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

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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.

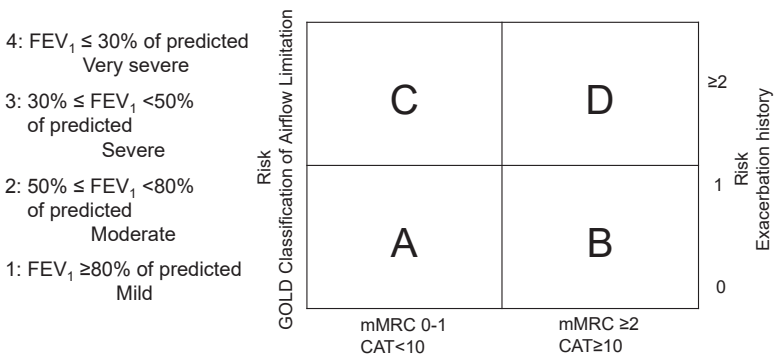

Figure 1: COPD assessment of severity. Adapted from Vestbo, et al. [1].

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

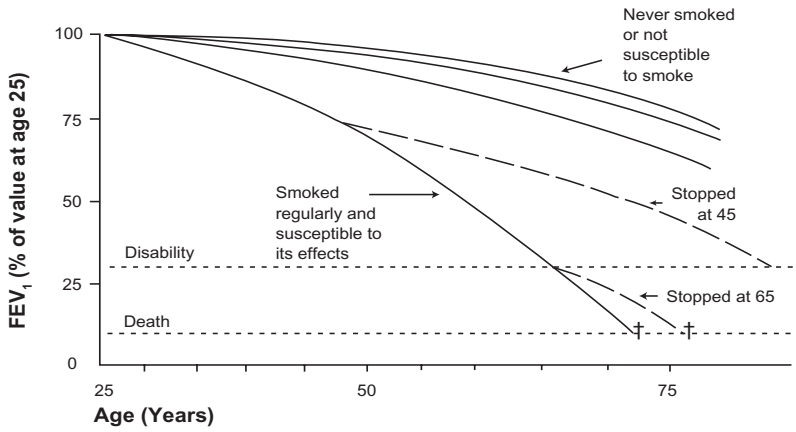

Figure 2: Effect of smoking cessation on lung function decline by Fletcher and Peto [30].

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.

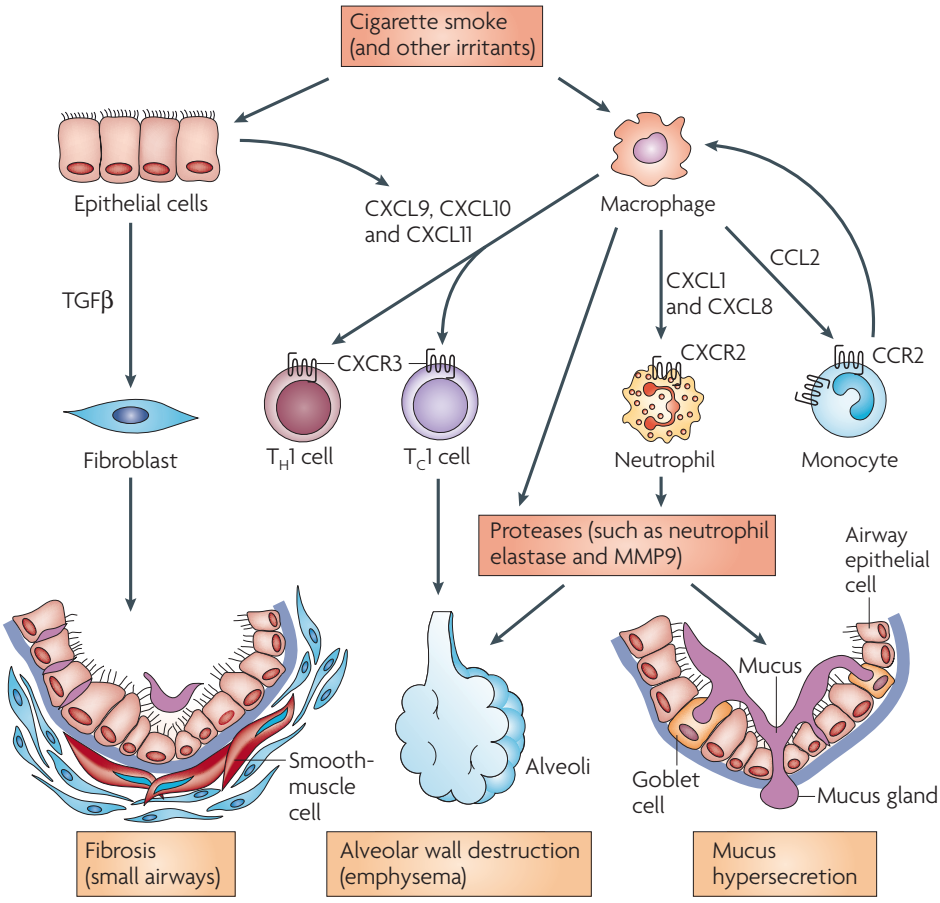

Figure 3: Pathological changes due to cigarette smoking in the airways in COPD. Reproduced from Barnes [61].

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.

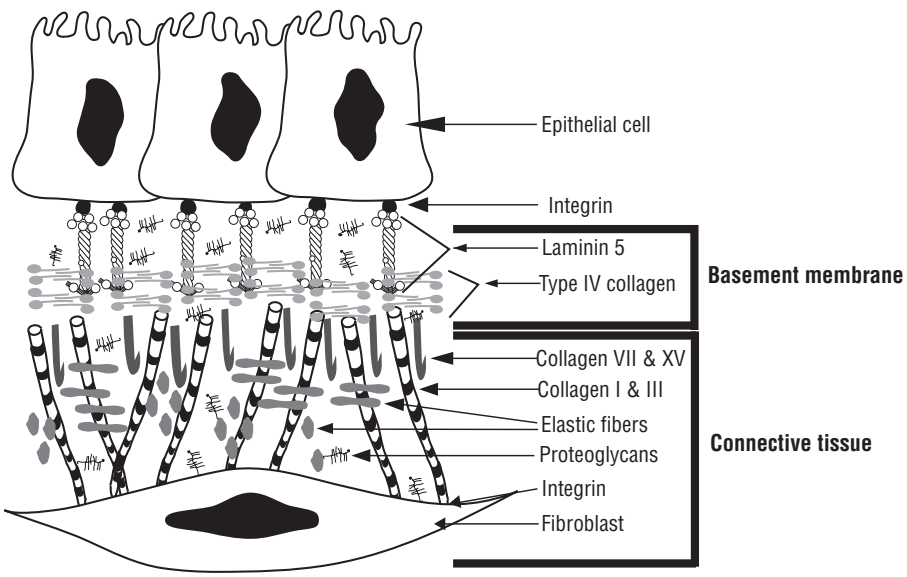

Figure 4: Schematic presentation of the airway wall and its extracellular matrix components. Reproduced from Dunsmore [186].

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 or SAMA	LABA or LAMA or SABA and SAMA	Theophylline
B	LABA or LAMA	LABA and LAMA	SABA and/or SAMA Theophylline
C	ICS with LABA or LAMA	LABA and LAMA	SABA and/or SAMA Theophylline
D	ICS with LABA And/or LAMA	ICS with LABA and LAMA or LABA and LAMA	N-acetylcysteine SABA and/or SAMA 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.

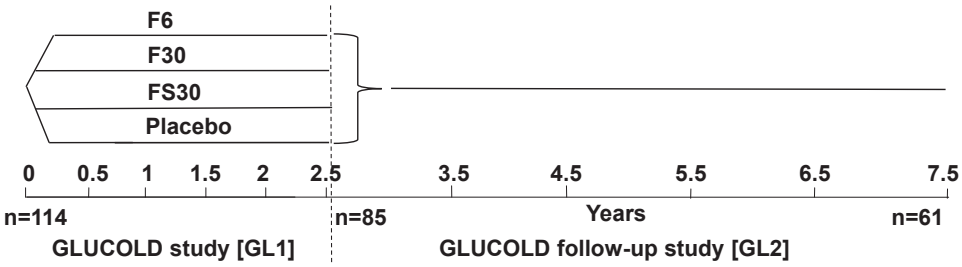

Figure 5: Study design during first (GL1) and second (follow-up, GL2) part of the GLUCOLD study.

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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