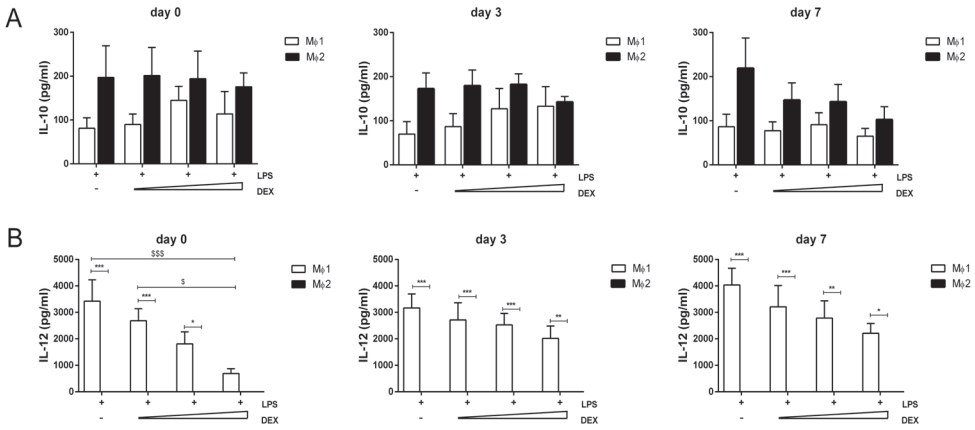


**Figure 4:** YKL-40 expression and protein secretion is inhibited by dexamethasone. YKL-40 mRNA levels (normalized expression) and YKL-40 protein secretion (figure 4A and B, respectively) in MΦ1 and MΦ2 with addition of dexamethasone at day 0, 3 and 7 of differentiation (0.1, 0.3 and 1nM) (left, middle and right panel, respectively). LL-37 mRNA levels (normalized expression) after 0, 3 and 7 days of differentiation with dexamethasone (0.1, 0.3 and 1nM) (figure 4C left, middle and right panel, respectively). Data represent means and SEM (n= 3 donors). * P≤0.05, ** P<0.01 or *** P<0.001 compared to MΦ2 with corresponding stimulus. $= p≤0.05, $$ P<0.01 or $$$ P<0.001 compared to corresponding unstimulated cells. ##: P<0.01 or ### P<0.001 compared to corresponding LPS stimulated cells.

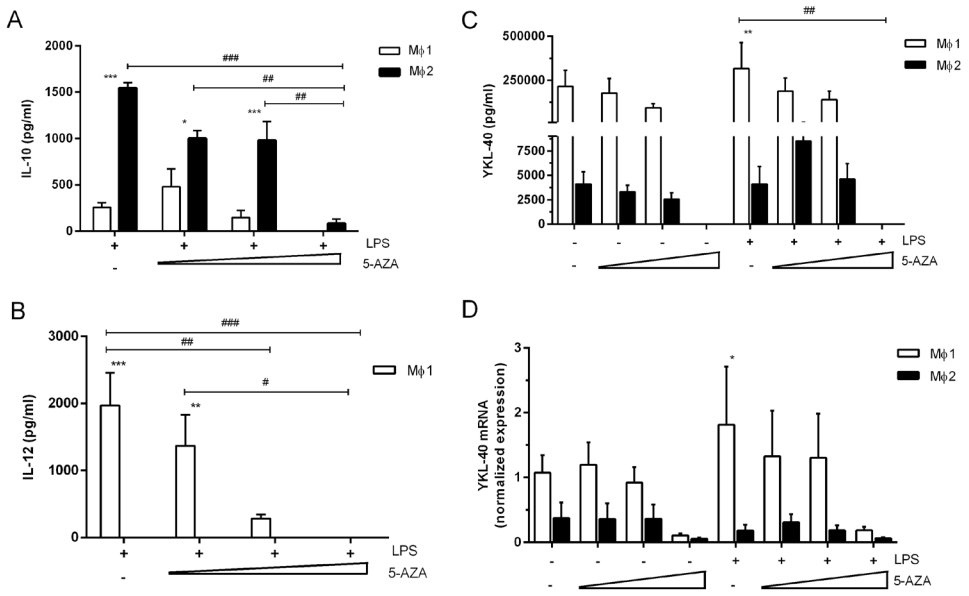


**Figure 5:** YKL-40 protein secretion and mRNA expression is inhibited by the demethylating agent 5-AZA. IL-10 and IL-12p40 secretion after stimulation with 5-AZA (concentration 0.1, 1 and 10μM) (Figures 5A and B, respectively). YKL-40 protein secretion and mRNA expression in MΦ1 and MΦ2 after culturing with 5-AZA (concentration 0.1, 0.3 and 1nM) (Figure 5C and D, respectively). Differentiated cells are stimulated with 100ng/ml LPS for 24 hours. Data represent mean and SEM (n=3 donors). * P≤0.05, ** P<0.01 or *** P<0.001 compared to MΦ2 with corresponding stimulus. # P≤0.05, ##: P<0.01 or ### P<0.001 compared to corresponding LPS stimulated cells.

## YKL-40 levels in serum and sputum of COPD patients are not changed by treatment with inhaled corticosteroids

We next used samples of the GLUCOLD study to investigate the effects of ICS treatment on serum and sputum YKL-40 levels in COPD patients. Baseline characteristics between the group of moderate to severe COPD patients treated with ICS and placebo were not significantly different as shown in Table 2. Of the 75 compliant patients, 70 serum samples and 59 induced sputum samples at baseline were available and suitable for analysis. Serum YKL-40 levels at baseline were significantly higher compared to sputum levels at baseline (respectively median 71 ng/ml versus 29 ng/ml, P<0.001). ICS treatment did not significantly change YKL-40 levels in serum and sputum compared to placebo (both p>0.05; Figure 6).

**Table 2:** Patient characteristics of compliant patients at baseline of the GLUCOLD study. The ICS group is a combination of the original 30-month fluticasone and 30-month fluticasone with salmeterol groups.
Data represent mean with SD, median with interquartile range or numbers.
GLUCOLD: Groningen and Leiden Universities Corticosteroids in Obstructive Lung Disease. ICS: Inhaled corticosteroids. $FEV_1$: Forced Expiratory Volume in 1 second. IVC: Inspiratory vital capacity. Pred: predicted.

| | ICS (n=51) | Placebo (n=24) |
|---|---|---|
| **Gender (M/F) (n)** | 45/6 | 20/4 |
| **Age (yr)** | 61.7 (7.8) | 59.4 (8.1) |
| **Smoking (y/n) (n)** | 33/18 | 17/7 |
| **Packyears (yr)** | 48 (31-56) | 42 (34-54) |
| **Post-bronchodilator $FEV_1$ (% pred)** | 62.5 (9.2) | 61.2 (8.3) |
| **Post-bronchodilator $FEV_1$ (L)** | 2.02 (0.41) | 2.00 (0.55) |
| **Post-bronchodilator $FEV_1$/IVC (%)** | 47.3 (8.8) | 46.7 (9.0) |
| **Serum YKL-40 (ng/ml)** | 66 (49-119) | 78 (60-118) |
| **Sputum YKL-40 (ng/ml)** | 52 (20-79) | 18 (12-40) |

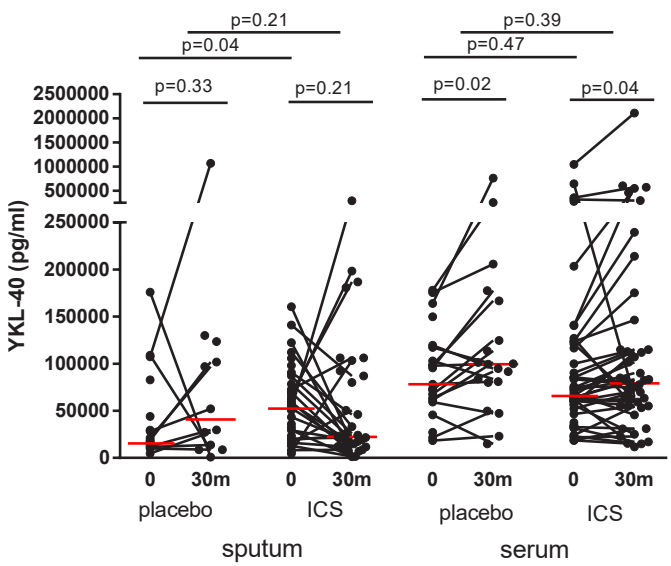


**Figure 6:** Sputum and serum YKL-40 protein levels of COPD patients before and after ICS treatment. YKL-40 levels in sputum and serum at baseline (0) and after 30 months (30m) of inhaled corticosteroids (ICS) and placebo. For comparison of the levels between baseline and 30 months, we only included patients from whom samples were available at both time points (paired data). Each dot represent a single patient, red horizontal bars represent medians.

# DISCUSSION

This study shows that secretion and expression of YKL-40, a chitinase-like protein, is higher in *in vitro* generated monocyte-derived MΦ1 than in MΦ2, and that YKL-40 expression is not further increased upon stimulation with several pro-inflammatory stimuli. In addition, YKL-40 release *in vitro* is strongly inhibited by dexamethasone especially in MΦ1, most likely due to an effect on differentiation. Addition of the demethylating agent 5-AZA did not significantly decrease YKL-40 release, but did decrease IL-12p40 production by MΦ1 and to a smaller extent IL-10 production by MΦ2 cells. YKL-40 levels in serum were significantly higher in serum than in sputum of COPD patients. Treatment of these patients for 2.5 years with inhaled corticosteroids did not significantly change serum and sputum YKL-40 levels compared to placebo. These results suggest that YKL-40 is a promising pro-inflammatory marker in *in vitro* cultured pro-inflammatory macrophages, but is less suitable for monitoring *in vivo* effect of treatment with steroids on YKL-40 in serum and sputum of COPD patients.

We show that YKL-40 is a novel marker of *in vitro* cultured monocyte-derived MΦ1, which is independent of LPS, OSM and TNF-α. This is an important observation, since many established MΦ1 markers require additional stimulation to induce expression. Our data confirm and extend previous results [16, 17, 29], reporting higher CHI3L1 expression in classically activated macrophages compared to alternatively activated macrophages. In the latter studies, in contrast to our study, interferon-gamma (IFN-γ) and IL-4 were used for MΦ1 and MΦ2 polarization, respectively [16, 17]. We extended these data by differentiating monocytes with GM-CSF and M-CSF into MΦ1 and MΦ2, respectively, and explored the effect of further stimulation after differentiation with several pro-inflammatory stimuli. We found that dexamethasone efficiently suppressed YKL-40 expression and secretion in MΦ1, but that this was mainly explained by an inhibitory effect of dexamethasone on MΦ1differentiation, thus extending previous results [17].

We found that YKL-40 levels in serum were higher than in sputum of COPD patients. Serum YKL-40 levels of our group of patients were comparable with previous studies [13, 30, 31]. However, sputum YKL-40 levels with sputum processed using the whole sample method, were considerably lower than in studies using the selected plug method [12]. This is most likely due to dilution which is inherent to the whole sample method. After long-term treatment with ICS, we did not detect a significant change in YKL-40 levels in serum and sputum compared to placebo. To our knowledge, this is the first study that evaluates the

long-term effect of ICS in serum and sputum YKL-40 levels of COPD patients. Therefore, this study presents new *in vitro* and *in vivo* data that may help to provide insight in the function of YKL-40.

The mechanisms that regulate YKL-40 expression in health and disease are partly understood. It has been demonstrated that YKL-40 expression is absent in monocytes, and markedly induced during macrophage differentiation, especially during the later stages of differentiation [2]. Promotor analysis of the *CHI3L1* gene revealed that especially Sp1, an ubiquitous transcription factor, is important for *CHI3L1* gene expression [6]. Possibly epigenetic mechanisms also contribute to CHI3L1 expression. This is supported by the finding of a SNP localized near the CpG island in the promoter region of the *CHI3L1* gene, which is associated with YKL-40 expression [19, 20, 32]. Furthermore, hypomethylation of the *CHI3L1* gene in rheumatoid arthritis is associated with increased expression of YKL-40 [33, 34]. Therefore, *CHI3L1* gene expression may be regulated by transcription factors such as Sp1 and by DNA methylation status. Our observations with 5-AZA treatment do not support a role for DNA methylation in the expression of YKL-40 in MΦ1. Further studies into methylation status of the promotor of YKL-40, the role of histone modification and microRNAs are needed to define a role of epigenetic mechanisms in the expression of YKL-40 in (lung) macrophages.

The strength of our study is that it describes a thorough evaluation of a novel, potential pro-inflammatory macrophage marker using both *in vitro* and *in vivo* approaches. Well-characterized patients with COPD used long-term, randomized, placebo-controlled treatment with ICS. However, we were unable to detect an effect of a randomized treatment with ICS on YKL-40 serum and sputum levels. Nevertheless, our study has some limitations. First, we used *in vitro* cultured monocyte-derived macrophages from whole blood of healthy subjects that were differentiated towards MΦ1 and MΦ2 instead of lung-derived (e.g. alveolar) macrophages that were differentiated under the influence of the local environment. Since the culture systems do not fully reflect *in vitro* differentiation of macrophage subsets [35, 36], it needs to be noted that the effect of steroids on lung macrophages may differ from that on *in vitro* differentiated macrophages. Furthermore, *in vivo* a heterogeneous and intermediate macrophage population exists [37], which complicates the comparison with *in vitro* generated MΦ subsets. We therefore cannot formally exclude the possibility that this has contributed to our inability to detect an effect of inhaled corticosteroids on serum and sputum YKL-40 levels. Second, the demethylating agent 5-AZA demonstrated cell toxicity which might have influenced our results. However, we found a dose-dependent inhibition of YKL-40 expression and secretion, suggesting that DNA methylation status may contribute to regulation of YKL-40 expression in MΦ1, which is in line with studies posing that methylation

of a part of the CpG island of the *CHI3L1* gene is associated with YKL-40 levels [19, 32].

How can we explain that serum and sputum YKL-40 levels of COPD patients were not significantly changed after long-term ICS treatment compared to placebo? This is unexpected since serum YKL-40 levels of rheumatoid arthritis patients rapidly decreased after one week of prednisolone [18]. However, the amount of inhaled fluticasone that reaches the systemic circulation is low [38], which could explain why serum YKL-40 levels did not significantly change with ICS therapy. In addition, lung macrophages in COPD have reduced glucocorticoid sensitivity [39, 40].

## CONCLUSION

YKL-40 is mainly expressed and secreted by MΦ1 and is not further increased by pro-inflammatory stimuli. YKL-40 release is inhibited by dexamethasone in MΦ1 *in vitro*, whereas long-term treatment of COPD patients with inhaled corticosteroids did not significantly change YKL-40 levels in serum and sputum. This suggests that YKL-40 is a potential marker for *in vitro* cultured pro-inflammatory macrophages and is not a valuable biomarker in serum and sputum of patients with COPD treated with inhaled corticosteroids.

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# CHAPTER 4

# Inhaled steroids modulate extracellular matrix composition in bronchial biopsies of COPD patients: a randomized, controlled trial

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# ABSTRACT

**Background**: Smoking and inflammation contribute to the pathogenesis of chronic obstructive pulmonary disease (COPD), which involves changes in extracellular matrix. This is thought to contribute to airway remodeling and airflow obstruction. We have previously observed that long-term treatment with inhaled corticosteroids can not only reduce bronchial inflammation, but can also attenuate lung function decline in moderate to severe COPD. We hypothesized that inhaled corticosteroids and current smoking modulate bronchial extracellular matrix components in COPD.

**Objective**: To compare major extracellular matrix components (elastic fibers; proteoglycans [versican, decorin]; collagens type I and III) in bronchial biopsies a) after 30-months inhaled steroids treatment or placebo; and b) between current and ex-smokers with COPD.

**Methods**: We included 64 moderate to severe, steroid-naive COPD patients (24/40 (ex)-smokers, 62±7 years, 46 (31-54) packyears, post-bronchodilator forced expiratory volume in one second ($FEV_1$) 62±9% predicted) at baseline in this randomized, controlled trial. 19 and 13 patients received 30-months treatment with fluticasone or placebo, respectively. Bronchial biopsies collected at baseline and after 30 months were studied using (immuno) histochemistry to evaluate extracellular matrix content. Percentage and density of stained area were calculated by digital image analysis.

**Results**: 30-Months inhaled steroids increased the percentage stained area of versican (9.6% [CI 0.9 to 18.3%]; P=0.03) and collagen III (20.6% [CI 3.8 to 37.4%]; P=0.02) compared to placebo. Increased collagen I staining density correlated with increased post-bronchodilator $FEV_1$ after inhaled steroids treatment (Rs=0.45, P=0.04). There were no differences between smokers and ex-smokers with COPD in percentages and densities for all extracellular matrix proteins.

**Conclusions**: These data show that long-term inhaled corticosteroids treatment partially changes the composition of extracellular matrix in moderate to severe COPD. This is associated with increased lung function, suggesting that long-term inhaled steroids modulate airway remodeling thereby potentially preventing airway collapse in COPD. Smoking status is not associated with bronchial extracellular matrix proteins.

# INTRODUCTION

Chronic Obstructive Pulmonary Disease (COPD) is characterized by an abnormal inflammatory response and structural alterations of the bronchial wall and parenchyma [1]. This pulmonary remodeling has been linked to airflow limitation in COPD [2, 3]. Changes in the extracellular matrix (ECM), produced by (myo)fibroblasts, epithelial cells and airway smooth muscle cells, contribute to this remodeling process and alter airway mechanics and dynamics [4, 5]. The ECM consists of three major components: elastic fibers, proteoglycans and collagens, which are involved in cell migration, proliferation, adhesion, water balance and regulation of inflammatory mediators [4].



The composition of the pulmonary ECM is different in subjects with and without COPD. Fewer elastic fibers are found in small airways and alveoli of COPD patients than in healthy controls [6, 7]. Furthermore, versican, a large proteoglycan is more abundant, while the small proteoglycan decorin is reduced in small airways in COPD compared to healthy subjects [8-10]. Collagens are the main component of the ECM, and collagen composition differs between COPD patients and healthy controls as shown by the observation that collagen type I is lower in the large and small airways [11] and collagen type III expression is lower in the small airways of COPD patients than in healthy controls [3].

Since smoking is a risk factor for COPD, this may also influence ECM composition. Indeed, cigarette smoke has been shown to induce secretion of several profibrotic growth factors, including transforming growth factor-beta (TGF-β), both in human lung fibroblasts and in lung tissue of COPD patients [12, 13]. Rodent models exposed to cigarette smoke had less lung elastic fibers, but more collagens than sham-smoked animals [14]. Others even reported an increased elastic fibers gene expression in lung tissue of severe COPD patients [15]. Smoke exposure decreased proteoglycan expression as demonstrated by a study with pulmonary fibroblasts from moderate and very severe COPD patients [16].

Although generally (neutrophil dominated) inflammation in COPD is considered to be resistant to steroids treatment, we recently observed that long-term inhaled corticosteroids (ICS) treatment partially decreased bronchial inflammation ($CD3^+$, $CD4^+$, $CD8^+$ and mast cells, without effects on neutrophils, and attenuated lung function decline in moderate to severe COPD patients participating in the GLUCOLD (Groningen Leiden Universities Corticosteroids

in Obstructive Lung Disease) study [17]. ICS may affect ECM through various mechanisms, including modulation of inflammation by profibrotic mediators and targeting ECM genes directly. This may explain differences in the effects of steroids that are observed in *in vivo* and *in vitro* studies. Whereas steroid treatment of asthmatics did not change elastic fibers and collagens in bronchial biopsies [18], steroids did inhibit serum-induced proteoglycan production in fetal lung fibroblasts [19]. In contrast to asthma, to the authors' knowledge, effects of ICS on ECM composition in COPD patients have not been described.

We hypothesized that inhaled steroids treatment modulates bronchial ECM components in COPD. In addition, we hypothesized that current smoking affects bronchial ECM.

# MATERIALS AND METHODS

## Subjects and study design

The current study is a substudy of the GLUCOLD (Groningen Leiden Universities Corticosteroids in Obstructive Lung Disease) study, a double-blind, placebo-controlled randomized trial in which 114 moderate to severe COPD steroid-naive patients were included [17]. Clinically stable subjects participating in the GLUCOLD study were aged 45-75 years, smoked ≥10 packyears, were current or ex-smokers with ≥1 month of smoking cessation and were allowed to use short-acting bronchodilators. Exclusion criteria were asthma and ICS use in the previous 6 months. Patients were randomly assigned to receive one of four treatments for 30 months: 1) fluticasone propionate 500 µg bid; 2) fluticasone/salmeterol 500/50 µg bid; 3) fluticasone 500 µg bid (6 months) and followed by placebo (24 months); or 4) placebo bid. Diskus dry-powder inhalers (GlaxoSmithKline, Zeist, The Netherlands), were used for inhalation of the study medication and placebo, and both had equal appearance. For the current study we used tissue and data of group 1 and 4. Spirometry, reversibility to salbutamol and airway hyperresonsiveness ($PC_{20}$) were determined according to international guidelines [20, 21]. Approval of the medical ethics committees of both centers was obtained: all subjects provided written informed consent [17].

## Bronchoscopy and bronchial biopsies

A fiberoptic bronchoscopy was performed at baseline and after 30 months according to standardized protocols [22]. Six bronchial biopsies per patient per visit were collected at the 3rd-5th bronchial level, one with the best morphology being used. Tissue of 64 out of 114 patients was available due to use in previous studies [17, 22, 23].

## (Immuno)histochemical stainings

Processing and analysis of bronchial biopsies was performed in line with the recommendations of the ATS/ERS task force [24] by using an internal reference parameter in the analysis. We did not take specific precautions to orientate the samples during processing to assure that the orientation of the biopsies is randomized [25]. However, since biopsies tend to curl after sampling, a random orientation of the tissue structures is favored during embedding [26]. Sections of 4 µm thickness of paraffin-embedded bronchial biopsies were used for histochemistry (elastic fibers) and immunohistochemistry for proteoglycans and collagens. Elastic fibers were stained according to Weigert's protocol [27]. Versican, decorin, collagen I and III antibodies were used after appropriate antigen retrieval, followed by horseradish peroxidase-conjugated anti-mouse or anti-rabbit EnVision system (DAKO, Glostrup, Denmark) and the chromogen NovaRed (Vector, Burlingame, CA). Images of stained biopsies are presented in Figure 1.



## Digital image analysis

Tissue samples were analyzed in a blinded manner by independent observers, unaware of the subjects' clinical data (LK, JS). Total biopsy images were acquired using a color camera (200x magnification) and analyzed with image analysis software (CellD, Olympus, Zoeterwoude, The Netherlands). The lamina propria was selected per biopsy (minimum area 0.09 $mm^2$). The percentage stained area for a specific ECM component was calculated dividing the stained area by the total selected area (volume fraction; used as an internal reference parameter; [24]). Staining intensity was further analyzed by densitometry (weighted mean per biopsy) and presented as gray value (black: gray value=0; white: gray value=255). Only immunohistochemical stainings can represent density, therefore density was not calculated for elastic fibers.

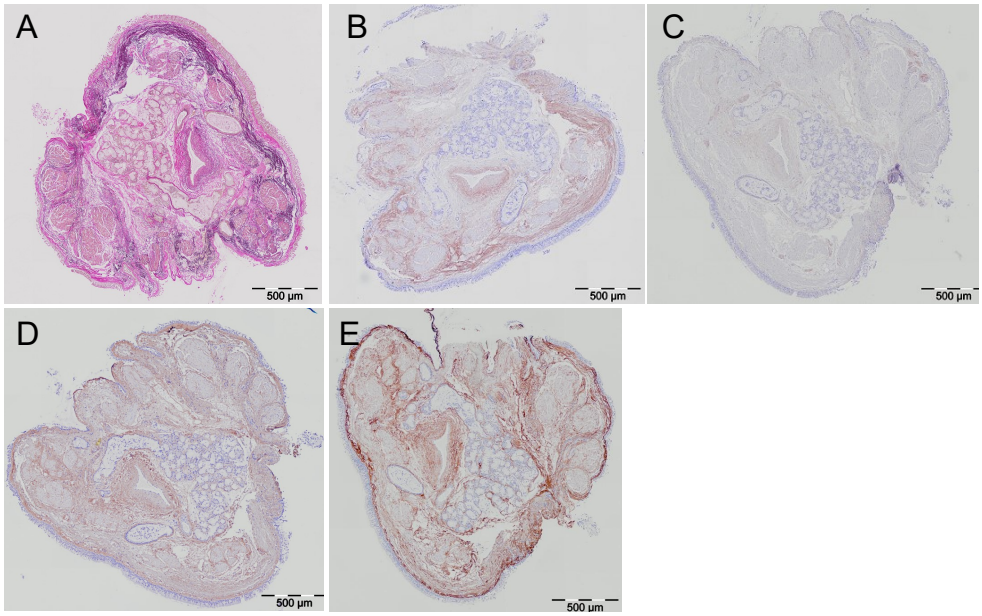


**Figure 1:** Examples of (immuno)histochemical stainings.

The same bronchial biopsy section is shown for the histochemical staining for elastic fibers (A) and the immunohistochemical stainings for versican (B), decorin (C), collagen type I (D) and collagen type III (E). Original magnification 200x. Scale bar represents 500 µm.

## Statistical analysis

Only biopsies from compliant subjects using ≥70% of the prescribed dose were analyzed (per-protocol analysis). Means with standard deviations (SD) and 95% confidence intervals (CI) or medians with interquartile range (IQR) are presented. Differences between smokers and ex-smokers were explored using Mann-Whitney tests. Paired and independent t-tests were used for evaluating the effect of ICS on ECM proteins within and between treatments, respectively. Correlations were analyzed using Spearman correlation coefficient (Rs). Statistical analysis was performed with SPSS 17.0 software (SPSS Inc., Chicago, IL). Significance was inferred at $P \leq 0.05$.

# RESULTS

## Patient characteristics

At baseline, bronchial biopsies of 64 of 114 unselected moderate to severe COPD patients [24/40 (ex-)smokers] were included. Patient characteristics of the whole group have previously been published [17, 22, 28]. 33 Patients were treated with either fluticasone or placebo for 30 months (19/19 and 13/14 adherent in fluticasone and placebo group, respectively). Mean post-bronchodilator $FEV_1$ was 62% predicted (SD 9.9%). Ex-smokers were older at baseline compared to current smokers, as is shown in Table 1. Baseline characteristics of the entire group, groups with available and unavailable bronchial biopsies, and the number of available biopsies were not significantly different between both treatment arms. During the study, six patients changed their smoking habits (balanced among groups).



## Inhaled corticosteroids and extracellular matrix proteins

Adjusted for baseline values, we found that ICS significantly increased percentage versican (9.6% [CI 0.9 to 18.3%]; P=0.03) and collagen III (20.6% [CI 3.8 to 37.4%]; P=0.02) compared to placebo (Figure 2); a trend was seen for the density of decorin (3.9 [CI -0.7 to 8.6]; P=0.09) and collagen III (8.4 [CI -1.1 to 17.9]; P=0.09). Baseline percentage and density of versican (17% [CI 3.5 to 30.6%]; P=0.02 and 8.0 [CI 2.7 to 13.3]; P=0.006, respectively) and collagen III (10.7% [0.1 to 21.4%]; P=0.03 and 7.9 [CI 0.9 to 15.0]; P=0.05, respectively) and percentage of decorin (2.0% [CI 0.5 to 3.5%]; P=0.02) were significantly higher in the placebo group than the fluticasone group. Change in smoking status was not included into our analysis, because current and ex-smokers with COPD had similar ECM composition. An increase in density of collagen I was associated with improvements in post-bronchodilator $FEV_1$ (l) (Rs=0.45, P=0.037) when we analyzed both fluticasone and placebo treated groups combined (Figure 3). No correlations were found for other ECM proteins and lung function.

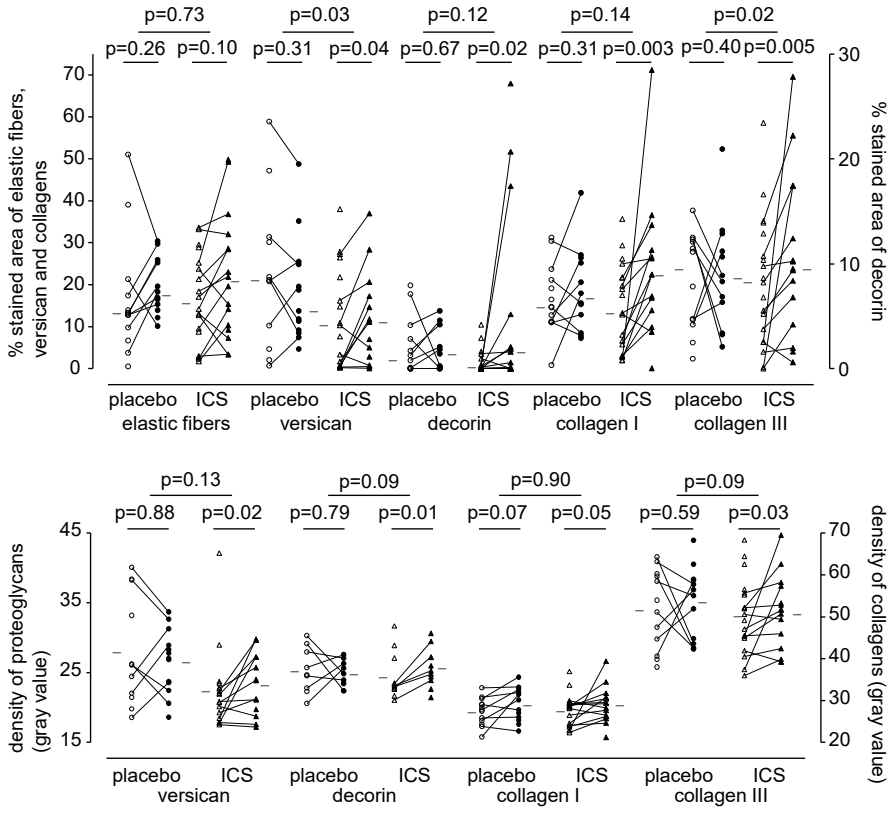


**Figure 2:** Percentage and density of stained area for placebo and fluticasone for all ECM proteins.
Percentage (upper panel) and density (lower panel) of stained area in bronchial biopsies is presented. Open figures: baseline percentage stained area, closed figures: percentage stained area after 30 months. Horizontal bars represent medians.

## Smoking status and extracellular matrix proteins at baseline

No significant differences in percentage of the area being positively stained and density of ECM proteins were found between current smokers and former smokers with COPD (Figure 4). Long-term ex-smokers (≥5.5 years, our median value) had similar percentage and density of all ECM proteins compared to short term ex-smokers (<5.5 years) and current smokers (all P>0.05). Furthermore, no relation was found between packyears and percentage or density of all ECM proteins.

**Table 1:** Patient characteristics at baseline. Patient characteristics for current smokers and ex-smokers with COPD and groups treated with placebo and fluticasone (only compliant patients). Bronchial biopsies were available at baseline of 64 (elastic fibers), 56 (versican), 61 (decorin), 61 (collagen I) and 64 (collagen III) patients. After 30 months, bronchial biopsies of 32 compliant patients were available, tissue from 29 (elastic fibers), 26 (versican), 27 (decorin), 28 (collagen I) and 28 (collagen III) patients had sufficient surface area for analysis (≥0.09 mm2) (fluticasone and placebo groups combined).

Data are presented as mean (SD) or median (IQR), unless otherwise stated. Methacholine $PC_{20}$: provocative concentration of methacholine that causes a 20% decrease in $FEV_1$, expressed as mean doubling doses. Part of the data have been published previously [17, 22, 28].

| | Smokers (n=40) | Ex-smokers (n=24) | Placebo (n=13) | Fluticasone (n=19) |
|---|---|---|---|---|
| **Males [n (%)]** | 37 (92.5) | 23 (95.8) | 12 (92.3) | 17 (89.5) |
| **Age (years)** | 60.9 (7.2) | 65.1 (6.6)* | 62.5 (7.9) | 62.0 (7.4) |
| **Current/ ex-smoker (n)** | | | 9/4 | 11/8 |
| **Packyears** | 46.8 (30.9-55.0) | 37.5 (32.1-52.5) | 42.0 (28.4-58.0) | 44.9 (31.2-51.0) |
| **Smoking cessation (years)** | | 5.5 (1.3-10.0) | 0.0 (0.0-1.5) | 0.0 (0.0-5.0) |
| **$FEV_1$ post-bronchodilator (l)** | 2.05 (0.44) | 1.94 (0.46) | 1.95 (0.61) | 2.03 (0.42) |
| **$FEV_1$ post-bronchodilator (%pred)** | 63.0 (8.7) | 59.6 (9.9) | 59.9 (9.8) | 62.5 (9.5) |
| **$FEV_1$/IVC% post-bronchodilator** | 48.7 (8.9) | 44.2 (8.9) | 44.3 (9.5) | 47.7 (8.6) |
| **Geometric mean methacholine $PC_{20}$ (mg/ml)** | 0.76 (2.9) | 0.39 (3.0) | 0.67 (1.9) | 0.41 (2.4) |

* P<0.05 compared to current smokers (two tailed unpaired t-tests).

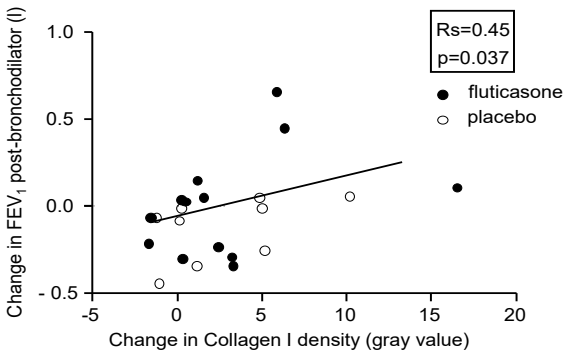


**Figure 3:** Correlation between change in post-bronchodilator $FEV_1$ (L) and change in density of collagen I. Both values represent values after 30 months minus values at baseline.
Closed circles represent fluticasone treated subjects, open circles represent placebo treated subjects.

## Correlations between extracellular matrix and lung function at baseline

Correlations between extracellular matrix and lung function at baseline percentage collagen I correlated positively with $FEV_1$ (% predicted) post-bronchodilator (Rs=0.31, P=0.015) (Figure 5, left panel) and $FEV_1$/IVC% (Rs=0.38, P=0.003). In addition, percentage collagen type I and III correlated with $PC_{20}$ (Rs=0.33, P=0.012; Rs=0.37, P=0.004, respectively) (Figure 5, right panel). Percentage collagen I, but not collagen III, was significantly lower in GOLD stage III (n=9) than GOLD stage II (n=55) (medians 5.5% and 17.7%, respectively, P=0.01). No significant correlations were found between lung function at baseline and densities of all ECM proteins.

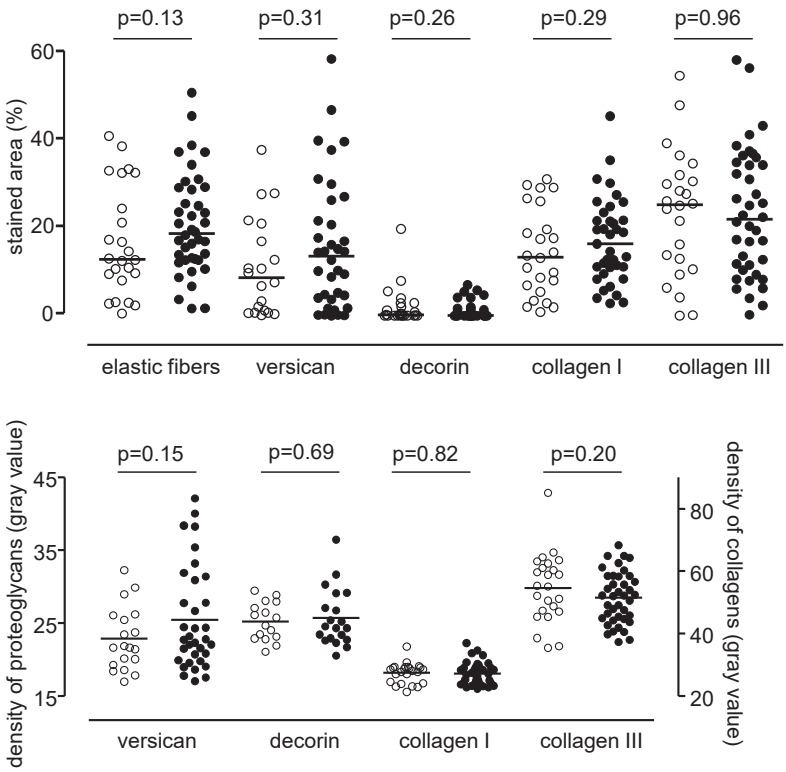


**Figure 4:** Percentage and density of stained area at baseline of ex-smokers and smokers with COPD. Percentage (upper panel) and density (lower panel) of stained area in bronchial biopsies is presented. Ex-smokers are presented as open circles, current smokers as closed circles. Horizontal bars represent medians. No significant differences were found for all studied extracellular matrix proteins (both percentage stained area and density).

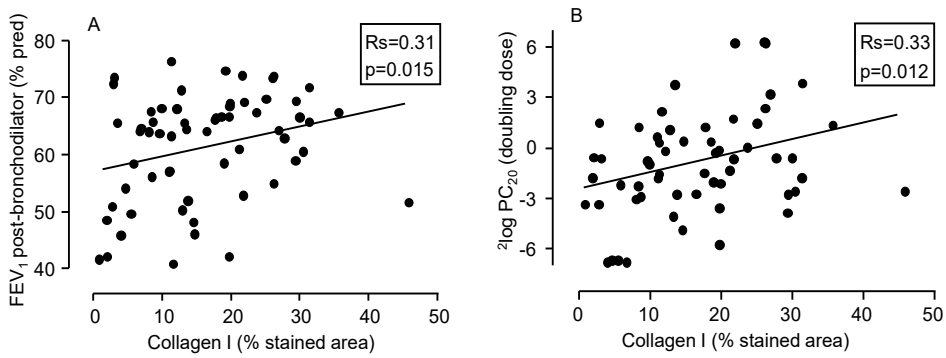


**Figure 5:** Correlation between percentage collagen type I at baseline and lung function parameters.
Panel A presents post-bronchodilator $FEV_1$ (% predicted) and panel B shows $PC_{20}$ (in doubling dose). Each dot represents a single patient.

# DISCUSSION

Our results show that 30-month treatment with inhaled corticosteroids increases the percentage stained area of versican and collagen III, indicating that long-term treatment possibly influences the remodeling process in the airways. Furthermore, lung function is weakly, but positively correlated with collagen I both at baseline and with regard to changes in $FEV_1$ and collagen I that occurred after treatment. In addition, we show that the content of ECM proteins in bronchial biopsies did not significantly differ between smokers and ex-smokers with moderate to severe COPD.

This study shows that the content of elastic fibers, major proteoglycans and collagens in the bronchial mucosa are similar in current and ex-smokers with COPD. Our findings extend previous observations, showing no difference in the percentage elastic fibers in COPD patients and smokers without airway obstruction [11, 29]. We observed no significant difference in versican and decorin content between current and ex-smokers with COPD, which is in line with an *in vitro* study with cultured lung fibroblasts of moderate COPD patients and control subjects. Cigarette smoke extract (CSE) exposure of these cells did not affect versican gene expression, but decreased decorin gene expression [16]. This apparent difference with our findings could be explained by the fact that smoke-exposed fibroblasts

are only selectively triggered compared to a multifactorial environment *in vivo*. Finally, in our study collagen type I and III were not significantly different between current and ex-smokers with COPD, which is similar to recent observations in cultured fibroblasts of COPD and non-COPD patients [30].

The percentage of versican and collagen III increased with long-term ICS treatment compared to placebo, without significant changes in elastic fibers, decorin and collagen I. In line with this, ICS for four weeks or 3.5 years did not affect elastic fibers content in bronchial biopsies of asthmatics compared to healthy controls [18]. Notably, we found a significant increase in collagen III, but not collagen I, after 2.5 years of ICS treatment compared to placebo, which was associated with lung function. Previous studies in COPD patients showed that gene expression of collagen 1α1 and collagen 3α1 in small airways and parenchyma was decreased in association with lower $FEV_1$ [3, 31]. Thus, collagen may have stabilizing effects on the collapsible airways in patients with COPD, which could be further enhanced by long-term use of ICS.



Our study has various strong points. We included only steroid-naive COPD patients, excluding possible influences of steroids on ECM components at baseline. Both the percentage and density of the stained area in bronchial biopsies were analyzed: the percentage corresponds to the presence of the ECM protein, whereas density represents the local amount of ECM protein. For the analysis of the percentage, we used the total selected tissue area for analysis as an internal reference parameter according to the recommendations of the Joint ATS/ERS Task Force [24]. We considered the possibility that part of our changes is explained by an effect of ICS on edema. However, less edema resulting from ICS treatment would probably have increased percentage and density all studied ECM proteins, whereas in our study the percentage of only some ECM proteins was affected. Furthermore, we previously found lower numbers of selected bronchial inflammatory cells after ICS treatment in the current study [17]. We did not find correlations between the effect of ICS treatment on inflammatory cells and ECM components (data not shown).

There are some considerations when interpreting our results. Matched bronchial biopsies both at baseline and follow-up were available from approximately half of our COPD patients, because part of the tissue was no longer available. This could have negatively affected the power of our study. Still, the number of available biopsies was similar among both groups. Furthermore, since one biopsy per patient per visit was studied, we cannot exclude that local heterogeneity of ECM proteins has affected our results. To minimize selection bias, we

only selected biopsies with the largest lamina propria. Lung tissue specimens from healthy or never-smokers were not available, but comparisons with these groups were beyond the objectives of this study. Furthermore, features of remodeling in COPD are different between large and small airways, nevertheless we evaluated the ECM in the central airways only [2] and important correlations with lung function could still be observed. Finally, despite treatment randomization, we accidentally found that the percentage and densities of versican, decorin and collagen III at baseline were significantly higher in the placebo than the fluticasone group. Not withstanding this, when still adjusted for the baseline values, we observed effect of ICS therapy. Taken together, we do not believe that the above limitations largely affected our results.

How can we explain that smoking has no effect on ECM? Exposure of cultured pulmonary fibroblasts of moderate and very severe COPD patients to CSE resulted in downregulation of decorin, but not versican and collagen type I and III expression [16, 30]. In addition, collagen I and tropoelastin were dose-dependently inhibited by CSE in rat fetal lung fibroblasts [32]. Mice with long-term exposure to cigarette smoke showed a decrease in elastic fibers and collagen type III, without significant effect on collagen I [33]. We could only partially confirm these *in vitro* and animal studies. Inflammation and remodeling in the lung *in vivo* are simultaneous and complex ongoing processes and may not be mimicked by studies in isolated fibroblasts and inbred animals kept under specific conditions. Furthermore, after smoking cessation bronchial inflammation (at least) partially persists [22], which is in line with our finding of similar ECM composition between smokers and ex-smokers.

We showed a positive correlation between the content of collagen and lung function after treatment with inhaled steroids. However, the current opinion of remodeling is that airway wall thickening is strongly associated with progression of COPD [2], suggesting that increased ECM deposition is related to a decreased lung function. How can we explain this apparent contradiction? In COPD, an imbalance between proteases and anti-proteases is present, as shown by an excess of matrix metalloproteinases (MMP) and a relative shortage of tissue inhibitor of metalloproteinases (TIMP) [34]. MMP degrade both collagens and proteoglycans [2, 34, 35]. Dexamethasone can reduce MMP-9 and increase TIMP-1 release from alveolar macrophages of COPD patients [36], which may result in a decreased capacity to degrade ECM. This is in line with our observation that ICS increase collagen and versican. Also the observation from Annoni et al [11], showing that patients with COPD have lower collagen I densities in their airways, is in line with the speculation that an increase in collagen I induced by ICS could stabilize the airways. Furthermore, the observed positive correlation between collagen with lung function and $PC_{20}$ before and after long-term ICS therapy also

suggests that increased airway wall fibrosis is actually preventing both airway collapse and attenuating airway smooth muscle contractions in COPD. Besides airway remodeling, emphysema might also influence airway collapse, which could contribute to the airflow obstruction. Unfortunately, no data were collected to quantify the extent of emphysema in our cohort of COPD patients.

Although smoking cessation shows positive clinical effects [1], smoking status was not significantly correlated with our studied ECM components. Treatment with ICS increased the percentage versican and collagen III. We found positive correlations between ECM proteins and several lung function parameters at baseline and after treatment with ICS. Therefore, our data may implicate that steroids alter airway structure by increasing ECM content in COPD which is associated with preserved lung function. This suggests that increased presence ECM proteins do not by themselves lead to detrimental consequences, but instead can prevent airway collapse.



In conclusion, we showed that treatment for 30 months with inhaled corticosteroids increased the relative content of versican and collagen III in the large airways of patients with moderate to severe COPD. Our data suggest that steroids not only prevent bronchial inflammation but possibly also alter airway structure by increasing specific ECM proteins in COPD that are associated with improvements in lung function. Further studies are needed to confirm these findings in other studies, and to understand the possible implications of these findings for current treatment strategies and for the development of future, targeted anti-remodeling medication in COPD.

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# CHAPTER 5

# Relapse in $FEV_1$-Decline after Steroid Withdrawal in Chronic Obstructive Pulmonary Disease

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# ABSTRACT

**Background**: We previously observed that 30 months of inhaled corticosteroids (ICS) can attenuate $FEV_1$-decline in COPD, but it is unclear whether withdrawal induces a relapse. We hypothesized that $FEV_1$-decline, airway hyperresponsiveness (AHR) and quality of life (QOL) deteriorate after ICS cessation even after prolonged use.

**Methods**: 114 moderate to severe COPD patients finished randomized treatment with 6-month (F6) or 30-month fluticasone (F30) (500µg, bid), 30-month fluticasone/salmeterol (FS30) (500/50µg, bid) or placebo during GLUCOLD-1 (GL1). The subsequent 5 years [GLUCOLD-2 (GL-2)], patients were prospectively followed annually, treated by their physician. Post-bronchodilator $FEV_1$, AHR and QOL were initially recorded at baseline, 30 months (GL1) and annually during GL2. Analysis was performed by linear mixed-effects models.

**Results**: Amongst 101 adherent patients during GL1, 79 patients started and 58 completed GL2. Patients using ICS during GL1, but only using ICS 0-50% of time during GL2 (n=56/79) had significantly accelerated annual $FEV_1$-decline compared to GL1 (difference GL2-GL1 [95%CI]: FS30 -68ml/year [-112 to -25], P=0.002; F30 -73ml/year [-119 to -26], P=0.002), accompanied by deterioration in AHR and QOL.

**Conclusions**: ICS discontinuation after 30-month in COPD can worsen lung function decline, AHR and QOL during 5-year follow-up. This suggests that ICS treatment lacks sustained disease modifying effect after treatment cessation.

# INTRODUCTION

Patients with stable Chronic Obstructive Pulmonary Disease (COPD) are currently treated with long-acting bronchodilators and in case of frequent exacerbations with inhaled corticosteroids (ICS) [1]. In these patients, ICS use reduces exacerbations, the rate of decline in quality of life (QOL) and the risk of death and hospitalization [2, 3]. However, the effect of ICS on lung function decline remains controversial.

Several studies in COPD presented transient improvements in lung function with ICS, whereas others failed to show benefits on $FEV_1$, QOL and frequency of exacerbations [4-6]. We have previously reported that 30-month treatment with fluticasone and salmeterol in 114 well-characterized, moderate to severe COPD patients decreased inflammation, attenuated lung function decline and improved QOL [7].

In contrast, long-term effects after ICS withdrawal on lung function and QOL have been little studied. Recent research indicates that discontinuation after 6 weeks of ICS in severe-to-very-severe COPD patients leads to a greater decrease in $FEV_1$ without effect on the number of exacerbations during a 1-year follow-up compared to the ICS continuation group [8]. Other ICS withdrawal studies found a deterioration in lung function, increased frequency of exacerbations and a lower QOL during 6-12 month follow-up compared to the non-ICS group [9, 10]. Thus, there is a clinical need for careful monitoring disease outcomes after withdrawal of long-term ICS treatment in COPD [11].

We hypothesized that lung function decline, airway hyperresponsiveness (AHR) and health-related QOL deteriorates after withdrawal of ICS in COPD patients who had previously been randomized to 30-month ICS treatment, but had no or reduced ICS treatment during 5 subsequent years of prospective follow-up.

# MATERIALS AND METHODS

## Patients and design

For the first interventional part of the GLUCOLD [Groningen Leiden Universities

Corticosteroids in Obstructive Lung Disease] study (GL1), a double-blind, placebo-controlled randomized trial, 114 stable, moderate to severe, steroid-naive COPD patients were included [7]. Participants were randomized to receive one of the following twice daily treatments as dry-powder inhaler: 6-month (F6) or 30-month fluticasone propionate (F30; 500µg), 30-month fluticasone with salmeterol (FS30; 500/50µg; single Diskus), or 30-month placebo.

During the present observational, prospective GLUCOLD follow-up study (GL2), participants visited the outpatient clinic annually for 5 consecutive years. At the start of GL2, the participants' physicians were recommended to treat the patients according to the guidelines [12]. This implies that some patients stopped using ICS, whereas others intermittently or continuously used ICS during GL2.

After completion of GL2, the patients' pharmacy presented an overview of delivered medications during the past 5 years of inhaled/oral steroids and antibiotics. Treatment adherence to the prescribed medication during GL2 was not checked.

Post-bronchodilator spirometry and measures of QOL were recorded at baseline and after 30 months (GL1) and subsequently yearly during follow-up (GL2) [7]. AHR to methacholine ($PC_{20}$) was measured at baseline, after 30 months, 2 and 5 years follow-up. The ethics committees of Leiden University Medical Center and University Medical Center Groningen approved the original and follow-up study. All patients provided new written informed consent for GL2.

## Outcomes

The primary outcome was the difference in the annual decline in post-bronchodilator $FEV_1$ during 5-year of follow-up (GL2) compared to the first 30 months (GL1). Secondary outcomes were differences between GL2 and GL1 in AHR and QOL, measured by the MRC dyspnea score (Medical Research Council), St. George's Respiratory Questionnaire (SGRQ) and Clinical COPD Questionnaire (CCQ) [13-14].

## Statistical Analysis

We used only data of adherent patients in GL1 (using ≥70% of the prescribed dose) [7]. Data of participants who did not complete GL2 were also used for analysis and analyzed with SPSS 22.0 software (SPSS Inc. Chicago, IL). The analysis was stratified for original treatment group

and ICS use during GL2. We used linear mixed-effect models with a random intercept for each subject using all $FEV_1$ measurements during the entire study as outcome variable and an unstructured covariance matrix. To assess the difference in $FEV_1$-decline between GL1 and GL2 we included two time variables in the models; time1: time since start of GL1 (range 0-7.5 years); time2: time since start of GL2 (range 0-5 years, during GL1 this value is zero). ICS use during GL2, based on delivered prescriptions by the pharmacies, was divided in the following groups: all (compliant) patients; patients without ICS use; patients with 0-50% of the time ICS use (which included the group without ICS use) and patients with 50-100% of the time ICS use. The daily dose of ICS (in µg, in beclomethasone dipropionate (BDP) equivalents) during 5 years was calculated as daily sum of the different doses of ICS (in µg/day) divided by the total time that ICS were used (days). For selected analyses, we combined the original FS30 and F30 groups to increase power. Given the limited sample size of patients completing the 7.5 years of prospective follow-up, possible confounders (smoking, age, sex and center) were not included in the model. A previous post-hoc analysis showed that smoking was unlikely to be a major confounder [7]. Baseline patient characteristics and daily dose of ICS dose were analyzed by Kruskal-Wallis tests, analysis of variance or $X^2$ tests. Data are presented as change in estimates between GL2 and GL1 with 95% confidence interval (CI), means with standard deviations or medians with interquartile range. Statistical significance was inferred at $P \leq 0.05$ (two-sided).



## RESULTS

At the start of the GLUCOLD study (GL1), 114 patients had been randomized to receive one of the above mentioned randomized 30-month treatments; 101 participants were adherent during GL1 [7]. Eighty-six patients completed GL1, 79 started the GLUCOLD follow-up study (GL2) and 58 patients completed GL2 (Figure 1). Patient characteristics at baseline and at start of GL2 (Table 1) were similar among the original treatment groups, except for a significantly higher post-bronchodilator $FEV_1$ after 30-month treatment among the FS30 and F30 groups, compared to the F6 and placebo groups [7]. Most patients (56/79 patients) did not use any or used 0-50% of the time ICS during GL2. The mean daily ICS dose during 5 years was 960µg (SD 496µg, in BDP equivalents, Table 2), which was not significantly different between the original treatment groups.

| | Baseline GL1 | | | | Start of GL2 | | | |
|---|---|---|---|---|---|---|---|---|
| | F6 (n=26) | F30 (n=26) | FS30 (n=25) | placebo (n=24) | F6 (n=23) | F30 (n=22) | FS30 (n=21) | placebo (n=20) |
| **Gender (M/F) (n)** | 22/4 | 23/3 | 22/3 | 20/4 | 20/3 | 219/3 | 20/1 | 18/2 |
| **Age (yr)** | 64.1 (7.4) | 62.3 (7.7) | 62.1 (7.7) | 59.8 (8.2) | 65.8 (7.7) | 63.1 (7.3) | 64.6 (7.1) | 63.5 (7.4) |
| **Smoking (y/n) (n)** | 14/12 | 16/10 | 17/8 | 17/7 | 10/13 | 11/11 | 12/9 | 11/9 |
| **Packyears (yr)** | 41 (29-57) | 44 (31-55) | 47 (31-56) | 42 (34-54) | 42 (28-54) | 44 (32-56) | 52 (35-57) | 46 (37-58) |
| **Post -bronchodilator $FEV_1$ (%pred)** | 64.6 (8.6) | 63.7 (9.1) | 61.2 (9.4) | 61.2 (8.3) | 63.7 (12.5) | 65.0 (11.1) | 61.9 (12.1) | 57.3 (8.7) |
| **Post-bronchodilator $FEV_1$ (L)** | 2.01 (0.47) | 2.04 (0.42) | 2.01 (0.40) | 2.00 (0.55) | 1.96 (0.60) | 2.06 (0.51) | 2.02 (0.51) | 1.85 (0.56) |
| **Post-bronchodilator $FEV_1$/FVC (%)** | 50.8 (8 | 48.9 (9.0) | 45.6 (8.4) | 46.7 (9.0) | 50.0 (9.8) | 49.1 (9.9) | 45.5 (9.7) | 42.1 (9.9) |
| **Geometric mean $PC_{20}$ (mg/ml)** | 0.71 (3.2) | 0.41 (2.4) | 0.72 (2.7) | 0.66 (2.0) | 0.94 (2.9) | 2.29 (2.9) | 1.33 (3.0) | 0.36 (2.4) |
| **MRC dyspnea score** | 2.46 (0.65) | 2.62 (0.57) | 2.88 (0.97) | 2.71 (0.81) | 2.61 (1.12) | 2.45 (0.67) | 2.43 (1.03) | 2.60 (1.05) |
| **CCQ total score** | 1.10 (0.56) | 1.26 (0.58) | 1.53 (0.76) | 1.62 (1.24) | 1.37 (0.82) | 1.29 (0.57) | 1.60 (1.02) | 1.49 (1.09) |
| **SGRQ total score** | 25.7 (15.2) | 32.9 (10.9) | 28.1 (13.2) | 33.4 (18.5) | 24.8 (16.9) | 28.2 (14.5) | 25.7 (13.3) | 31.3 (20.0) |

**Table 1:** Patient characteristics at baseline of randomized therapy (GL1) and at the start of 5 years post-treatment follow-up (GL2) for the original GL1 treatment groups. Data are presented as mean and standard deviation, median with interquartile range (for packyears) or numbers. $PC_{20}$ is expressed as mean doubling doses. F6: 6 months treatment with fluticasone and 24 months with placebo; F30: 30 months treatment with fluticasone; FS30: 30 months treatment with fluticasone and salmeterol; $PC_{20}$: provocative concentration of methacholine that causes a 20% decrease in $FEV_1$; MRC: Medical Research Council; SGRQ: St. George's Respiratory Questionnaire; CCQ: Clinical COPD Questionnaire.

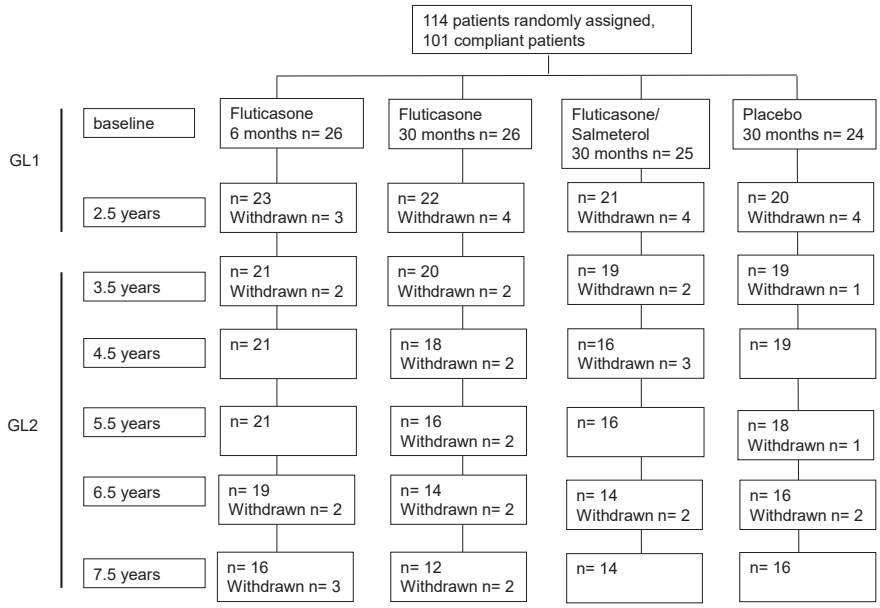


**Figure 1:** Study flow diagram. Number of randomly assigned patients who were adherent to the original therapy (used ≥70% of prescribed dose during GLUCOLD study (GL1)). After 2.5 years, the GLUCOLD follow-up study (GL2) started. The number of patients who remained in GL2 and those withdrawn are presented.

## Lung function decline

Annual $FEV_1$-decline was significantly faster during GL2 than GL1 in patients who used ICS 0-50% of the time during GL2 in the original FS30 group (difference in $FEV_1$-decline between GL2 and GL1 (95% CI)) (-68 ml/year [-112 to -25 ml/year]; P=0.002) and F30 group (-73 ml/year [-119 to -26 ml/year]; P=0.002) (Table 3 and Figure 2). When analyzing patients without ICS use during GL2, $FEV_1$-decline during GL2 compared to GL1 was even more pronounced (original FS30 group: -106 ml/year [-171 to -41 ml/year]; P=0.002; F30 group: -84 ml/year [-149 to -18 ml/year]; P=0.01). Patients in the original combined FS30/F30 groups using ICS 50-100% of the time during GL2 had a decline in $FEV_1$ of -59 ml/year ([-106 to -11 ml/year]; P=0.02) in GL2 compared to GL1.

**Table 2:** Number of compliant patients at the start of the GLUCOLD follow-up study (GL2) using ICS and daily dose of ICS (in µg) during 5 years in those patients who used ICS during GL2.
The daily dose of ICS (in µg, in BDP equivalents) during 5 years was calculated by the sum of the different doses of ICS per day (in µg/day), divided by the total time that ICS were used (in days). Doses were based on data provided by the patients' pharmacy.

| Original treatment group | No ICS use | ≤50% use of ICS (n) | >50% use of ICS (n) | 100% use of ICS (n) | Daily dose ICS (µg) |
|---|---|---|---|---|---|
| **F6** | 8 | 7 | 6 | 0 | 671 (383) |
| **F30** | 10 | 5 | 3 | 2 | 1025 (590) |
| **FS30** | 5 | 6 | 5 | 3 | 1132 (490) |
| **Placebo** | 11 | 4 | 3 | 1 | 1049 (418) |
| **Total** | 34 | 22 | 17 | 6 | 960 (496) |

Daily dose of ICS is presented as means with standard deviations. F6: 6 months treatment with fluticasone and 24 months with placebo; F30: 30 months treatment with fluticasone; FS30: 30 months treatment with fluticasone and salmeterol.

## Airway hyperresponsiveness

Patients of the combined original FS30/F30 groups without ICS use and patients who used ICS 0-50% of time during GL2 showed a deterioration in methacholine $PC_{20}$ in GL2 compared to GL1 (-1.3 doubling dose/year [-2.0 to -0.5], P=0.002; -1.1 doubling dose/year [-1.8 to -0.5], P=0.001, respectively). Patients in the original placebo group who used 50-100% of the time ICS during GL2 had an increase in $PC_{20}$ in GL2 compared to GL1 (1.5 doubling dose/year [0.06 to 2.9]; P=0.04).

**Table 3:** Annual decline in post-bronchodilator $FEV_1$ (ml/year) during GL2 compared to GL1. Treatment in GL1 (first column) and use of inhaled corticosteroids (ICS) during GL2 (upper row). * P<0.05

| Treatment in GL1 | all patients (n=79) | no ICS use (n=34) | 0-50% ICS use (n=56) | 50-100% ICS use (n=23) |
|---|---|---|---|---|
| **F6** | -19 (-49 to 11) | -19 (-71 to 33) | -26 (-63 to 10) | -11 (-71 to 50) |
| **F30** | -67* (-103 to -30) | -84* (-149 to -18) | -73* (-119 to -26) | -37 (-122 to 48) |
| **FS30** | -65* (-98 to -31) | -106* (-171 to -41) | -68* (-112 to -25) | -72* (-128 to -16) |
| **placebo** | 10 (-18 to 39) | 13 (-28 to 54) | -3 (-36 to 31) | 34 (-15 to 83) |

Data presented as estimates and 95% CI. F6: 6 months treatment with fluticasone and 24 months with placebo; F30: 30 months treatment with fluticasone; FS30: 30 months treatment with fluticasone and salmeterol.



## Health-related quality of life

Complete withdrawal of ICS during GL2 in the original combined FS30/F30 groups was accompanied by worsening of the MRC dyspnea score in GL2 by 0.2 points/year (0.06 to 0.3 points/year, P=0.006), SGRQ total score by 2.5 points/year (0.2 to 4.7 points/year, P=0.03), CCQ total score by 0.1 point/year (0.008 to 0.2 points/year, P=0.03) and CCQ symptom score by 0.2 points/year (0.05 to 0.3 points/year, P=0.008) compared to GL1.

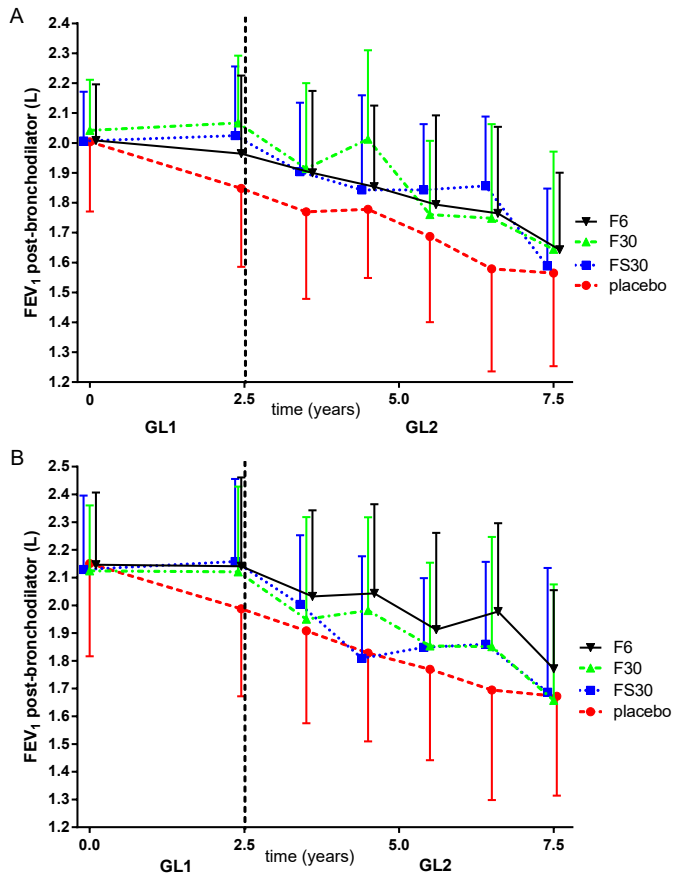


**Figure 2:** Mean post-bronchodilator $FEV_1$ (L) and 95% CI during GL1 and GL2 over time for all compliant patients (A, upper part) and those using 0-50% ICS during GL2 (B, lower part) of the four original treatment groups.

F6: 6months treatment with fluticasone and 24 months with placebo; F30: 30 months treatment with fluticasone; FS30: 30 months treatment with fluticasone and salmeterol.

# DISCUSSION

This study shows that discontinuation of ICS after long-term use in COPD seems to accelerate lung function decline during subsequent follow-up together with deterioration in AHR and health-related QOL. This indicates that the initial benefits of 30-month ICS treatment on COPD progression are confined to active treatment and are not sustained after long-term cessation of ICS.

This is the first observation that lung function decline significantly accelerates during 5-year follow-up in moderate to severe COPD patients who did not use or intermittently continued ICS after 30-month randomized ICS treatment. Our results on $FEV_1$ decline are in line with those of previous trials [8-10, 15]. However, different study designs, follow-up time, disease severities, sample sizes and definitions of ICS withdrawal makes it difficult to compare the annual decline in $FEV_1$ between these studies. Furthermore, QOL measured by MRC, SGRQ and CCQ, deteriorated during long-term follow-up compared to the previous randomized treatment period, although the minimal clinically important difference was not reached [16, 17]. SGRQ total score gradually declines over time after withdrawal of ICS in patients who previously used long-term ICS, which is similar to the decline in SGRQ found in the ISOLDE trial [15]. Our data extends previous observations, showing a deterioration in QOL after discontinuation of ICS compared to salmeterol or placebo during follow-up in COPD patients [9, 10]. Finally, we observed a deterioration in AHR during 5 years after ICS cessation, which thus far had only been described in asthmatics and after short-term treatment in COPD [7, 18, 19]. In contrast to asthma, where AHR is mostly related to the degree of inflammation [20], we previously showed that AHR in this group of COPD patients is associated with both airflow limitation and airway inflammation [7, 21]. Taken together, the present study provides novel data on relapse of $FEV_1$ decline and AHR after discontinuation of ICS after long-term use in COPD patients.



The strength of our study is represented by its long-term prospective design with repeated monitoring during 5 years of observational follow-up. In addition, the concordance of the currently observed changes in lung function decline, AHR and QOL further contributes to the confidence in our data. Nevertheless, this study had some limitations. First, only half of the patients randomized at baseline completed the entire 7.5-year follow-up, mostly due to the natural course of the disease with associated mortality and comorbidities. This could have led to a loss of statistical power compared to GL1 and a selection bias, even though the number of withdrawn patients during GL2 was similar among the original treatment groups. Second, only few patients used ICS 50-100% of the time during GL2 (n=23). Although only adherent patients during GL1 were used for the follow-up analysis, compliance to inhalation medication was not checked during GL2, thereby reflecting adherence in daily practice [22]. Creating small subgroups of patients using steroids during GL2 made it difficult to detect a difference in annual decline of $FEV_1$ in the FS30 group between those who used ICS 0-50% and 50-100% of the time during GL2. Third, pneumonia and exacerbation rates were not recorded during GL2, though prolonged use of ICS in COPD may have adverse effects, like the risk of serious pneumonia, especially with high-dose fluticasone [23]. However, retrospectively retrieved rates of antibiotics and prednisolone courses were similar between the groups. Amongst the 26 patients who died during the 7.5-year follow-up only one

patient in the original F6 group (not participating in GL2) died of a pneumonia, yet occurring as complication of a lung carcinoma. Furthermore, overall survival was not statistically different between the original treatment groups. Hence, our data do not allow conclusions on the incidence of pneumonia or exacerbations during or after ICS usage.

How can we interpret our results? Effects of ICS in COPD are affected by the complexity of this heterogeneous disease, classified by clinical, physiologic, pathological and radiologic variables and varies by host susceptibility and/or cigarette smoking [24, 25]. Previously, we described that ICS treatment attenuates lung function decline and decreases inflammation in this group of COPD patients [7]. The present study shows a relapse in lung function decline after discontinuation of ICS. The presently observed rate of decline is higher compared to that in the recent WISDOM study [8]. Moderate to severe COPD patients as in our study are representing a pathophysiological distinct group and thereby potentially more responsive to ICS [4]. The annual rate of decline after ICS discontinuation could therefore be larger compared to severe airflow obstruction [9, 10, 15]. This suggests (at least) temporary disease modification of COPD, especially during active and prolonged periods of ICS use. Future analyses should focus on inflammatory outcomes to determine whether our previously observed anti-inflammatory effects of ICS in COPD are also ablated.

Our results may have consequences for future treatment of COPD patients. Although meta-analyses show limited benefits of ICS in COPD [3], the original GLUCOLD study suggests that maintenance use of ICS can lead to attenuated lung function decline at least in this subset of COPD patients [7]. Notably, the current long-term follow-up study indicates that such benefits are not maintained after prolonged cessation of treatment. Though these data may suggest that ICS treatment in COPD should not be discontinued, this study was not designed to show evidence of any continued benefits of prolonged ICS therapy.

## CONCLUSIONS

The present data indicate that discontinuation after 30-month use of ICS in this group of moderate to severe COPD deteriorate lung function decline during 5-years follow-up. This is accompanied by worsening in AHR and a small drop in QOL. These results suggest that, whereas initial long-term ICS use can have a disease modifying effect in particular COPD patients, such benefits disappear when ICS are discontinued.

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# CHAPTER 6

# Airway Inflammation in COPD after Long-term Withdrawal of Inhaled Corticosteroids

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**Eur Respir J, in revision**

# ABSTRACT

**Background**: Long-term treatment with inhaled corticosteroids (ICS) might attenuate lung function decline and decrease airway inflammation in a COPD subset, whereas ICS discontinuation relapses lung function decline. We hypothesized that airway inflammation increases after ICS withdrawal following long-term ICS treatment in COPD.

**Methods:** In the GLUCOLD-1 study (GL1) 114 patients with moderate to severe COPD were treated randomized to 6-month or 30-month fluticasone propionate (500µg bid), 30-month fluticasone/salmeterol (500/50µg, bid) or placebo. In the follow-up study (GL2), patients were followed prospectively for 5 consecutive years, treated by their physician. Bronchial biopsies and induced sputum were collected at baseline (GL1), 30-month (end GL1) and 7.5-year (end GL2) to assess inflammatory cell counts. Analysis was performed by linear mixed-effects models.

**Results**: In patients using ICS during GL1 and using ICS 0-50% of time during GL2 (n=61/85), bronchial cells increased in GL2 significantly: $CD3^+$ (fold change/year GL2-GL1 [95%CI] 2.68 [1.87-3.84]), $CD4^+$ (1.91 [1.33-2.75]), $CD8^+$ cells (1.71 [1.15-2.53]) and mast cells (1.91 [1.36-2.68]). Additionally, sputum total cell counts increased significantly in GL2 (1.90 [1.42-2.54]), macrophage (2.10 [1.55-2.86]) neutrophil (1.92 [1.39-2.65]) and lymphocyte counts (2.01 [1.46-2.78]).

**Conclusions**: ICS discontinuation increases airway inflammation in moderate to severe COPD patients, suggesting that anti-inflammatory effects of ICS in COPD are not maintained after ICS discontinuation.

# INTRODUCTION

Chronic Obstructive Pulmonary Disease (COPD) is characterized by chronic inflammation in the airways, with neutrophils, macrophages and $CD8^+$ T-cells as major inflammatory cell types [1]. More severe airflow limitation is associated with more severe airway inflammation. As the progressive course of the disease continues to more severe airflow limitation, airway inflammation increases over time [2-4]. Except for smoking cessation, there is currently no therapy that halts the inflammatory process in the airways.

According to current guidelines, treatment with inhaled corticosteroids (ICS) is recommended for severe and very severe COPD patients in case of frequent exacerbations. Up to now only a few trials have evaluated the anti-inflammatory effect of ICS in bronchial biopsies and bronchoalveolar lavage (BAL) in COPD. A recent meta-analysis showed that 12- to 26-week ICS treatment in COPD reduced $CD4^+$ and $CD8^+$ cells in bronchial biopsies [5-10]. In addition, ICS reduced neutrophil and lymphocyte counts in BAL, but increased macrophage counts [9, 11-13]. Our study group has previously shown that 30-month treatment with inhaled fluticasone decreased the number of bronchial $CD3^+$, $CD4^+$, $CD8^+$ cells and mast cells, and reduced the sputum neutrophil, macrophage and lymphocyte counts [8].



Discontinuation of ICS may increase the number of exacerbations [14-16] and accelerate lung function decline in patients with COPD [17-22]. However, little is known about the effect of ICS discontinuation on airway inflammation. An increase in percentage sputum neutrophils was found in COPD patients randomized to ICS withdrawal when compared to neutrophils in case of 6-week ICS continuation [23]. We previously showed that discontinuation after 6-month ICS increased bronchial $CD3^+$ cells, mast cells and plasma cells at 2.5 years compared to continued therapy, without significant effect on sputum inflammatory cells [8]. However, the effects of withdrawal of ICS after long-term treatment on airway inflammation have not been investigated, but are highly relevant to evaluate the sustained reductions in bronchial inflammation and possible disease-modifying effects.

We hypothesized that inflammatory cell counts in bronchial biopsies and sputum increase after withdrawal of ICS in COPD patients who had previously been randomized to 30-month ICS treatment during 5 subsequent years of prospective follow-up. Additionally, we examined whether the changes in inflammation after ICS withdrawal were associated with

changes in lung function decline.

# MATERIALS AND METHODS

## Study design and participants

Patients of the GLUCOLD (Groningen and Leiden Universities Corticosteroids in Obstructive Lung Disease) study (GL1) were enrolled in the observational, follow-up study (GL2). Details of the study design were previously described [8, 21]. In short, in GL1 114 moderate to severe, steroid-naive COPD patients were randomized to one of the four treatment arms, with Diskus dry-powder inhalers (GlaxoSmithKline, Zeist, The Netherlands) each twice daily for 30 months: 1) fluticasone propionate (FP) 500µg (F30); 2) FP with salmeterol, 500/50µg, single inhaler (FS30); 3) 6-month FP followed by 24-month placebo (F6); and 4) placebo. During GL2, patients were treated by their own physician according to current guidelines [24], which implied that the majority of patients intermittently used or did not use ICS. At the end of GL2, a list of delivered medications was provided by the patients' pharmacy. The ethics committees of Leiden University Medical Center and University Medical Center Groningen approved both GL1 and GL2. Separate written informed consent for GL2 was provided by all patients.

## Outcomes and measurements

The primary outcome of the present study was the effect of ICS withdrawal on inflammatory cell counts in the lamina propria of bronchial biopsies. Therefore, a fiberoptic bronchoscopy was performed after 5 years of follow-up (GL2) according to standardized protocols, consistent with bronchoscopies in GL1 [25]. Processing of bronchial biopsies was performed according to present recommendations [26], and two biopsies per patient were selected based on the largest lamina propria by evaluaton of hematoxylin-eosin stained sections. Immunohistochemical stainings on 4µm sections of paraffin-embedded bronchial biopsies were performed with specific antibodies against T-lymphocytes (CD3, CD4, and CD8), macrophages (CD68), neutrophil elastase (NE), mast cell tryptase (AA1) and eosinophils (EG2), according to the previous protocols [25]. Due to a lack of significant ICS-induced changes in plasma cells in GL1, we did not include these cells in our current analysis. Bronchial cells were counted using image analysis software (ImageJ, version 1.48i, National Institutes of Public Health). Subepithelial cells were calculated as weighted means and expressed as number of cells per 0.1mm$^2$. The minimal selected area of lamina propria for analysis per biopsy was 0.02mm$^2$. Data from bronchial cell counts of baseline and after 30 months (GL1) were

previously reported [8].

The secondary outcome was the effect of ICS discontinuation on inflammatory cell counts in induced sputum. A sputum induction was performed at year two and five of GL2. For safety reasons, sputum was only induced in patients with a post-bronchodilator $FEV_1$ ≥1.2l. Induced sputum was processed according to the full sample method [27]. Two cytospins per sample were stained with May-Grünwald Giemsa (MGG) to obtain differential cell counts. A sputum sample was considered adequate if ≤80% squamous cells were present. Differential cell counts were expressed as cell count per $10^4$ml non-squamous nucleated cells. Sputum cell counts at baseline and after 30 months (GL1) were previously reported [8].

## Statistical analysis

Data from all patients were used for the analysis and the statistical analysis was performed with SPSS 22.0 software (SPSS Inc., Chicago, IL). Because there were no differences in inflammatory cell counts after 30 months of treatment in the F6 and placebo groups, and in the FS30 and F30 groups [8], we combined these to increase power into two groups: F6/placebo and FS30/F30. ICS use during GL2 was retrospectively divided into the following groups: patients who used ICS 0-50% or 50-100% of the time. For the analysis of GL2, we focused on those patients using 0-50% ICS during GL2, being the largest subgroup (61 out of 85 patients). Based on the information of the patients' pharmacy, the daily dose of ICS (in µg, in beclomethasone dipropionate [BDP] equivalents) during 5 years was calculated as daily sum of the different doses of ICS (in µg/day) divided by the total time that ICS were used (days).

A linear mixed-effect model with a random intercept for each subject was applied, using all natural-log transformed inflammatory measurements from GL1 and GL2 as outcome variable and an identity covariance matrix. The analysis was stratified for original combined treatment groups and ICS use during GL2. The change in inflammatory cell counts in GL2 compared to GL1 (GL2 minus GL1) was assessed by two time variables in the models: time 1 (time since start of GL1: range 0-7.5 years) and time 2 (time since start of GL2: range 0-5 years; during GL1 this value is zero). To assess the change in cell counts between the original FS30/F30 groups compared to the original F6/placebo groups, a linear mixed-effect model with the same time variables was used including an interaction term between these time variables with the original combined treatment groups. Given the limited sample size of patients completing the 5 years of prospective follow-up, possible confounders (age, sex and center) were not included in the model. Smoking was unlikely to be a major confounder as shown in

a previous post-hoc analysis [8]. By Spearmans' correlation coefficient, we assessed whether the changes in inflammation in GL2 versus GL1 were associated with change in lung function in the same period.

Since the number of cells decreased during GL1 and increased at the end of GL2, and therefore represent a difference in slope, we calculated the rate of change. Therefore, data are presented as fold change per year between GL2 minus GL1 with 95% confidence interval (CI), calculated by taking the antilog of estimates from the linear mixed-effects models. Statistical significance was inferred at P≤0.05.

**Table 1.** Patient characteristics at baseline of randomized treatment (GL1) and at the start of 5 years of follow-up (GL2) for the original FS30/F30 and F6/placebo groups.

| | **Baseline GL1** | | **Start of GL2** | |
|---|---|---|---|---|
| | F6/placebo (n=60) | FS30/F30 (n=54) | F6/placebo (n=46) | FS30/F30 (n=46) |
| **Gender (M/F) (n)** | 51/9 | 48/6 | 41/5 | 42/4 |
| **Age (yr)** | 61 (7.7) | 62 (7.8) | 64 (7.7) | 64 (7.3) |
| **Smoking (y/n) (n)** | 36/24 | 36/18 | 22/24 | 26/20 |
| **Packyears (yr)** | 41 (31-54) | 47 (31-56) | 43 (31-58) | 49 (34-57) |
| **Post-bronchodilator $FEV_1$ (% pred)** | 64 (8.3) | 62 (9.3) | 61 (11.1) | 63 (11.7) |
| **Post-bronchodilator $FEV_1$ (L)** | 2.05 (0.5) | 2.06 (0.4) | 1.92 (0.6)* | 2.03 (0.5) |
| **Post-bronchodilator $FEV_1$/IVC (%)** | 49 (8.5) | 47 (8.6) | 46 (10.6) | 47 (9.9) |

Data of the 2 combined groups are derived from the original 4 treatment groups in GL1 are presented as means and standard deviation, median with interquartile range (packyears) or numbers. F6: 6 months treatment with fluticasone, followed by 24 months of placebo; FS30: 30 months treatment with fluticasone and salmeterol; F30: 30 months treatment with fluticasone. Data for the individual 4 treatment groups have been previously reported [21].

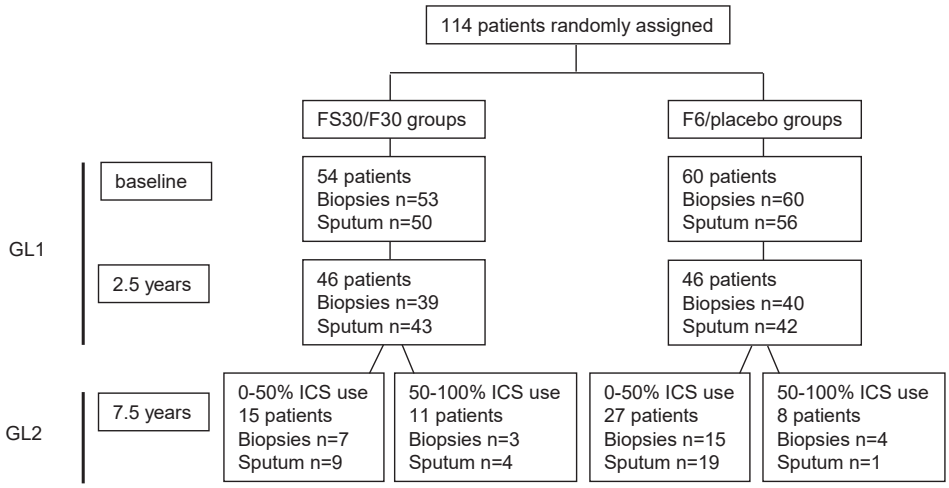
* P≤0.001 compared to original combined F6/placebo groups at baseline GL1 (calculated with paired samples T-test).

# RESULTS

Data from 114 patients were used for the analysis of GL1, 92 patients completed GL1, 85 patients started and 61 completed GL2 [8, 21]. Patient characteristics at both baseline and at start of GL2 were similar among the original combined treatment groups, except for post-bronchodilator $FEV_1$, which was significantly lower at the start of GL2 in the original combined F6/placebo groups compared to the original combined FS30/F30 groups (Table 1).

Most patients (61/85) used ICS 0-50% of time during GL2, with a mean daily ICS dose of 1019µg (SD 554µg) in BDP equivalents during GL2 (Table 2), which was not significantly different between the original combined treatment groups. Bronchial biopsies of 29 patients were available at the end of GL2 (Table 3), and 21 of these 29 patients used 0-50% of time ICS during GL2. Sputum samples suitable for analysis were available from 47 and 33 patients after 2 and 5-year follow-up, respectively (Table 3). A diagram with the number of patients per group and available number of samples is presented in Figure 1.

**Figure 1:** Diagram presenting the number of patients and available bronchial biopsies and sputum samples in GL1 and GL2 in the original combined treatment groups.




GL1: GLUCOLD 1 study, first part of the study. GL2: GLUCOLD 2 study, follow-up study. F6: 6 months treatment with fluticasone, followed by 24 months of placebo; FS30: 30 months treatment with fluticasone and salmeterol; F30: 30 months treatment with fluticasone.

**Table 2:** Number of patients at the start of the GLUCOLD follow-up study (GL2) using ICS and daily dose of ICS (in µg) during 5 years in those patients who used ICS during GL2.

| Original treatment group | No ICS use (n) | ≤50% use of ICS (n) | >50% use of ICS (n) | 100% use of ICS (n) | Daily dose ICS (µg) |
|---|---|---|---|---|---|
| **F6/placebo** | 20 | 12 | 10 | 1 | 875 (479) |
| **FS30/F30** | 15 | 14 | 8 | 5 | 1141 (591) |
| **Total** | 35 | 26 | 18 | 6 | 1019 (554) |

The daily dose of ICS (in µg, in BDP equivalents) during 5 years was calculated by the sum of the different doses of ICS per day (in µg/day), divided by the total time that ICS were used (in days). Doses were based on data provided by the patients' pharmacy.

Daily dose of ICS is presented as means with standard deviations. F6: 6 months treatment with fluticasone and 24 months with placebo; FS30: 30 months treatment with fluticasone and salmeterol; F30: 30 months treatment with fluticasone.

## Bronchial biopsies

Compared to GL1, patients within the combined original FS30/F30 groups, who used ICS 0-50% of time during GL2 showed an increase in bronchial $CD3^+$ [GL2-GL1, expressed a fold change/year: 2.68 (1.87-3.84); P<0.001], $CD4^+$ [1.91 (1.33-2.75); P=0.001], $CD8^+$ cells [1.71 (1.15-2.53); P=0.008] and mast cells [1.91 (1.36-2.68); P<0.001] at the end of GL2 (Figure 2). Discontinuation of ICS or use of ICS 0-50% of time during GL2 in the original combined FS30/F30 groups increased the number of $CD3^+$ cells (expressed as fold change/year) [1.78 (1.21-2.64); P=0.04], $CD8^+$ cells [1.73 [1.05-2.85); P=0.033] and mast cells [1.52 (1.06-2.17); P=0.023] compared to the original combined F6/placebo groups.

**Table 3:** Sputum samples and bronchial biopsies at year 2 and year 5 of GL2, presented by original combined treatment groups and use of ICS during GL2.

| | | | No ICS use | <50% ICS use | >50% ICS use | 100% ICS use | total |
|---|---|---|---|---|---|---|---|
| Sputum | Year 2 | F6/placebo | 10 | 10 | 3 | 1 | 24 |
| | | FS30/F30 | 8 | 7 | 5 | 3 | 23 |
| | Year 5 | F6/placebo | 12 | 7 | 1 | 0 | 20 |
| | | FS30/F30 | 4 | 5 | 3 | 1 | 13 |
| Bronchial biopsies | Year 5 | F6/placebo | 10 | 5 | 3 | 1 | 19 |
| | | FS30/F30 | 3 | 3 | 4 | 0 | 10 |

Sputum samples were collected from 63 and 51 patients after 2 and 5 years of follow-up (suitable for analysis 47 and 33), respectively. Data presented as number of samples.

GL2: follow-up study; ICS: Inhaled corticosteroids. F6: 6-month ICS followed by 24-month placebo. FS30: 30-month fluticasone with salmeterol; F30: 30-month fluticasone.

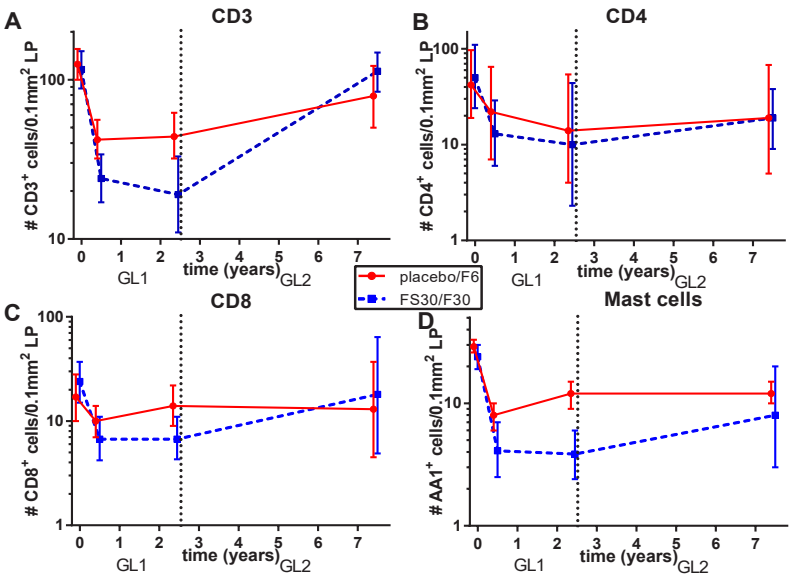



**Figure 2:** Geometric mean cell counts in bronchial biopsies (per 0.1 mm$^2$ lamina propria) for those patients who used ICS 0-50% of the time during GL2 in the original combined treatment groups FS30/F30 and F6/placebo. Error bars represent 95% confidence interval. Data of bronchial CD3$^+$ cells (Figure 2A), CD4$^+$ cells (2B), CD8$^+$ cells (2C) and mast cells (2D) are presented. Data were calculated by taking the antilog of the means of natural log-transformed number of cells. The group of patients who used ICS 50-100% of time during GL2 was too small, and is therefore not shown in the figures.

## Sputum

At the end of GL2, patients of the original FS30/F30 groups who used 0-50% of time during GL2 had a higher total sputum cell count [GL2-GL1, expressed as fold change/year: 1.90 (1.42-2.54); P<0.001], as well as higher counts of sputum macrophages [2.10 (1.55-2.86)]; P<0.001), neutrophils [1.92 (1.39-2.65)]; P<0.001) and lymphocytes [2.01 (1.46-2.78)]; P<0.001), at the end of GL2 compared to during GL1 (Figure 3). Discontinuation of ICS or use of ICS 0-50% of time during GL2 in the original combined FS30/F30 groups increased the total number of sputum cells (expressed as fold change/year) [1.66 (1.12-2.46); P=0.012], sputum neutrophils [1.68 [1.09-2.58); P=0.018], macrophages (1.90 [1.26-2.85]; P=0.002) and lymphocytes [1.73 (1.09-2.74); P=0.020] compared to the original combined F6/placebo groups.

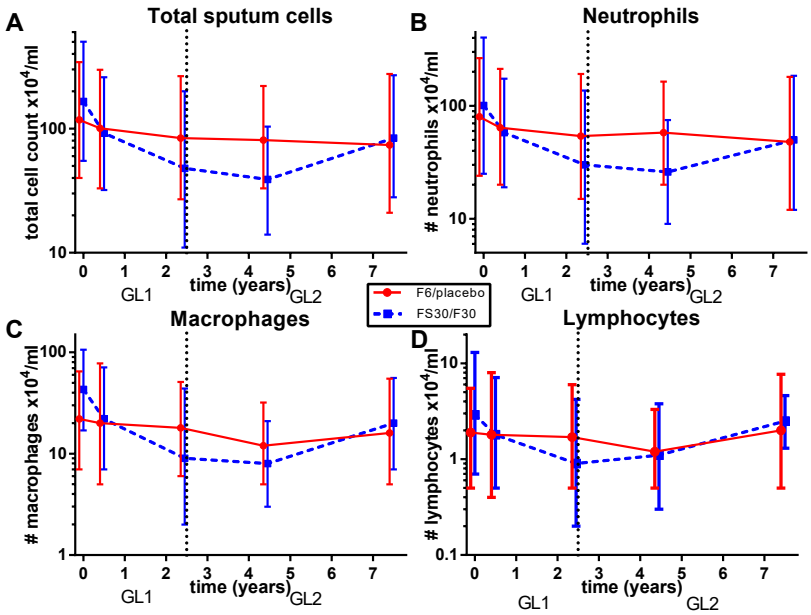



**Figure 3:** Geometric mean sputum cell counts (x $10^4$ per ml) for original combined treatment groups FS30/F30 and F6/placebo. Error bars represent 95% confidence interval. Data of patients who used ICS 0-50% of the time during GL2 are presented for total sputum cells (Figure 3A), neutrophils (3B), macrophages (3C) and lymphocytes (3D). Data were calculated by taking the antilog of the means of natural log-transformed number of cells. The group of patients who used ICS 50-100% of time during GL2 was too small, and is therefore not shown in the figures.

## Relation between lung function decline and inflammatory cells

The accelerated rate of decline in post-bronchodilator $FEV_1$ during GL2 was associated with an increase in sputum macrophages in patients of the original FS30/F30 groups (Rs=-0.63, P=0.04) and with a trend towards an increase in bronchial neutrophil counts (Rs=-0.60, P=0.07) (Figures 4A and B, respectively).

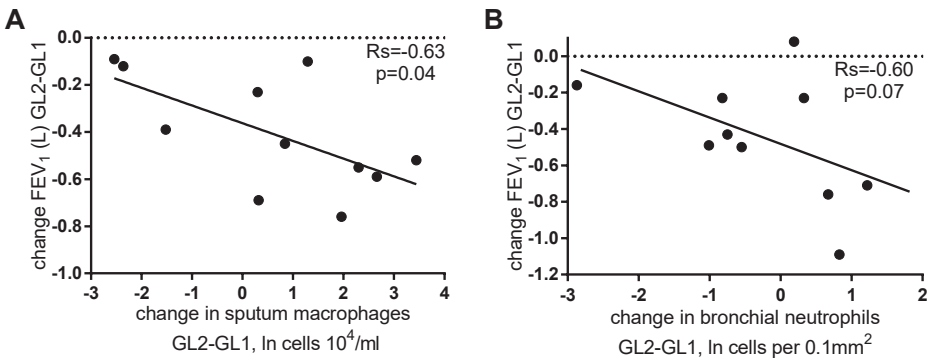



**Figure 4:** Correlation between change (end GL2-end GL1) in post-bronchodilator $FEV_1$ (L) and change in natural log-transformed sputum macrophages (end GL2-end GL1, expressed as number of cells x $10^4$ per ml) (Figure 4A, left panel) and changes in natural log-transformed bronchial neutrophils (per 0.1 $mm^2$ lamina propria, Figure 4B, right panel). Each dot represents a single patient.



# DISCUSSION

The present study shows that withdrawal of ICS after previous long-term ICS treatment in patients with moderate to severe COPD, is accompanied with increased bronchial T-lymphocytes and mast cells as well as several sputum cell counts. In addition, we found an association between the accelerated rate of lung function decline and increase in sputum macrophages during GL2, and a trend with bronchial neutrophils. These results suggest that airway inflammation is suppressed during active treatment with ICS and might relapse after long-term discontinuation of ICS.

We observed that several inflammatory cells in bronchial biopsies and sputum significantly

increased during 5-year follow-up in patients with moderate to severe COPD who did not use or only intermittently used ICS after previous 30-month randomized ICS treatment. These unique data confirm and extend previous findings of our study group, showing that withdrawal of ICS after 6-month ICS treatment increases bronchial $CD3^+$ cells, mast cells and plasma cells when followed up to 30 months compared to those who continued ICS therapy, without a significant effect on sputum inflammatory cells [8]. Another open-labelled pilot study with only 6-week ICS withdrawal showed an increase in the percentage of sputum neutrophils compared to ICS continuation [23]. Taken together, the present study provides novel data on a relapse of airway inflammation after prolonged ICS discontinuation with previous long-term ICS use in COPD.

In this longitudinal study, we found that lung function decline seems to be related to an increase in sputum macrophage counts in moderate to severe COPD patients, the majority being without ICS treatment. Furthermore, a trend for an association with higher bronchial neutrophils was found. A previous study showed that higher sputum neutrophils were associated with faster $FEV_1$ decline in patients with severe COPD using ICS [3]. Furthermore, a weak association between sputum percentage neutrophils and $FEV_1$ percentage predicted was found in a cross-sectional study [4]. These findings in sputum neutrophils are likely not only explained by differences in study design, but also by differences among the studies in number of patients, severity or phenotype of COPD, and duration of treatment and withdrawal of ICS. A recent study by Barnes et al. found that COPD patients treated with fluticasone and a high percentage blood eosinophils at baseline have a slower rate of $FEV_1$ decline compared to placebo treated patients [28]. However, we could not detect a relation between baseline blood eosinophilia and lung function decline during GL2 in those who stopped or continued using ICS during GL2. Taken together, until now, this is the only long-term study that suggests an association between lung function decline and change in inflammation after prolonged ICS withdrawal.

A strength of our study is the long-term follow-up with monitoring of lung function and availability of sputum and bronchial biopsies during 5-year follow-up. This is unique, since no previous studies had such a prolonged treatment period as well as long follow-up period with treatment according to the current guidelines in a real life setting. It needs to be noted that the effect of ICS withdrawal in the present study was more pronounced in bronchial biopsies than in sputum, stressing the importance to not only study sputum cell counts when investigating COPD. Nevertheless, our study has some limitations. First, expectedly, the number of patients that finished the complete study is limited, particularly when considering the original treatment groups separately. Therefore, we chose to combine the original FS30/

F30 and F6/placebo groups to increase power as there were no differences between the F6 and placebo groups at the start of the GL2 but only following first 6-months treatment during GL1. The small groups of patients from whom bronchial biopsies and sputum samples were available, make the correlation with lung function decline less strong. Despite these relatively low numbers, we still detected associations between clinical and histological outcomes. Second, GL2 was a prospective, (non-randomized) observational study and the majority of patients (61 of 85) discontinued their ICS or used 0-50% of time of observation ICS during GL2 (Table 2). Adherence to medication was not checked, reflecting daily practice. This could have led to a misclassification of ICS use during GL2 and therefore might have influenced out outcomes. Nevertheless, when only compliant patients during GL1 were included in the current analysis, similar results were found (data not shown). Due to the limited number of patients in whom a bronchoscopy was performed (Figure 1), a comparison between those with continuous ICS use versus ICS withdrawal during GL2 was not possible. Third, inflammatory cells were stained by immunohistochemistry with different batches of antibodies and counted using a different camera and image analysis software compared to GL1 [28]. We cannot rule out that these differences could have influenced our data. However, the bronchial inflammatory cell counts found in GL1 and GL2 were in a comparable range and cannot explain the observed difference found between and within the groups. Finally, bronchial inflammation is unequally distributed along the airways [29]. Since we were only able to collect samples from the central airways, the effect of ICS withdrawal in the small airways could not be investigated in this study. Taken into account these considerations, we are nevertheless confident that our data provide a novel view on relevant changes in airway inflammation after long-term cessation of prolonged ICS treatment.



How can we interpret our results? In the first part of the study (GL1), we found a reduction in bronchial inflammation and attenuation of lung function decline during 30-month ICS treatment in patients with moderate to severe COPD [8]. In the current study (GL2), we observed the expected opposite in that ICS withdrawal after previous long-term treatment resulted in an increase in $CD3^+$, $CD4^+$, $CD8^+$ T-cells and mast cells in this selected group of patients, which further extends our previous observation that withdrawal of ICS relapses lung function decline [21]. The opposite outcome of ICS therapy in the first phase when compared to the effects of ICS withdrawal on airway inflammation in the second observational phase of our study can be regarded as a validation and strongly supports the plausibility of our findings. Taken together, our data suggest that during active treatment with ICS effects on lung function and inflammation are transient, without persistent disease modification.

During the past 25 years numerous studies have been published concerning the question whether or not ICS are beneficial in COPD patients [30]. Our study shows that in this group of moderate to severe COPD, patients experience transient positive effects on lung function and on airway inflammation during ICS therapy, which are not maintained after ICS withdrawal [21]. The present selection of COPD patients may be representative of a particular phenotype that is responsive to steroid treatment [31], in whom long-term ICS therapy may need to be continued to maintain the observed beneficial effects on the course of lung function and airway inflammation over time.

## CONCLUSION

In conclusion, the present data indicate that ICS discontinuation during 5 years following 30 months use of ICS, in a group of moderate to severe COPD patients, induces a relapse in bronchial and sputum inflammatory cells which is partially accompanied by a more rapid decline in lung function. These data suggest that ICS do not have a sustained disease modifying activity after ICS withdrawal in this group of COPD patients which is in line with observations in asthmatic patients.

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# CHAPTER 7

# Summary and general discussion

# INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is a common disease, which is currently the third leading cause of death worldwide [1], and is mainly caused by cigarette smoking. It is characterized by a progressive airflow limitation in most patients with COPD, structural changes in lung tissue (remodeling) and chronic airway inflammation. Anti-inflammatory treatment, such as inhaled corticosteroids (ICS), is currently recommended for patients with severe and very severe COPD with frequent exacerbations with the aim to inhibit the airway inflammation to prevent exacerbations. Previously, our study group showed that ICS treatment causes attenuation of lung function decline in a subgroup of moderate to severe COPD patients, accompanied by less airway inflammation and a better quality of life [2]. Although many patients use inhaled treatments, compliance to any long-term prescribed therapy is unfortunately poor [3]. Therefore it is important that both the treating physician and patient are informed about the clinical benefits of the particular treatment and understand what occurs after treatment withdrawal.

This thesis focusses on airway inflammation in COPD, with a specific focus on macrophages, macrophage phenotypes and their markers and airway remodeling in COPD. Another focus is the effects of long-term discontinuation of ICS on lung function decline and airway inflammation following 30 months of treatment with ICS in patients with moderate to severe COPD.

For the studies described in this thesis, data and samples from the GLUCOLD (Groningen and Leiden Universities Corticosteroids in Obstructive Lung Disease) study were used. The GLUCOLD study is a randomized, double-blind, placebo-controlled trial with four treatment arms. During the first part of the study, moderate to severe COPD patients were randomized to receive an inhaled treatment with 30-month fluticasone propionate with or without salmeterol, 6-month fluticasone (followed by 24-month placebo) or 30-month placebo. During the second part of the study (GLUCOLD follow-up study), patients were followed annually for 5 years while being treated by their own physician. During this period the majority of the patients were not at all or intermittently treated with ICS.

In this chapter, first the conclusions of the separate studies are presented below, followed by a general discussion and directions for future research.

# CONCLUSIONS OF THIS THESIS

## Relation between smoking, ICS and macrophage heterogeneity in COPD

- Smoking cessation changes the macrophage phenotype *in vivo* in the peripheral lungs, sampled by bronchoalveolar lavage (BAL), towards an anti-inflammatory phenotype, which is not associated with a decrease in pro-inflammatory mediators in patients with COPD *(Chapter 2)*.

- The pro-inflammatory biomarker YKL-40 is mainly secreted by pro-inflammatory MΦ1 macrophages derived from *in vitro* cultured monocyte-derived macrophages, and its release is dose-dependently inhibited by dexamethasone *(Chapter 3)*.

- Serum and sputum YKL-40 levels of COPD patients are not affected by 30 months of treatment with ICS *(Chapter 3)*.

## Effect of smoking and ICS on airway remodeling in COPD

- No differences are observed in bronchial extracellular matrix proteins, such as collagen I and III, the proteoglycan versican and decorin and elastic fibers between current and ex-smokers with COPD *(Chapter 4)*.

- Long-term ICS treatment in COPD increases the percentage stained area of versican and collagen III compared to placebo *(Chapter 4)*.

- Increased density of collagen type I is associated with an increase in lung function in patients with moderate to severe COPD *(Chapter 4)*.

## Clinical and pathological outcomes after long-term withdrawal of ICS in COPD

- Discontinuation of ICS after 30-month ICS treatment relapses lung function decline, airway hyperresponsiveness and quality of life during 5 years of follow-up in patients with COPD *(Chapter 5)*.

- Withdrawal of ICS after 30-month treatment in patients with COPD increases bronchial $CD3^+$, $CD4^+$, $CD8^+$ T-cells and mast cells, together with a higher total sputum cell counts, sputum macrophages, neutrophils and lymphocytes. Increased sputum macrophages are associated with an accelerated lung function decline *(Chapter 6)*.

# GENERAL DISCUSSION

## Macrophage heterogeneity is a complex phenomenon

Macrophages have important functions in innate and adaptive immunity and play a central role in the pathogenesis of COPD. They constitute a heterogeneous cell population with classically activated or pro-inflammatory MΦ1 cells and alternatively activated or anti-inflammatory MΦ2 cells, analogous to the Th1 and Th2 dichotomy [4, 5]. MΦ1 secrete pro-inflammatory cytokines, such as TNF-α and IL-12, promote Th1 immunity and have a good antigen presenting capacity. In contrast, MΦ2 cells are characterized by a role in Th2 immunity, promote T-regulatory cells and efferocytosis (removal of apoptotic cells), have a poor antigen presenting capacity and secrete anti-inflammatory cytokines, such as IL-10 [6, 7]. Monocyte-derived macrophages differentiated into MΦ1 or MΦ2 can switch from a certain subtype into another due to local environmental stimuli, a feature which is called plasticity [8, 9]. MΦ2 cells can even be further divided in three subsets: MΦ2a facilitate parasite encapsulation and destruction; MΦ2b are important for immunoregulation; and MΦ2c promote tissue remodeling and matrix deposition [10]. In this thesis, the subdivision between pro- and anti-inflammatory macrophages is used. Various well-validated various macrophage markers have thus far been identified mainly based on *in vitro* studies. However, skewing of macrophage phenotypes depends on activation after a certain environmental condition and stimulation, which is different in *in vitro* and *in vivo* conditions. CD163 is considered as a specific marker for M-CSF generated MΦ2, whereas CD80 seems a marker for IFN-γ polarized MΦ1 [11]. Although there are suggestions that macrophage markers in humans are different compared to murine markers [12-14], one study found that there are at least some similarities in macrophage markers between humans and mice [15].

A positive relation has been found between the increasing severity of COPD and an increased percentage of macrophages in the small airways of COPD compared to smokers and ex-smokers with a normal lung function [16], which even (partially) persists after smoking cessation [17, 18]. Although the above-mentioned characteristics of macrophage

phenotypes suggest a MΦ1 predominance in COPD, the role of macrophage phenotypes in COPD is still not fully clear. Some authors even suggest that in COPD patients *in vivo* an intermediate phenotype of macrophages, with characteristics of both MΦ1 and MΦ2 cells, can be found [19, 20]. In this thesis we evaluated the MΦ2 marker CD163 (Chapter 2) and the novel MΦ1 marker YKL-40 (Chapter 3). The relative abundant presence of $CD163^+$ macrophages in bronchoalveolar lavage (BAL) fluid in moderate to severe COPD patients suggests that the peripheral lung can be considered to be a more anti-inflammatory environment compared to the central airways (Chapter 2). Furthermore, we found that former smokers compared to current smokers have a higher percentage of anti-inflammatory macrophages in BAL, but not in sputum, indicating that removal of the pro-inflammatory stimulus of cigarette smoking in the peripheral airways changes the environment to a more anti-inflammatory character. Others confirmed this finding by showing that the chemokine ligand 18 (CCL18), a marker of alternatively activated macrophages is lower in BAL from current smokers compared to never smokers [21]. In addition, reduced efferocytosis has been found in alveolar macrophages from smoking patients with COPD compared to ex-smokers, suggesting that smoking cessation induces mostly a MΦ2 phenotype in the peripheral airways [22]. The observation that serum and sputum levels of YKL-40 are elevated in current smokers as well as in smoking and ex-smoking COPD patients compared to never smokers without COPD, is in line with this finding [23-25]. We confirmed that levels and expression of YKL-40 are higher in *in vitro* cultured monocyte-derived MΦ1 compared to MΦ2. YKL-40 levels were higher in sputum compared to BAL from the GLUCOLD patients, but no differences were found between current and ex-smokers with COPD (data not shown), which is in line with findings from a recent study [25]. In contrast to the anticipated predominance of MΦ1 macrophages in COPD, some studies have even suggested the contrary. An analysis of alveolar macrophages from moderate to severe, mostly smoking COPD patients demonstrated a lower expression of CD86 and CD11a (markers for co-stimulation and adhesion, respectively, needed for antigen presentation) compared to asymptomatic smokers and nonsmokers, suggesting a decreased MΦ1 phenotype in COPD [26]. However, this study did not evaluate the effect of smoking cessation on macrophage phenotypes. The MΦ2 predominance in murine alveolar macrophages and peripheral blood mononuclear cells (PBMCs) after cigarette smoke exposure, shown by increased CD163 expression and anti-inflammatory cytokines, has been confirmed in *in vivo* and *in vitro* mouse studies [27]. Furthermore, it is interesting to study which component(s) in cigarette smoke is responsible for the skewing of macrophages. One study found that nicotine can skew MΦ1 cells, derived from PBMCs of healthy donors, partially into a MΦ2 phenotype [28].

Increased numbers of macrophages found in small airways in COPD, which are associated with airflow limitation [16]. In addition, some studies suggest that macrophage phenotypes

are related to the degree of airflow limitation. One study found a correlation between increased serum YKL-40 and decreased lung function in patients with COPD [23], indicating that in severe stages of COPD more MΦ1 cells are present. In contrast to this, a small study found that in alveolar macrophages from smoking patients with mild COPD compared to asymptomatic smokers and nonsmokers, expression of genes related to MΦ2 was upregulated, whereas MΦ1-related genes were downregulated [29]. This suggests that in addition to smoking, also the degree of airflow limitation could explain skewing of macrophage phenotypes. The presented data above remain hard to interpret. Mouse models with only exposure to cigarette smoke are not fully comparable with humans exposed to multifactorial environmental factors, including cigarette smoke. In addition, very little is known about diversity, phenotypes and function of macrophages, such as luminal versus tissue macrophages in the large and small airways [19]. To summarize, cigarette smoking causes a disturbance in macrophage phenotypes in COPD, which depends on the type of macrophage, disease severity and the location of the respiratory tract studied.

Possible options for treatment of COPD include the restoration of this imbalance [30]. Several possibilities have been evaluated. One study evaluated the effect of procysteine, a glutathione precursor, which is essential for both MΦ1 and MΦ2 function. The authors found that in alveolar macrophages and lung tissue derived macrophages from a murine COPD model, efferocytosis significantly increased after treatment with oral procysteine [31]. This group also found that after long-term use of low-dose azithromycin by COPD patients, phagocytosis of bacteria by alveolar macrophages was improved in combination with a higher expression of the mannose receptor (marker of MΦ2) [32, 33]. This suggests that after treatment with procysteine and azithromycin the macrophage phenotype is skewed towards MΦ2. However, these medications are not often prescribed in patients with COPD.

More importantly, treatment with (inhaled) corticosteroids is frequently prescribed in COPD. Steroids have been shown to adapt the disturbed balance of macrophage phenotypes. *In vitro* cultured PBMCs, differentiated with IL-4 followed by stimulation with fluticasone propionate, showed reduced T-cell proliferation and RFD1 expression (marker for inductive or pro-inflammatory macrophages), whereas RFD7 expression (marker for suppressive or anti-inflammatory macrophages) was increased [34]. In addition, they found that the effect of fluticasone remained active for at least 24 hours after steroid removal. In line with this, dexamethasone treatment of *in vitro* differentiated macrophages results in a MΦ2 morphology and increased percentage $CD163^+$ cells [9]. These studies all suggest a skewing phenomenon to a MΦ2 phenotype after corticosteroid treatment. We found that YKL-40 expression and secretion by *in vitro* cultured MΦ1 cells was dose-dependently inhibited

by dexamethasone (Chapter 3). However, we could not detect a significant difference in serum and sputum levels of the MΦ1 marker YKL-40 after 30-month ICS in our group of COPD patients (Chapter 3). This is in line with a recent paper that showed no difference in serum YKL-40 in COPD patients treated with oral or inhaled corticosteroids [25]. Thus, it seems that ICS do not influence macrophage phenotypes *in vivo* in COPD as evaluated by YKL-40 [35]. A possible explanation is that certain phenotypes of macrophages are steroid resistant, as was observed in healthy subjects after LPS-inhalation [36]. HLA-DR$^+$ inducible pulmonary monocyte-like cells (suggestive for MΦ1 cells), obtained from BAL fluid of these subjects, produced pro-inflammatory cytokines that did not respond on *in vitro* stimulation with dexamethasone, whereas alveolar macrophages responded by lower levels of IL-6 and IL-8. Another recent study showed that a subset of pulmonary tissue macrophages from COPD patients are less responsive to budesonide compared to macrophages of non-smokers and healthy smokers, measured by CXCL8 and TNF-α release after LPS-stimulation [20]. A possible mechanism of steroid resistance involves reduced expression of histone deacetylase 2 (HDAC2), which can deactivate inflammatory gene expression in COPD. Smoking and oxidative stress may reduce HDAC2 activity and expression [37]. In addition, HDAC3 activity is inhibited by cigarette smoke exposure in PBMCs differentiated MΦ1, which show an increased production of IL-8 and IL-1β, thereby promoting chronic inflammation [38].

Finally, in patients with chronic kidney allograft injury who are treated with immunosuppressive treatment, including steroids, kidney biopsies showed areas with high numbers of CD163$^+$ MΦ2 cells which correlated with the amount of collagen I deposition [39]. This study group also showed that in *in vitro* cultured PBMCs, stimulated with dexamethasone, gene expression of CD163 and TGF-β were both induced, suggesting that macrophages are skewed to an anti-inflammatory (MΦ2) and possible pro-fibrotic phenotype [39]. MΦ2-like cells are also present in mouse models of bleomycin-induced lung fibrosis, in which IL-10 attenuates bleomycin-induced inflammation and fibrosis [12]. In contrast, overexpression of IL-10 in this model induces extracellular matrix deposition in the lung [40]. Furthermore, corticosteroids can induce MΦ2-like cells and thereby promote fibrosis [41]. This observation is in line with our findings that after long-term treatment with ICS more collagen III and versican in large airways of COPD patients was found (as described in Chapter 4 and discussed in paragraph below), which was associated with an improved lung function. These observations show that macrophage heterogeneity is a complex process, which can be markedly influenced by a local and multifactorial environment. Steroids might influence the composition of this heterogeneous cell population, but may also have effects on extracellular matrix deposition.

## Inhaled corticosteroids and extracellular matrix production in COPD

Smoking and chronic airway inflammation both contribute to structural changes and remodeling in the airway wall and lung parenchyma in COPD [42, 43]. Remodeling is a result of changes in the extracellular matrix (ECM), a three dimensional structure which acts as the backbone of the lung tissue. It provides a scaffold for the cells and contributes to regulation of their activity, and its composition is an important determinant of lung function. The major ECM components are collagens, proteoglycans and elastic fibers [44]. The ECM has a high turnover even in healthy subjects, thereby maintaining its stability and functions. However, remodeling is a complex process, which results from many small changes in tissue structure that lead to tissue degrading and/or accumulation of proteins. Several mechanisms play a role in remodeling, such as cell proliferation, increasing cell volume, modified synthesis and deposition of ECM proteins and a disturbed balance between matrix metalloproteinases (MMPs) and tissue inhibitor of metalloproteinases (TIMP) [45]. A relative excess of MMPs and shortage of TIMPs, as in COPD, can result in irreversible pathological changes [46]. An example of the imbalance between MMPs and TIMPs is during exacerbations of COPD, as elevated plasma and serum levels of protein fragments of collagen type III, IV and VI and elastic fibers and decreased levels of versican were found, compared to stable COPD [47]. In addition, elevated levels of MMP-9 and decreased levels of TIMP-1 in sputum of patients with COPD have been found during an exacerbation [48]. This suggests that during an exacerbation the ECM is actively degraded.

The composition of the ECM is regulated by several cells types, such as macrophages, fibrocytes and fibroblasts. Macrophages produce several growth factors, such as transforming growth factor β1 (TGF-β1), which contribute in wound repair and tissue regeneration. Macrophages are in close contact with the ECM proteins and interaction with these proteins can improve their phagocytic capacity [49]. However, cigarette smoke modifies the ECM proteins, and as a result the phagocytic capacity of macrophages is critically diminished [49, 50]. Furthermore, MΦ2 cells, especially MΦ2c, are important in wound healing and fibrosis [14]. When the process of wound healing is almost completed, MΦ2 cells turn into a suppressive phenotype, by the expression of e.g. arginase-1, and IL-10, enhance the resolution of wound healing and restore homeostasis, thereby suppressing fibrosis [51, 52]. Although the pathogenesis of fibrosis is currently not fully understood, these data suggest that a disturbed balance between macrophage phenotypes can induce or inhibit fibrosis.

Bone-marrow derived fibrocytes produce collagens along with inflammatory cytokines. They are induced by pro-fibrotic mediators like TGF-β, and have been found in areas of wound healing, as well as in lung tissue from asthmatic patients [53]. Even in young children with asthma, chronic inflammation causes thickening of the reticular basement membrane in combination with subepithelial fibrosis [54, 55]. The ECM components that are mainly changed in patients with asthma are collagens and the proteoglycans laminin, tenascin and fibronectin [56-59]. The increased amount of collagens and fibronectin in the airways make them stiffer, which may prevent collapse and thereby protect the bronchi from airway hyperreactivity or airway narrowing as has been found in animal models [60, 61]. This reduced airway hyperresponsiveness and the association with increased airway wall thickness and subepithelial collagen deposition has also been found in asthmatic patients [62, 63]. Also in mild COPD patients, higher numbers of fibrocytes have been found compared to healthy subjects and patients with more advanced stages of COPD [64]. In addition, circulating fibrocytes are increased during acute exacerbations of COPD and were associated with a lower lung function and a higher mortality [65].

ICS have been found to significantly decrease the number of fibrocytes in mild COPD patients compared to non-treated patients. In addition, ICS inhibited the release of MMPs and prevented collagen degradation in *in vitro* culture systems [64, 66]. Although less fibrocytes are found after treatment with ICS in COPD, these results may suggest that corticosteroids inhibit ECM breakdown. Furthermore, some studies suggest increased matrix deposition after treatment with corticosteroids, which has already been suggested some decades ago by Torry et al. [67], showing that the number of colony-forming human fibrotic lung fibroblasts increases after stimulation with dexamethasone. This implies that fibroblasts obtained from chronically inflamed, fibrotic lung tissue behave differently under growth conditions compared to fibroblasts obtained from healthy lung tissue. The increased collagen deposition under inflammatory conditions, such as in COPD, has been confirmed in *in vitro* cultured human lung fibroblasts [68]. These observations are in line with the GLUCOLD study in which COPD patients treated with 30-month ICS, had increased content of collagen III and versican in large airways (Chapter 4). However, some studies suggest that only some components of the ECM is steroid-sensitive. A previous study in a rat model sensitized to ovalbumin, fluticasone prevented remodeling of the airways when given simultaneously with this allergen, but the structural changes remained when fluticasone was given post-allergen exposure [69]. Furthermore, asthmatics treated for 6 weeks with ICS showed no change in collagen deposition in the airway wall [70]. Collagen remodeling in human airway smooth muscle cells (ASM) in a gel area was steroid resistant [71]. Another study showed that collagen I and fibronectin expression in ASM cells from asthmatics were unchanged by corticosteroids, whereas corticosteroids induced the expression of collagen I and fibronectin

in ASM cells from non-asthmatics [72]. A possible explanation for the partially steroid resistance of the remodeling process, is that altered ECM components, such as collagen type I, are less responsive to dexamethasone [73]. This may to some extent explain why in our group of COPD patients, collagen I content was not affected by treatment with ICS.

Another reason for altered ECM production is epithelial-mesenchymal transition (EMT), a process in which epithelial cells acquire properties of mesenchymal cells. It has been suggested that EMT is active in the airways of COPD, as the mesenchymal markers S100A4 and vimentin have been found in the airway epithelium [74]. The GLUCOLD study group showed previously that after 30-month treatment with fluticasone, expression of genes involved in epithelial cell signaling, oxidative stress, remodeling and apoptosis was decreased in patients with moderate to severe COPD [75]. This study demonstrated a reduced expression of transmembrane serine protease (TMPRSS)-4, which is an important protein in EMT. Another study found that epithelial activation, basement membrane fragmentation and mesenchymal biomarkers were reduced in bronchial biopsies of mild to moderate COPD after treatment with 6 months of inhaled corticosteroids [74]. In contrast, other genes involved in focal adhesion, gap junction and extracellular matrix deposition were increased after treatment with ICS [75]. These results suggests that ICS alters the gene expression profile involved in EMT and in remodeling of COPD.

In summary, the above presented results suggest that whereas (inhaled) corticosteroids may have pro-fibrotic properties, their effect differs between the various extracellular matrix proteins. In our study we found an increased deposition of collagen type I after long-term treatment with ICS, which was associated with a better lung function, suggesting that ICS induced changes in ECM contributes to this. However, it remains the question whether airway remodeling is (partially) reversible, which mechanism is most susceptible and at which time point therapeutic intervention should be given to prevent the progressive course of the disease.

## Withdrawal of ICS relapses lung function decline and airway inflammation

Inhaled corticosteroids are currently widely prescribed for prolonged periods in patients with all stages of COPD, although compliance to any (chronically) prescribed treatment is

approximately relatively low (30-50%) [3]. According to current guidelines, ICS should only be prescribed to patients who will benefit most, namely symptomatic patients with severe and very severe COPD with frequent exacerbations [76]. This implies that many patients who are currently using ICS should actually discontinue their ICS.

What happens when long-term used ICS are withdrawn? The most feared adverse event, namely adrenal insufficiency, develops only in approximately 7% of asthmatic patients [77]. Several studies have evaluated the effect of discontinuation of ICS. One of the most recent studies, the WISDOM trial, found a similar risk of exacerbations in severe and very severe COPD patients who continued or discontinued ICS. However, lung function decline was significantly greater after one year of ICS withdrawal compared to those who continued ICS [77, 78]. Another study in patients with symptomatic mild and moderate COPD with less than 2 exacerbations each year, showed no deterioration in symptoms and exacerbations and a stable lung function after 6 months of follow-up [79]. A disadvantage of these studies is that they did not have a prolonged randomized treatment period with ICS before withdrawal, which may differentially affect the outcome of withdrawal, nor a long-term follow-up. In addition, patient characteristics at baseline and during follow-up regarding clinical and inflammatory parameters, quality of life and airway hyperresponsiveness are only partially available in the studies. This difference in disease severity of COPD, duration of steroid treatment and follow-up time makes it difficult to compare the currently available withdrawal studies [80-82].

In the first part of the GLUCOLD study, ICS withdrawal after 6 months of treatment induced increased bronchial inflammatory cells, airway hyperresponsiveness and worsened quality of life, compared to 30-month continued treatment with ICS, without effect on lung function decline [2]. These effects on inflammation and clinical parameters have been confirmed by others [82-85]. In the GLUCOLD follow-up study (Chapter 5), we found that 5-year discontinuation of ICS after previous 30-month treatment with ICS induces a relapse in lung function decline, in combination with deterioration in airway hyperresponsiveness and quality of life. Furthermore, bronchial T-cells and mast cells and several sputum cell counts increased after ICS cessation, which was associated with accelerated lung function decline (Chapter 6). Therefore, the relapse in clinical and pathological parameters after long-term withdrawal of ICS as described in the present study, confirmed the results found during the first part of the GLUCOLD study.

Why is it important to study the long-term effect of withdrawal of ICS, when already by ICS withdrawal after 6-month treatment with ICS a relapse in bronchial inflammation and quality of life was found? By studying long-term ICS treatment, we speculated that disease modification could have been reached. Although no official definition exists, disease modification can be described as 'an improvement in, or stabilization of, structural or functional parameters as a result of reduction in the rate of progression of these parameters which occurs whilst an intervention is applied and may persist even if the intervention is withdrawn [86]. Only functional parameters, such as $FEV_1$, exacerbations and health-related quality of life, are easily measured to monitor disease modification. However, our longitudinal study shows that withdrawal of ICS even after previous long-term treatment with ICS, does not lead to a sustained disease modifying effect, as we found a relapse in lung function decline, quality of life, airway hyperresponsiveness and an increase in airway inflammation (Chapter 5 and 6). Thus, ICS does not influence the disease progression; instead, the positive effects of ICS fade out after withdrawal. It is interesting to speculate on the reason for the progressive course of COPD. Options could be the persistent smoking habits, a chronic inflammatory process that continues despite smoking cessation, or auto-immunity. As ICS seem beneficial in a subgroup of COPD patients [87], our results warrant studies in large cohorts of patients with substantial disease heterogeneity that are treated for a prolonged period with ICS compared to ICS withdrawal.

This increase in inflammatory parameters after discontinuation of ICS is not a specific feature of COPD, but has also been found in asthma. Mild to moderate asthmatics treated with mometasone for 8 weeks followed by 4 weeks of withdrawal, showed an increase in exhaled nitric oxide (FeNO) already in the first week after discontinuation of ICS [88]. Another study in adults with severe asthma showed that tapering of ICS resulted in an increase in airway hyperreponsiveness to methacholine and hypertonic saline and an increase in sputum eosinophils [89]. In asthmatic children, it has been found that withdrawal of ICS for 4 months increased FeNO, peripheral blood eosinophils and serum eosinophil cationic protein (ECP), compared to the group that continued with ICS [90]. Predictors of loss of asthma control were high blood eosinophils during ICS treatment, variability of peak expiratory flow (PEF) and increased sputum eosinophils during tapering of ICS [91, 92]. Small-sized particle ICS might be more effective in reducing systemic and pulmonary inflammatory parameters in asthma compared to normal sized particle ICS [93]. In COPD patients treated with lower doses of small-sized ICS equal exacerbation rates were found compared to the group treated with higher doses with conventionally sized ICS [94].

Should all COPD patients be treated for a prolonged time with ICS? When only considering

the results from the first part of the GLUCOLD study, showing an attenuation in lung function decline, improvement in quality of life and airway hyperresponsiveness and decreased bronchial inflammation [2], one might conclude that ICS have many beneficial effects in at least some subgroups. However, the prescribing physician should keep in mind that ICS use may have potential serious adverse effects, such as pneumonia and fractures, especially in long-term users, which is dose-related and depending on the type of ICS [95]. Patients with long-term use of fluticasone have been suggested to be more at risk compared to those who use budesonide [96]. Most importantly, no conclusions can be drawn from the presented data in *Chapter 5 and 6* whether very long-term ICS use prevents decline in lung function and airway inflammation compared to patients who discontinued ICS, as the number of patients in the subgroup that continued ICS treatment was too small to draw firm conclusions. In addition, data from the GLUCOLD study showed that a subset of COPD patients with specific features, such as less severe emphysema, less hyperinflation, less inflammation and fewer packyears, will benefit most from treatment with ICS [87]. This emphasizes the importance of proper phenotyping of patients with COPD, to identify those who may benefit most from anti-inflammatory treatment, taking components such as physiological, radiological, genetic and environmental factors into account, as was already suggested by Orie and colleagues in 1961 [97].

## Limitations of the studies and methodological considerations

The GLUCOLD study started with 114 steroid-naive patients. After the first, randomized part of the trial 85 patients continued and at the end of the follow-up study only 61 patients completed the study, which is approximately half of the patients that started. The main reason for patient withdrawal was disease progression, comorbidity of patients and health problems of their relatives. After 7.5 years, 29 patients underwent the last, fourth bronchoscopy. The relatively low number of patients per original treatment group was further split in subgroups who used ICS during the GLUCOLD follow-up study. Therefore, our group of patients and related outcomes are difficult to compare to other studies. Still, the sample size was sufficient to find associations between clinical and inflammatory outcomes *(Chapter 6)*.

In the present cross-sectional studies *(Chapter 2 and 4)*, current and ex-smokers with COPD were included for the analysis. This could have led to a selection bias, since ex-smokers could have experienced more smoking-related symptoms and therefore quitted smoking.

Whether patients had quitted smoking was only based on information provided by the patient, as was in line with other studies [17, 98], and not confirmed by e.g. a cotinine urine test. Hence, there is a possibility that ex-smokers were actually still smoking. Besides, ex-smokers had similar packyears and lung function compared to current smokers. In addition, a *post-hoc* analysis showed that smoking was unlikely to be a major confounder for airway inflammation and effects of ICS [2].

Monocyte-derived macrophages from healthy subjects were cultured *in vitro* and differentiated towards classical MΦ1 and alternatively MΦ2. This is an oversimplification of the heterogeneity of macrophages, as at least other subtypes of especially MΦ2 can be found [12]. However, the exact role of these subsets in respiratory diseases, especially COPD remains to be established. In addition, well-defined MΦ1 markers are still under debate. Furthermore, *in vitro* cultured macrophages differentiate under the influence of specific stimuli that may differ from those that regulate differentiation *in vivo* in the local environment in the lung. Indeed, a heterogeneous lung-derived macrophage population is present in the lung that is not restricted to classically defined MΦ1 and MΦ2 subsets [19]. Therefore, macrophage phenotypes *in vitro* and *in vivo* are not fully comparable [30, 99]. It requires further studies towards specific (human) macrophage markers and determination of the functions of the heterogeneous macrophage populations.

COPD is a very heterogeneous disease, which is more complex than the conventional proposed entities of emphysema and chronic bronchitis. The central airways have a different composition of e.g. cellular inflammation, the epithelium and of extracellular matrix components compared to the small airways and lung parenchyma [43, 100]. In the first part of the GLUCOLD study, we collected bronchoalveolar lavage (BAL) samples, but this had to be stopped due to ethical considerations as some patients had complications (fever and pneumonia in one patient) of the procedure. During the bronchoscopies, we are (logically) only able to sample the large airways for bronchial biopsies. Furthermore, as we used one or two biopsies of the large airways, we cannot exclude the possibility that local heterogeneity of bronchial cells and extracellular matrix proteins caused a bias in our results. In addition, we can only speculate on the effect of inhaled corticosteroids in the small airways.

## Directions for future research

- Which pro- and anti-inflammatory macrophage markers are suitable for both analysis *in vitro* and *in vivo* in COPD? Can these markers be used to monitor treatment effects?
- What is the effect of ICS on MΦ1 and MΦ2 macrophages in bronchial biopsies and peripheral airways in COPD?
- Can we use a personalized medicine approach, based on e.g. gene expression profiles, proteomics or metabolomics analysis, to improve the treatment of COPD patients?
- Are there particular stages of the disease that are most sensitive to the clinical and pathological benefits of corticosteroids?
- Is there a renewed place for systemic drugs in COPD?
- Which other potential anti-inflammatory treatments are beneficial for COPD? Does this require targeting highly selected pathways or does not this suffice given the biological complexity of the disease?
- Does continuation of dual (combination of anticholinergic and β2-agonists) bronchodilating agents after withdrawal of ICS prevent lung function decline?
- When is a deterioration of lung function decline detectable, following withdrawal of inhaled corticosteroids after previous long-term treatment?
- How can primary and secondary prevention of COPD be further accomplished?

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# CHAPTER 8

# Nederlandse samenvatting

In dit proefschrift staan de effecten van sigarettenrook op ontstekingscellen (en in het bijzonder: macrofagen) en de behandeling met geïnhaleerde ontstekingsremmers (inhalatiecorticosteroïden) bij patiënten met chronische obstructieve longziekte (COPD) centraal. In het eerste deel van deze samenvatting wordt deze ziekte, de achtergrond en de behandeling besproken; in het tweede deel van deze samenvatting wordt ingegaan op de resultaten die in dit proefschrift zijn beschreven.

# COPD

COPD is een acroniem voor de Engelse term 'Chronic Obstructive Pulmonary Disease' en is een langdurige en blijvende ziekte. Vooral vroeger werd COPD ook wel chronische bronchitis en emfyseem genoemd, hoewel vooral de term emfyseem nog regelmatig wordt gehanteerd. Patiënten met deze aandoening hebben in toenemende mate last van bijvoorbeeld kortademigheid in rust en bij inspanning, hoesten, opgeven van slijm (sputum) en reageren op bepaalde prikkels, zoals weersveranderingen of rook. De ene patiënt heeft meer of andere klachten dan de andere patiënt. Een fase waarin er een toename is van de klachten, wordt een longaanval of exacerbatie genoemd en wordt vaak uitgelokt door een luchtweginfectie. COPD wordt gekenmerkt door een versnelde achteruitgang van de longfunctie. Volgens het Rijksinstituut voor de Volksgezondheid en Milieu (RIVM) waren er in 2011 in ons land meer dan 350.000 mensen die lijden aan COPD en het is momenteel de derde doodsoorzaak wereldwijd.

## Risicofactoren voor COPD

De belangrijkste risicofactor voor het ontwikkelen van COPD is het roken van sigaretten. Langer roken en meer sigaretten per dag leidt tot een hogere kans op het ontwikkelen van COPD. Ongeveer 10-20% van alle rokers ontwikkelt COPD; omgekeerd heeft 90% van alle COPD patiënten gerookt. Een belangrijke vraag is waarom sommige rokers wel COPD ontwikkelen, en anderen niet. Dit heeft te maken met een complex geheel van erfelijke factoren, in combinatie met omgevingsfactoren waar het lichaam aan wordt blootgesteld. Daarnaast kan meeroken, luchtverontreiniging en beroepsmatige blootstelling aan bepaalde stoffen, het risico op het krijgen van COPD verhogen.

### Heterogeniteit van COPD

Hoewel COPD nu onder één noemer valt, zijn er veel verschillen tussen patiënten. Dat komt doordat COPD een heterogene ziekte is, wat inhoudt dat onder andere door de hoeveelheid roken, lichaamsgewicht, aantal exacerbaties, inspanningsbeperking en psychische status er verschillende subgroepen van patiënten zijn. Daarnaast zijn er ook inwendige verschillen, zoals afwijkingen in de luchtwegen, verhoogde vatbaarheid voor infecties en veranderde cellulaire afweer die er voor zorgen dat het beloop van COPD zich op verschillende manieren kan presenteren. Deze heterogeniteit impliceert dat subgroepen van COPD patiënten gerichter behandeld zouden moeten worden.

## De longen en luchtwegen bij COPD

### Longfunctie

Vanaf de leeftijd van ongeveer 20-25 jaar gaat bij iedereen jaarlijks de longfunctie achteruit met zo'n 25-30ml. Patiënten met COPD hebben met 60ml per jaar een versnelde achteruitgang van de longfunctie. Rokende COPD patiënten hebben een snellere achteruitgang van de longfunctie ten opzichte van ex-rokers met COPD; het 'stoppen met roken'-advies is daarom voor elk stadium van COPD relevant. Longfunctiemetingen, waarbij de hoeveelheid uitgeademde lucht wordt gemeten, zijn cruciaal voor het stellen van de diagnose COPD. Door luchtwegvernauwing, ook wel (luchtweg)obstructie genoemd, ontstaat een vermindering in de geforceerde hoeveelheid uitgeademde lucht, gemeten met de één-seconde-waarde (de maximale hoeveelheid lucht die in één seconde kan worden uitgeblazen, $FEV_1$). Als er luchtwegobstructie bestaat, is het uitademen van lucht bemoeilijkt door een verhoogde luchtwegweerstand, vergelijkbaar met het effect van het uitademen door een rietje. De hoogte van de $FEV_1$ is een maat voor de ernst van de luchtwegobstructie en kan geclassificeerd worden in mild (GOLD klasse 1), matig (GOLD 2), ernstig (GOLD 3) of zeer ernstig (GOLD 4). Hierbij staat GOLD voor de internationale consensus op het gebied van COPD. Omdat alleen de uitademing moeilijker gaat, maar de inademing niet, kan bij ernstig COPD lucht in de longen achterblijven na volledige uitademing, vergelijkbaar met de lucht in een fietsband (lucht kan er wel in, maar via het ventiel niet eruit). Dit wordt hyperinflatie genoemd.

Door het roken ontstaat schade aan de longblaasjes, ook wel de alveoli genoemd, waar de gaswisseling van zuurstof ($O_2$) en koolzuur ($CO_2$) plaatsvindt. De ernst van deze schade kan gemeten worden met de diffusiecapaciteit. Patiënten met COPD kunnen meer last krijgen

van kortademigheid als zij in contact komen met bepaalde prikkels, zoals vochtig weer, bepaalde geuren of sigarettenrook. Dit wordt hyperreactiviteit van de luchtwegen genoemd. Een methacholine provocatietest kan gebruikt worden om te meten in welke mate de luchtwegen gevoelig zijn voor prikkels.

## Luchtwegontsteking

Door de blootstelling aan sigarettenrook ontstaat er een chronische ontstekingsreactie in de luchtwegen. Welk component of welke componenten van de sigarettenrook daar verantwoordelijk voor zijn is tot nu toe onbekend, aangezien er circa 5000 verschillende stoffen in sigarettenrook zitten. De rook komt in eerste instantie in contact met de luchtwegwand (epitheelcellen) en het aangeboren, niet-specifieke afweersysteem. De rook zorgt ervoor dat er een cascade van reacties op gang komt, waarbij verschillende ontstekingscellen betrokken zijn, zoals macrofagen, $CD8^+$ T-cellen en neutrofiele granulocyten, onder invloed van bepaalde stoffen, zoals chemokines en cytokines, worden aangetrokken worden vanuit het bloed naar de longen met als doel om de schadelijke effecten tegen te gaan. De cellen raken 'geactiveerd' door contact met rook, waardoor de cytokines en chemokines vrijkomen, en kunnen bijdragen aan schade aan de luchtwegen en alveoli. Hoewel het stoppen met roken de achteruitgang van de longfunctie vermindert, blijkt uit onderzoek dat het stoppen met roken de luchtwegontsteking maar ten delen vermindert.

## Macrofagen

Macrofagen zijn afweercellen van het aangeboren immuunsysteem die een belangrijke rol spelen bij chronische ontstekingsprocessen, zoals bij COPD. Hoewel er meer macrofagen aanwezig zijn in de longen van COPD patiënten ten opzichte van gezonde controle personen, werken deze cellen minder effectief in het opruimen van bacteriën (fagocytose). Macrofagen blijken uit meerdere subtypen (fenotypes) te bestaan, die zowel in *in vitro* (buiten het lichaam) als *in vivo* (in het lichaam) gemakkelijk in elkaar kunnen overgaan (plasticiteit). Een simpele variant van deze subtypen zijn MΦ1 en MΦ2 macrofagen (ook wel Mf1 en Mf2): MΦ1 hebben eigenschappen die het ontstekingsproces bevorderen (pro-inflammatoir), zoals het afscheiden van cytokines die passen bij ontstekingen. MΦ2 cellen werken juist de ontsteking tegen (anti-inflammatoir), o.a. door het afscheiden van cytokines die ontsteking remmen. Er zijn aanwijzingen dat er bij COPD 'mengvormen' van macrofagen bestaan. Dit proefschrift

beschrijft de effecten van sigarettenrook op de macrofagen fenotypes bij patiënten met COPD. Tevens wordt een nieuwe marker voor MΦ1, namelijk YKL-40, onderzocht voor het gebruik bij *in vitro* gekweekte macrofagen en in serum en sputum van patiënten met COPD na behandeling met ontstekingsremmers.

## Remodellering van de luchtwegen

De extracellulaire matrix (ECM) is een driedimensionale structuur die als het ware het skelet van de longen vormt. Deze matrix bestaat uit drie belangrijke componenten, namelijk elastine, proteoglycanen en collagenen. Door de langdurige blootstelling aan omgevingsfactoren, zoals sigarettenrook, gaan de luchtwegen zich aanpassen, een proces dat 'remodellering' wordt genoemd. Deze remodellering vindt op meerdere plekken in de longen plaats, zowel in de grote en kleine luchtwegen als in het longweefsel. De eerste plek waar de schade door de geïnhaleerde rook ontstaat, is bij de epitheelcellen, de binnenbekleding van de luchtwegen. Door de rook raken de trilhaartjes, die bovenop de epitheelcellen zitten beschadigd, waardoor ze het slijm dat in de luchtwegen zit niet meer goed richting de keel kunnen verplaatsen. Door langdurige blootstelling aan rook ontstaan er epitheelcellen met een andere vorm zonder trilharen (ook wel metaplasie genoemd). Daarbij ontstaan door sigarettenrook meer cellen die slijm produceren (goblet cel hyperplasie). Deze twee mechanismen geven onder andere de typische rokershoest bij patiënten met COPD. Het relatieve 'stilstaan' van het sputum zorgt voor een toename in vatbaarheid voor luchtweginfecties. Daarnaast zorgt de rook voor een verdikking van het basale membraan, die laag die onder de epitheelcellaag ligt. Ook worden er stoffen afgescheiden die de luchtwegen dikker maken, wat kan bijdragen aan de luchtwegvernauwing. Tenslotte lopen de alveoli ook schade op door de sigarettenrook en ontstaat er emfyseem. De door sigarettenrook opgewekte ontstekingsreactie is de drijvende kracht voor de remodellering. Dit proefschrift beschrijft de effecten van sigarettenrook en geïnhaleerde ontstekingsremmers op de componenten van de ECM in de grote luchtwegen van patiënten met COPD.

## Behandeling van COPD

Tot nu is er geen behandeling die COPD kan genezen. De behandeling is er daarom op gericht om de klachten van kortademigheid en sputumvorming zo goed mogelijk te onderdrukken. Dit kan op twee manieren: een niet-medicamenteuze en een

medicamenteuze behandeling. Het belangrijkste niet-medicamenteuze advies aan rokende COPD patiënten is het stoppen met roken. Dit heeft positieve effecten op de klachten, de achteruitgang van de longfunctie en het aantal exacerbaties. Dit is voor veel patiënten een uitdaging en voor velen erg lastig, waarbij rokers die gestopt zijn vaak opnieuw gaan roken. Roken moet daarom ook worden gezien als chronische ziekte.

De medicamenteuze ingeademde behandeling (inhalatiemedicatie) heeft twee belangrijke pijlers: de luchtwegverwijders en de inhalatiecorticosteroïden. De luchtwegverwijders geven op diverse manieren verslapping van de gladde spiercellen rondom de luchtwegen en zijn de eerste stap in de behandeling van COPD. Ze zijn in kort- en langwerkende varianten beschikbaar en geven verlichting van de benauwdheid. Inhalatiecorticosteroïden zijn ontstekingsremmers (ook wel ICS genoemd) en kunnen de ontsteking in de luchtwegen remmen door hun lokale werking. ICS hebben dezelfde werkzaamheid als Predniso(lo) n, maar worden in een veel lagere dosering, lokaal en via inhalatie toegediend. ICS worden afgebroken in de maag, waardoor de concentratie steroïden in de bloedbaan erg laag is. Bijwerkingen van ICS zijn onder andere een schimmelinfectie in de mond en keel en heesheid. Eerder onderzoek heeft aangetoond dat ICS bij enkele patiënten ook een longontsteking kan veroorzaken. In de huidige richtlijnen worden ICS geadviseerd (in combinatie met of zonder luchtwegverwijders) bij patiënten met ernstig of zeer ernstig COPD (GOLD 3-4) met veel klachten en meer dan twee exacerbaties per jaar. Eerder onderzoek heeft laten zien dat ICS verbetering geeft van de klachten en van het aantal exacerbaties per jaar. Tot nu toe zijn er wisselende resultaten van ICS gezien op de achteruitgang van de longfunctie. Het GLUCOLD-onderzoek heeft dit ook onderzocht en vond juist dat 30 maanden behandeling met de ICS fluticason met of zonder luchtwegverwijder (salmeterol) een verminderde afname van de longfunctie geeft in combinatie met een vermindering van de ontstekingscellen in de luchtwegen, en betere kwaliteit van leven.

## Stoppen met ICS

In de dagelijkse praktijk worden ICS vaak voorgeschreven aan patiënten met COPD, ook als zij weinig klachten of minder dan twee exacerbaties per jaar hebben. Slechts een derde van de patiënten gebruikt hun medicatie dagelijks en regelmatig worden deze medicijnen slechts een paar maanden gebruikt. Tot nu toe is maar weinig onderzoek gedaan naar de effecten van het stoppen van ICS. Enkele onderzoeken vonden een toename in het aantal exacerbaties na het stoppen van ICS. Ook is het effect op de longfunctie na langdurig

stoppen van ICS nog niet goed in kaart gebracht. Dit proefschrift heeft de effecten van het langdurig stoppen van ICS onderzocht op de longfunctie en de ontstekingscellen in de luchtwegen na eerdere langdurige behandeling.

## Proefschrift

In dit proefschrift zijn de resultaten beschreven van onderzoek naar de effecten van roken en ICS op luchtwegontsteking (met nadruk op macrofagen) en de mate van remodellering van de luchtwegen. Daarnaast is onderzoek gedaan naar de effecten van stoppen van ICS op de longfunctie en luchtwegontsteking bij patiënten met COPD. Voor het onderzoek is gebruik gemaakt van de gegevens van de eerder verrichtte GLUCOLD* studie, en is ook aanvullend onderzoek gedaan bij de patiënten die de GLUCOLD studie hadden afgerond: de GLUCOLD vervolgstudie (ook wel GLUCOLD2 studie). Deze studie werd uitgevoerd in het Leids Universitair Medisch Centrum te Leiden en het Universitair Medisch Centrum Groningen te Groningen.

* Groningen and Leiden Universities Corticosteroids in Obstructive Lung Disease

De onderzoeksdoelen van het huidige proefschrift zijn:

1. Is er een verschil in het fenotype van macrofagen in sputum en bronchoalveolaire lavage (BAL, longspoeling) tussen rokende en ex-rokende COPD patiënten?

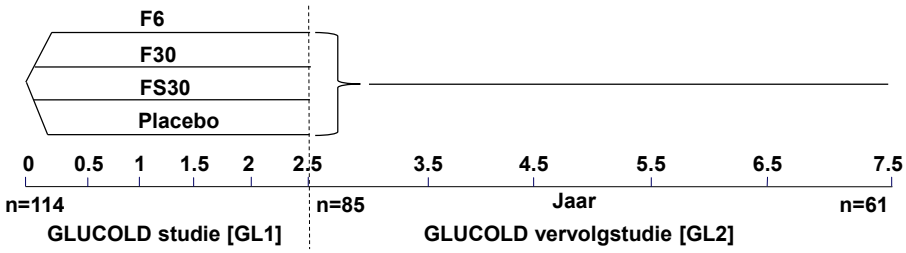
2. Wat is het effect van ontstekingsremmers op de MΦ1 marker YKL-40 bij *in vitro* (in het laboratorium gekweekte) pro-inflammatoire macrofagen, en in bloed en sputum van COPD patiënten *in vivo*?

3. Ontstaat er verandering in de structuur van de luchtwegwand (remodellering) na roken en na langdurig gebruik van ICS bij COPD?

4. Wat is het effect van langdurig stoppen van ICS op de achteruitgang van de longfunctie en mate van luchtwegontsteking na eerder langdurige behandeling (2,5 jaar) bij patiënten met COPD?

Voor de GLUCOLD studie zijn 114 mensen met matig tot ernstig COPD (longfunctie tussen 20 en 80% van voorspelde waarde) onderzocht, zowel rokende als niet-rokende mensen in de leeftijd tussen 45 en 75 jaar. Deze vrijwilligers mochten geen astma hebben (gehad). Bij aanvang van het eerste deel van de studie werden ze uitgebreid onderzocht op symptomen, longfunctie, hyperreactiviteit en luchtwegontsteking in sputum. Tevens werd een kijkonderzoek in de longen (bronchoscopie) verricht, waarbij een BAL en luchtwegweefsel (biopten) werden verkregen. Hierna werden patiënten door middel van loting in 4 behandelgroepen verdeeld (Figuur 1), 6 of 30 maanden behandeling met geïnhaleerde onstekingsremmers met en zonder luchtwegverwijder of placebo (nepmedicijn), waarbij de behandeling voor zowel de onderzoekers als de deelnemers onbekend was. Elke 3 maanden werd de longfunctie en kwaliteit van leven gemeten, en na 6 en 30 maanden werd opnieuw de luchtwegontsteking onderzocht (sputum, BAL, biopten). Na 30 maanden behandeling hadden 85 mensen het eerste deel van de studie doorlopen. De uitkomsten van de GLUCOLD studie (GL1) zijn eerder gepubliceerd door Thérèse Lapperre en Jiska Snoeck-Stroband.

Tijdens het tweede gedeelte van de GLUCOLD studie (vervolgstudie, GL2) werd de longfunctie en kwaliteit van leven jaarlijks gemeten. Hyperreactiviteit en sputum werd onderzocht na 2 en 5 jaar; biopten werden na 5 jaar opnieuw verkregen. De totale studieduur komt hiermee op 7,5 jaar. Tijdens de laatste 5 jaar werden de patiënten behandeld door hun eigen longarts of huisarts volgens de laatste richtlijnen. In de praktijk betekende dit dat het merendeel van de deelnemers van de studie geen of weinig ontstekingsremmers gebruikten tijdens GL2. Uiteindelijk hebben 61 patiënten de totale studie afgerond.




**Figuur 1:** Studie opzet tijdens eerste (GL1) en tweede (vervolg, GL2) deel van de GLUCOLD studie.

## Macrofaagheterogeniteit bij COPD

*Hoofdstuk 2: Anti-inflammatoire macrofagen in sputum en BAL van rokende en ex-rokende COPD patiënten.*

Macrofagen spelen als afweercellen een belangrijke rol bij de ontwikkeling van COPD. Macrofagen vormen een heterogene populatie met MΦ1 (pro-) en MΦ2 (anti-inflammatoire) cellen. Eenmaal gedifferentieerde macrofagen blijven de mogelijkheid behouden tot verandering van fenotype. Dit houdt in dat als macrofagen in aanraken komen met een inflammatoire prikkel, de MΦ2 cel kan veranderen in een MΦ1 cel, en andersom. Er wordt aangenomen dat in de grote luchtwegen, die als eerste in aanraking komen met onder andere bacteriën en omgevingsfactoren, meer MΦ1 cellen aanwezig zijn. In de kleinere luchtwegen en alveoli zijn juist meer MΦ2 cellen aanwezig om de gaswisseling goed te laten verlopen. In dit hoofdstuk is het effect van roken op de anti-inflammatoire macrofagen onderzocht. Hiervoor hebben we de MΦ2 marker CD163 gebruikt naast bepaalde pro-inflammatoire (interleukine 6 en 8) en anti-inflammatoire eiwitten (elafine en Secretory Leukocyte Protease Inhibitor [SLPI]), die aanwezig zijn in BAL en opgewerkt sputum van rokende COPD patiënten en COPD patiënten die gestopt zijn met roken. De belangrijkste resultaten van dit onderzoek zijn:

- Ex-rokers met COPD hebben een hoger percentage van CD163-positieve macrofagen en een groter totaal aantal cellen, maar een kleiner aantal in BAL ten opzichte van rokende COPD patiënten.

- Het percentage CD163-positieve MΦ2 macrofagen is hoger in BAL dan in sputum.

- Er is geen verschil tussen rokers en ex-rokers met COPD in de concentratie pro- en anti-inflammatoire eiwitten aanwezig in BAL en sputum.

Deze resultaten suggereren dat stoppen met roken het fenotype van macrofagen gedeeltelijk kan veranderen naar een anti-inflammatoire type in de longen van COPD patiënten. Dit gaat niet gepaard met een verandering in aantal neutrofielen, ontstekingsbevorderende of ontstekingsremmende eiwitten in het sputum of BAL. Tevens laten deze resultaten zien dat in de diepere luchtwegen meer MΦ2 cellen aanwezig zijn bij COPD patiënten.

*Hoofdstuk 3: Effect van ontstekingsremmers op YKL-40 op pro-inflammatoire macrofagen*

MΦ1 en MΦ2 macrofagen worden met verschillende markers gekarakteriseerd. In Hoofdstuk 2 werd CD163 beschreven als een marker voor MΦ2 cellen, in hoofdstuk 3 wordt de nieuwe, potentiële marker YKL-40 beschreven als marker voor MΦ1 cellen. YKL-40 is een chitinase-achtig eiwit dat onder andere door macrofagen en andere celtypes wordt afgescheiden en is geassocieerd met verschillende ontstekingsziekten, waaronder astma en COPD. Eerder onderzoek heeft laten zien dat YKL-40 sterk wordt onderdrukt door dexamethason (een sterke ontstekingsremmer), waardoor de indruk bestaat dat YKL-40 voornamelijk op MΦ1 cellen zit. In ons laboratorium hebben we dit verder onderzocht door monocyten (voorlopers van macrofagen) uit bloed te isoleren, *in vitro* te kweken in het laboratorium tot MΦ1 en MΦ2 cellen en deze vervolgens te stimuleren met stoffen die ontsteking opwekken. Tenslotte werden de cellen behandeld met dexamethason. Verder is onderzocht welk fenotype van gekweekte macrofagen het meest boodschapper RNA (mRNA) tot uiting brengt en YKL-40 eiwit uitscheidt. Ook is het effect van dexamethason op de fenotypen van macrofagen bestudeerd. Als laatste is het effect van ICS op YKL-40 in bloed en sputum van patiënten met COPD onderzocht. De bevindingen van dit onderzoek zijn als volgt:

- Gekweekte MΦ1 cellen maken meer YKL-40 mRNA en scheiden meer YKL-40 eiwit uit ten opzichte van MΦ2 cellen, onafhankelijk van de stimulatie van de cellen.

- Dexamethason remt dosis-afhankelijk YKL-40 mRNA expressie en eiwit uitscheiding in MΦ1 cellen.

- Hogere eiwitconcentraties YKL-40 wordt gevonden in bloed vergeleken met sputum van patiënten met COPD.

- Behandeling met ICS verandert de hoeveelheid YKL-40 eiwit in sputum en bloed van patiënten met COPD niet na 30 maanden behandeling met ICS.

Deze resultaten suggereren dat YKL-40 een geschikte marker is voor *in vitro* gekweekte MΦ1 cellen, maar niet gebruikt kan worden om het effect van ICS behandeling te meten in serum en sputum van patiënten met COPD. Een mogelijke verklaring hiervoor kan zijn dat MΦ1 cellen een specifieke stimulatie nodig hebben, die toegediend kan worden bij *in vitro* gekweekte macrofagen, maar die niet aanwezig is in het bloed of luchtwegen van patiënten met COPD.

## Ontstekingsremmers en luchtwegwand remodellering

*Hoofdstuk 4: Effect van roken en ontstekingsremmers op de samenstelling van extracellulaire matrix (ECM) in de luchtwegwand van COPD patiënten.*

Het weefsel rondom de luchtwegen is een driedimensionale structuur, die ook wel de extracellulaire matrix (ECM) wordt genoemd. Deze bestaat onder andere uit elastische vezels, proteoglycanen en collagenen. Eerder onderzoek heeft aangetoond dat langdurig roken onomkeerbare schade veroorzaakt aan de elastische vezels in de alveoli. Dit suggereert dat er door sigarettenrook ook veranderingen kunnen ontstaan in de structuur en componenten van de wand van de kleine en grote luchtwegen, een proces dat bijdraagt aan de luchtwegvernauwing. In dit hoofdstuk is onderzocht wat de effecten zijn van roken en ICS op verschillende componenten van de ECM in de grote luchtwegen, namelijk elastische vezels, proteoglycanen (versican en decorine) en collagenen (collageen I en III). De uitkomsten van dit onderzoek zijn:

- (Stoppen met) roken heeft geen effect op de diverse componenten van de ECM in de grote luchtwegen van COPD.
- Langdurig behandelen met ICS geeft een toename van versican en collageen III in de grote luchtwegen.
- Er is een relatie tussen de hoeveelheid collageen I en de longfunctie na langdurige behandeling met ICS.

Bovenstaande resultaten suggereren dat de samenstelling van de luchtwegwand verandert in patiënten met COPD na langdurige behandeling met ICS. Deze remodellering van de luchtwegen, kan er mogelijk voor zorgen dat ze stugger en stijver worden en voorkomen het samenvallen van de luchtwegen bij de ademhaling.

## Effect van stoppen met ICS op longfunctie en luchtwegontsteking

*Hoofdstuk 5: Langdurig stoppen met ICS na 2,5 jaar behandeling veroorzaakt versnelde achteruitgang in longfunctie bij patiënten met COPD.*

Het GLUCOLD onderzoek heeft eerder laten zien dan 2,5 jaar behandeling met ICS een verminderde achteruitgang van de longfunctie geeft, verminderde luchtweghyperreactiviteit en betere kwaliteit van leven. In de dagelijkse praktijk gebruiken veel patiënten de onstekingsremmers echter kortdurend of onregelmatig. Tot nu toe zijn de effecten van stoppen met ICS onvoldoende onderzocht. Dit is belangrijk om te weten, omdat ICS mogelijk ook de onderliggende mechanismen van COPD zou kunnen veranderen (ziekte modificatie). In dit hoofdstuk hebben we onderzocht wat de effecten zijn van langdurig stoppen (vijf jaar) van de ICS na eerder 2,5 jaar behandeling. Hiervoor zijn de patiënten van het eerste deel van het onderzoek gevraagd om mee te doen aan het vervolgonderzoek. Patiënten zijn behandeld door hun eigen longarts of huisarts. Het merendeel van de patiënten gebruikte geen of onregelmatig ontstekingsremmers. Jaarlijks werd de longfunctie en kwaliteit van leven gemeten evenals de hyperreactiviteit van de luchtwegen. De belangrijkste resultaten van dit onderzoek zijn:

- Langdurig stoppen met ICS leidt tot een versnelde jaarlijkse achteruitgang van de longfunctie ten opzichte van de periode dat wel ICS worden gebruikt.

- Patiënten die langdurig gestopt zijn met ICS hebben een toename in luchtweghyperreactiviteit ten opzichte van de periode dat ze wel ICS gebruikten.

- Langdurig stoppen van ICS geeft enige achteruitgang van de kwaliteit van leven ten opzichte van de periode dat de patiënten werden behandeld met ICS.

Bovenstaande resultaten suggereren dat actief gebruik van ICS alleen een positief effect heeft op de longfunctie, kwaliteit van leven en mate van luchtweghyperreactiviteit tijdens actief gebruik van de ICS, maar dat bij langdurig staken ervan deze effecten weer verdwijnen. Er lijken dus door ICS behandeling geen langdurige en blijvende veranderingen in het ziekteproces (geen ziektemodificatie) op te treden die persisteren na het stoppen met ICS. Patiënten met matig tot ernstig COPD is een aparte groep van patiënten, die de snelste achteruitgang van de longfunctie hebben. Juist daardoor valt er in deze groep de meeste winst op de longfunctie te behalen met de behandeling met ICS. Toch dient de conclusie van het onderzoek voorzichtig te worden geïnterpreteerd: het onderzoek heeft immers niet laten

zien dat COPD patiënten moeten doorgaan met de langdurige behandeling met ICS. Dit was ook niet het doel van het huidige onderzoek en zal verder onderzocht moeten worden in toekomstige onderzoeken.

### *Hoofdstuk 6: Langdurig stoppen met ICS na 2,5 jaar behandeling leidt tot toename van luchtweg ontsteking bij patiënten met COPD.*

Het GLUCOLD onderzoek heeft eerder laten zien dat 2,5 jaar behandeling met ICS zorgt voor een afname van het aantal CD3-, CD4-, en CD8-positieve cellen en mestcellen. Tevens werd een afname gevonden in het aantal neutrofielen, macrofagen en lymfocyten in het sputum. Wederom is tot nu toe onbekend wat de effecten zijn van het langdurig stoppen van de behandeling met ICS op het aantal ontstekingscellen in de luchtwegen. De patiënten die in Hoofdstuk 5 werden genoemd, werd gevraagd of zij ook sputum wilden inleveren (na 2 en 5 jaar vervolgonderzoek) en een kijkonderzoek weefselstukjes (biopten) uit de luchtwegwand wilden ondergaan (na 5 jaar vervolgonderzoek). De belangrijkste bevindingen van dit onderzoek zijn:

- Vijf jaar stoppen met ICS na eerdere 2,5 jaar behandeling geeft een toename van het aantal CD3, CD4, en CD8 positieve cellen en mestcellen in biopten.

- Langdurig stoppen met ICS geeft een toename van het totaal aantal cellen in sputum, en het aantal macrofagen, neutrofielen en lymfocyten in sputum.

- Er is een relatie tussen een versnelde afname van longfunctie en toename van aantal sputum macrofagen en luchtweg neutrofielen.

Deze resultaten laten zien dat ICS de luchtwegontsteking onderdrukken zolang deze inhalatiemedicatie wordt gebruikt. Wederom is er geen aanhoudende ziekte aanpassing door langdurig gebruik van ICS.

## Perspectief voor toekomstig onderzoek

De onderzoeken zoals beschreven in dit proefschrift hebben bijgedragen aan de kennis over macrofagen fenotypes, luchtweg remodellering en lange termijn effecten van stoppen met de behandeling met ICS. Uiteraard zijn dit slechts puzzelstukjes van de grote puzzel over ontsteking in de longen van COPD patiënten en de behandeling daarvan. De hier beschreven onderzoeken roepen daarom ook nieuwe vragen op, die in toekomstig onderzoek nader uitgezocht dienen te worden.

- Wat is het effect van ICS op CD163 (of andere MΦ2 markers) en YKL-40 (of andere MΦ1 markers) positieve macrofagen in grote en kleine luchtwegen van patiënten met COPD?

- Zijn andere pro- en anti-inflammatoire macrofagen markers wellicht geschikter om het effect van ontstekingsremmers te monitoren?

- Wat zijn de effecten van ICS op de remodellering van de kleine luchtwegen en alveoli bij COPD?

- Welke groepen van COPD patiënten hebben het meeste baat bij de behandeling met geïnhaleerde ontstekingsremmers? Kunnen bepaalde (epi)genetische profielen of gen expressie profielen herkend worden die reactie op ontstekingsremmers kunnen voorspellen?

- Blijven de positieve effecten op longfunctie, ontsteking en kwaliteit van leven aanhouden bij zeer langdurige (>2,5 jaar) behandeling met ICS?

- Zijn er andere potentiële ontstekingsremmende medicijnen beschikbaar om de progressie van COPD te verminderen? Zijn ICS met fijnere deeltjes meer geschikt om de luchtwegontsteking bij COPD te behandelen?

# Appendix

# Curriculum vitae

# Dankwoord

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# The GLUCOLD study group

# Curriculum vitae

Lisette Kunz was born on October 30th, 1978 in Voorburg, The Netherlands. In 1997 she graduated from secondary school at the Huygens Lyceum in Voorburg. In the same year, she started with her Pharmacy training at the University of Utrecht. In 1999 she switched to the University of Leiden for the study Medicine. During her medical school she participated for three months in a research project in north-east Ghana in 2002 (collaboration with the department of Parasitology of the Leiden University Medical Center; supervisor: Prof. Dr. A. Polderman) and in 2003 she did a nine months research internship at the department of Pulmonology of the Leiden University Medical Center (supervisor: Prof. Dr. P.J. Sterk), which resulted in her first publication. In 2005 she followed a clinical internship in Internal Medicine in the Diakonessenhuis in Paramaribo, Surinam. She graduated from medical school in 2006.

She began her first two year clinical training of Internal Medicine in the Bronovo Hospital in The Hague (head: Dr. J.W. van 't Wout). In 2008, she started with her PhD project at the department of Pulmonology at the Leiden University Medical Center, supervised by Prof. Dr. P.S. Hiemstra, Prof. Dr. D.S. Postma (University Medical Center Groningen, Groningen) and Prof. Dr. P.J. Sterk (Academic Medical Center, Amsterdam). During her PhD project, she won three Travel Awards from the American Thoracic Society and was nominated in 2015 for the Dutch 'Longdagen publieksprijs'. She started her clinical training to become a respiratory physician in June 2011 at the department of Pulmonology at the Leiden University Medical Center (head: Prof. Dr. K.F. Rabe). In 2012 she continued her clinical training in the Department of Respiratory Medicine at the Academic Medical Center in Amsterdam (head: Prof. Dr. E.H.D. Bel), and she finished her training in June 2016. Currently, she is working as a respiratory physician in the Diakonessenhuis in Utrecht.

# Dankwoord

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