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

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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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