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Asthma and COPD: Smoking, Atopy and Corticosteroid responsiveness

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Fatemeh Fattahi

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university of
groningen

Asthma and COPD: Smoking, Atopy and Corticosteroid responsiveness

PhD thesis

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by

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Chapter 1

General introduction

Asthma

Asthma is a chronic respiratory disorder characterized by airway hyperresponsiveness (AHR) and variable episodic, mostly reversible airway obstruction [1]. Nowadays asthma is not seen anymore as a single disease, but as collection of different phenotypes, with different underlying pathologic mechanisms [2, 3]. Not only a large number of risk factors and pathological mechanisms have been discovered, but also a large number of phenotypes have been described. In 2006 the Global Strategy for Asthma Management and Prevention defined asthma as follows: 'Asthma is a chronic inflammatory disorder of the airways in which many cells and cellular elements play a role. The chronic inflammation is associated with airway hyperresponsiveness that leads to recurrent episodes of wheezing, breathless, chest tightness, and coughing, particularly at night or in the morning. These episodes are usually associated with widespread, but variable, airflow obstruction within the lung that is often reversible either spontaneously or with treatment' [4]. This definition is purely descriptive and includes the broad range of phenotypic manifestations that are used for its clinical diagnosis. Identification of the underlying endotypes is one of the major challenges in the next years in asthma research [5], and this challenge starts with careful phenotyping of our patients. One of the oldest ways to categorize asthmatic patients is based on the presence or absence of atopy [6].

Asthma and atopy

The word "atopy," stems from the Greek language, refers to "special" or "unusual," and was for the first time used in the medical literature by Coca in 1923 [7]. "Special" or "unusual" referred to the fact that atopy was limited to only a small group of patients, with a certain hereditary predisposition. Atopy was clinically characterized by hay fever and bronchial asthma, and associated with immediate-type skin reactions. Currently, atopy has been defined as follows: "Atopy is a personal and/or familial tendency to become sensitized and produce IgE antibodies in response to ordinary exposures to allergens, usually proteins. As a consequence, these persons can develop typical symptoms of asthma, rhinoconjunctivitis, or eczema" [8]. Regarding the nomenclature a position paper in 2001 recommended that "a healthy asymptomatic person with a positive skin prick test or the presence of specific IgE antibodies should be referred to as "skin prick test positive" or "IgE sensitized," and the term atopic reserved for a person with this predisposition who is suffering from typical allergic symptoms" [9], whereas the revised nomenclature report concludes that "atopy is a clinical definition of an IgE-antibody high-responder, and allergic symptoms in a person of the atopic constitution may be referred to as atopic, as in atopic rhinitis" [8].

Atopy is a well-known risk factor for asthma since atopy is more frequently reported in asthma than non-atopy [10-12]. Studies estimating the percentage of asthma cases attributable to atopy have reported a population attributable risk (PAR) ranging between 33% and 56% [13-15]. In line, atopy in early life seems to be one of the important risk factors to develop asthma in later life [16], whereas atopic asthma in adulthood seems to have its roots in early childhood [3].
In adults, a classical way to phenotype asthma patients is on basis of atopic (extrinsic) versus non-atopic (intrinsic) asthma [10]. Atopy has often been associated with increased severity of asthma, especially among children [17]. Non-atopic asthma is more frequently present in adults and associates with increased lung function decline, yet less frequent exacerbations [18]. Similar immunological processes have been described in atopic and non-atopic asthma [19, 20], but there are also studies that have observed differences [21-23]. Regarding airway pathology, subjects with atopic asthma showed higher numbers of eosinophils, T lymphocytes, Th2 cytokines (interleukin (IL)-4 and IL-5) and lower numbers of neutrophils and non-Th2 cytokines (IL-8) than subjects with nonatopic asthma [24]. Definitive conclusions are not easy to draw at this moment because there is rather scarce information about the pathological distinctions between atopic and non-atopic asthma.

Asthma and smoking

During the last century, the harmful effects of tobacco smoking on the clinical expression of respiratory symptoms in asthmatics patients were increasingly documented. Smoking asthmatics have worse control of asthma independently of FEV1, show accelerated decline in lung function, and increased mortality rates [25-27]. Even passive smoking has been reported to increase asthma symptoms [28] and to worsen asthma control [29]. Several studies showed that tobacco smoking in asthmatic patients associates with the start and progression of asthma symptoms, accelerated lung function decline and impaired therapeutic response to corticosteroids [25, 30-35]. Not surprisingly, tobacco smoking in asthma patients may also induce a “COPD-like” airway obstruction and airway inflammatory changes [36]. In general, chronic exposure to cigarette smoke drives the airway luminal and tissue macrophages into an activated state, secreting proinflammatory cytokines such as IL-8, and promoting the influx of inflammatory cells, particularly neutrophils. In line, asthmatic smokers demonstrate higher numbers of neutrophils in induced sputum [37, 38], correlating with smoking history [37]. And airway biopsies from asthmatic smokers demonstrate increased numbers of mast cells, decreased numbers of eosinophils, and mucus-producing elements in the bronchial epithelium. Additionally, intraepithelial IL-8 expression is increased in airway biopsies [39], while in vitro

cigarette smoke induces the release of IL-8 from human bronchial epithelial cells [40]. This is in accordance with the observation that IL-8 correlates positively with the number of neutrophils in induced sputum, and associates with a higher number of pack years [37]. Similarly, our group showed that increased epithelial cell proliferation in asthmatic smokers associates with a higher number of pack-years [41]. Taken together, tobacco smoking in asthma patients may induce a "COPD-like" airway inflammation and obstruction [36, 42, 43].

Chronic Obstructive Pulmonary Disease (COPD)

COPD is defined by the Global Initiative for Chronic Obstructive Lung Disease (GOLD) as "a preventable and treatable disease with some significant extra pulmonary effects that may contribute to the severity of COPD in individual patients. Although COPD affects the lungs, it also produces significant systemic effects" [44]. Its pulmonary component is characterized by airflow limitation that is not fully reversible [42]. This airflow limitation is usually progressive and associated with an abnormal inflammatory response of the lung to noxious particles or gases [39, 40]. A spirometric definition of the disease is broadly used both in the clinical setting and for research, establishing COPD patients as those subjects with post-bronchodilator forced expiratory volume in the first second to forced vital capacity ratio (FEV_1/FVC) <0.70 or ≤0.70 [44]. The post bronchodilator FEV_1 % predicted is used for classification of the severity of airway obstruction in COPD (table 1).

Table 1. GOLD classification of COPD severity based on post-bronchodilator FEV1

Stage I: Mild COPD	FEV_1/FVC <70%; FEV_1> 80% predicted
Stage II: Moderate COPD	FEV_1/FVC <70%; 50%< FEV_1<80% pred.
Stage III: Severe COPD	FEV_1/FVC <70%; 30%< FEV_1<50% pred.
Stage IV: Very Severe COPD	FEV_1/FVC <70%; FEV_1<30% pred. or FEV_1< 50% pred. plus presence of chronic respiratory failure

FEV_1: Forced Expiratory Volume in second one; FVC: Forced Vital Capacity

Clinically COPD is characterized by symptoms of chronic and progressive dyspnea, cough and sputum production. Tobacco smoke is the main risk factor for COPD but nevertheless COPD is a complex multi-causal disease where e.g. air pollution may contribute to COPD development

as well [45]. Although some COPD definitions in the guidelines state that COPD is "primarily caused by cigarette smoking" [44], not all smokers develop the disease whereas some non-smokers present clinically significant COPD. Indeed, Fletcher et al. have shown that the decline in lung function is faster in smokers compared to non-smokers [46]. However, in a population of industrial smokers, only 15-20% of all smokers, and up to 50% of elderly (>75 years) smokers developed COPD [47] suggesting a role for age, individual susceptibility and other environmental triggers. The genetic risk factor that is best documented is a severe hereditary deficiency of alpha-1 antitrypsin, a major circulating inhibitor of chemicals, air pollution, reduced lung growth and development, oxidative stress, female gender, infections, low socioeconomic status, inadequate nutrition, cooking and heating in poorly ventilated spaces, and asthma [43, 49]. Whereas smoking cessation reduces respiratory symptoms and lung function decline in COPD, histopathological studies suggest that inflammation persists in ex-smokers [50]. Nevertheless, there are also indications that (components of) airway inflammation fades out. One study demonstrated that long-term smoking cessation (>3.5 years) resulted in less bronchial epithelial remodeling as compared to current smokers [51]. Additionally, with longer duration of smoking cessation, CD8 cell numbers decrease and plasma cell numbers increase in bronchial biopsies [52]. This indicates that bronchial T lymphocyte and plasma cell counts, but not other inflammatory cells, are related to duration of smoking cessation in patients with COPD [52].

Asthma and COPD differences and similarities/overlap

Asthma and COPD have important differences [53], yet there are also important similarities [54]. In clinical practice, the main feature that is generally used to distinguish asthma and COPD is the reversibility of airflow limitation in response to inhaled bronchodilators such as β-agonists, anticholinergics, methylxanthines, and corticosteroids. Table 2 lists the most important clinical features; and shows that there is frequent overlap. A proportion of the asthmatics has COPD-like features (chronic cough, sputum production, airway obstruction), whereas a proportion of the COPD patients have asthma-like features (good bronchodilator response, increased bronchial hyperresponsiveness).

Table 2. Clinical features of asthma and COPD

	Asthma	COPD
Age	Predominantly childhood and young adulthood, but may continue into elderly age	Predominantly in middle-aged and elderly people
Sex	Before puberty: male>female After puberty: female>male	Somewhat more in males (in the industrialized world)
Chronic cough and sputum	15%	80%
Dyspnoe	90% intermittent, 5% chronic	50% intermittent (exercise), 25% chronic
Airway obstruction	Mostly intermittent, but sometimes chronic	Chronic per definition (FEV1/FVC<0.7)
Reversibility to BD (>10% FEV1 baseline)	Mostly good	Mostly poor
Atopy	80%	20%: similar to population
Hyperresponsiveness		
Histamine	90%	40%
Metacholine	90%	70%
AMP	90%	40-90% (in non-smokers and smokers respectively)
Chest radiograph	Normal or hyperinflation	Normal or hyperinflation, tramlines, emphysema

AMP: adenosine-5-monophosphate

The question rises how frequently asthma and COPD occur together. A frequently quoted epidemiological study in that perspective is the study of Marsh et al [55]. In a random sample of adults in an urban New Zealand community the authors used detailed questionnaire data, pulmonary function tests and chest CT scans to determine the proportion of subjects within each phenotypic subgroup of COPD. The figure below depicts the relationship in a non-proportional Venn diagram of chronic airflow obstruction (figure 1) [55]. The overlap between asthma and COPD comprised about 20% of the population with chronic airflow obstruction (segment 6,8,7 below).

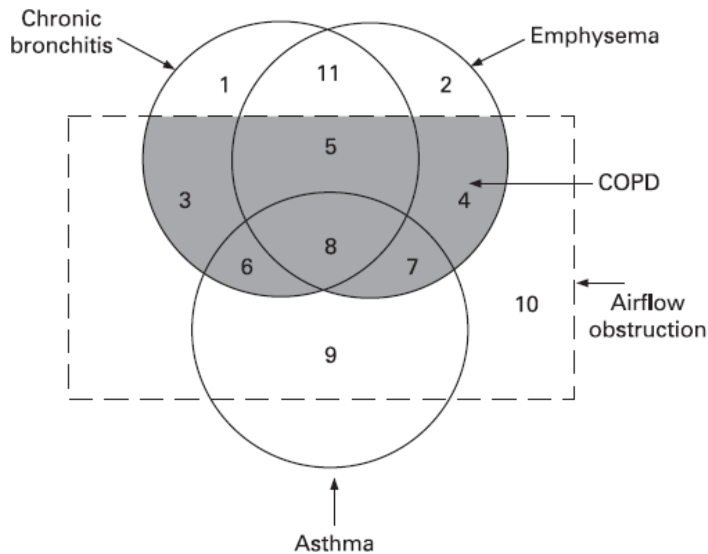

Figure 1. Non-proportional Venn diagram of chronic airflow obstruction

Although both diseases are clinically characterized by chronic or intermittent airway obstruction they represent different underlying complex inflammatory abnormalities, as the nature of asthma's inflammation is mainly eosinophilic and affecting the airway and peribronchial lung parenchyma [56, 57], whereas neutrophils are more important in COPD and affecting the total lung. This key difference may explain the different response to corticosteroid treatment in both diseases, as corticosteroids reduce eosinophil survival but prolong neutrophil survival [58]. Table 3 summarizes the main differences in the nature of the inflammation in both diseases [53].

As mentioned earlier, asthma and COPD represent two different classes of obstructive lung disorders with different diagnostic and management strategies [42, 43]. Nevertheless, the medical treatment may show important overlap. Asthma is optimally treated with regular anti-inflammatory medications (preferably ICS), and short-acting bronchodilators are used when needed [59]. COPD is usually treated with long-acting bronchodilators, which provide symptomatic benefits, and ICS to reduce the frequency of exacerbations [59]. Whilst chronic inflammation underlies both asthma and COPD, the nature of the inflammation differs, as does the response to anti-inflammatory medications [59]. The available evidence so far suggests that COPD cannot be sufficiently distinguished from asthma.

Table 3. Pathological features of Asthma and COPD

Inflammation	Asthma	COPD
Inflammatory cells	Eosinophils	Neutrophils
	CD4+ cells (Th2)	CD8+ cells (Tc)
	Macrophages **+**	Macrophages **++**
	Mast cells	Eosinophils during exacerbations
Inflammatory mediators	LTB4, histamine	LTB4
	IL-4, IL-5, IL-13	TNF-α
	Eotaxin, RANTES	IL-8, GRO-α
	Oxidative stress **+**	Oxidative stress **+++**
Inflammatory effects	All airways	Peripheral and central airways
	AHR **+++**	AHR **±**
	Epithelial shedding	Epithelial metaplasia
	Fibrosis **+**	Fibrosis **++**
	No parenchymal involvement	Parenchymal destruction
	Mucus secretion **++**	Mucus secretion **+++**
Response to corticosteroids	**+++**	**±**

Th2= T-helper type 2, Tc= T-cytotoxic, LTB4= Leukotriene B4, GRO-α= growth-related oncogene α, AHR= Airway hyperresponsiveness [53]

Back in the 60s, Orie et al [60] proposed what is known as the “Dutch hypothesis”: that COPD and asthma were manifestations of the same obstructive lung disease (OLD). Moreover, Orie proposed that asthma, as a form of OLD, could evolve into COPD, another form of OLD. The Dutch hypothesis, although controversial, could not be proven wrong and has been present in the scientific literature since then. In 1995, the American Thoracic Society stated that “it may be impossible to differentiate patients with asthma whose airflow obstruction does not remit completely from persons with chronic bronchitis and emphysema with partially reversible airflow obstruction and bronchial hyperresponsiveness” [55]. It is noteworthy that lung function impairment, as a precursor of COPD, has been suggested to increase the risk of asthma both in children and adults [61], and this fact together with other available evidences has attracted attention towards the potential early origins of COPD [62].

Many years after Orie and the postulation of the Dutch hypothesis the term ‘Asthma COPD overlap syndrome’ (ACOS) has been introduced by GINA and GOLD [63]. ACOS was defined

as persistent airflow limitation with several features usually associated with asthma and several features usually associated with COPD [63]. Because ACOS does not represent a single discrete disease entity GINA/GOLD recommended later to use the term 'Asthma COPD overlap' (ACO). However, it is widely recognized that asthma and COPD can coexist as asthma-COPD overlap, but the preliminary attempts at providing universal guidelines for the diagnosis of ACO still need to be improved [64].

In a recent review the prevalence of ACO was estimated to range from 0.9% to 11.1% in the general population, from 11.1% to 61% in asthma patients and from 4.2% to 66% in COPD patients [65]. The patients with ACO were generally older than patients with asthma and younger or similarly aged as compared to patients with COPD. The prevalence of ACO seems to increase as age rises, and this result is the same as that for COPD. The review reported contradictory findings for predominance of gender.

Corticosteroid responsiveness

Since the introduction of inhaled corticosteroids, they have become established as the cornerstone of asthma management due to their consistent ability to reduce symptoms, prevent asthma exacerbations, improve lung function, suppress non-invasive markers of airway inflammation, eosinophil numbers, inflammatory cell activation and inflammatory gene transcription in the majority of patients [43, 66]. It has also been shown that COPD patients can benefit from corticosteroid treatment, i.e. symptomatic patients with an $FEV_1 < 50\%$ predicted (stage III, severe COPD, and stage IV, very severe COPD) and repeated exacerbations [49].

It is known since more than 2 decades ago that corticosteroids have the capacity to inhibit the secretion of cytokines and consequently decrease inflammation [67]. Corticosteroids exert their effects on gene expression via many mechanisms. One important mechanism is the control of epigenetic changes e.g. changing protein acetylation through manipulation of HDACs. HDACs form part of several important intracellular multiprotein complexes that are involved in transcription control of DNA and are expressed throughout the lung, with the highest levels found in airway epithelial cells and alveolar macrophages [68]. A proposed mechanism for the reduced response to corticosteroids in steroid resistant asthma a nd COPD is that oxidative stress, either from active smoking or other sources, reduces HDAC activity impairing the ability of corticosteroids to reduce inflammation [66, 69]. Indeed, the reduction in HDAC activity in subjects with severe asthma which was associated with a reduced anti-inflammatory effect of dexamethasone *in vitro* [70]. Recently, the T lymphocyte subset T(H)17 was shown to play a role in regulating neutrophilic and macrophage inflammation in the lung, suggesting a

potential role for T(H)17 cells in severe, steroid-insensitive asthma and COPD [71]. However, mechanisms and pathways related to corticosteroid-insensitive airway inflammation in both asthma and COPD need to be further studied.

It is important to study inflammatory patterns and factors which affect the phenotype of the asthma and COPD and thereby change their response to corticosteroid treatment.

Scope of the thesis

The aim of this thesis was to describe, investigate and compare the effects of smoking and atopy in asthma and COPD. How do smoking and atopy change the clinical expression of asthma and COPD and the response to corticosteroids, in relation with underlying changes in pathology and immunology? A part of our research focused on asthmatic patients who developed with ageing and smoking a COPD-like phenotype, resulting in difficulties to discriminate between these two disorders. In chapter 2, we reviewed the literature regarding the differences made by smoking in asthma, both in clinical and pathological aspects. In chapter 3, we focused on this question and addressed whether smoking asthmatics with irreversible airway obstruction could be differentiated from matched COPD patients by comparing their airway wall biopsies. In chapter 4 and 5, atopy was studied as a modulating factor which may change the phenotype of asthma and COPD, resulting in different responses to corticosteroid treatment. Data from a large COPD population (EUROSCOP) was analysed to discover the effect of atopy on incidence/remission of respiratory symptoms in COPD patients after 3-year treatment of inhaled corticosteroids (ICS). In chapter 5, the effect of atopy and the response to ICS were studied in bronchial biopsies from asthma patients. In this population, the expression of the inflammatory markers and IL-17 was compared between ICS and non-ICS users, considering the atopy status. In chapter 6, effect of ICS on release of CCL20, as Th17 and neutrophil chemoattractant, from airway epithelial cells was investigated, as well as its implication in a decreased response to glucocorticoids in asthma. In chapter 7, the effect of ICS and smoking on HDAC-2 expression in the bronchial epithelial cells from asthma patients was studied. Finally, in chapter 8, the relationship between lysis of activated eosinophils in the airways of uncontrolled asthma was investigated.

The above described topics are listed in the following aims and chapters:

1. To compare smoking and non-smoking asthmatics and review their differences in clinical outcome and pathogenesis (Smoking and non-smoking asthma: differences in clinical outcome

and pathogenesis. Expert Rev Respir Med. 2011 Feb;5(1):93-105).

2. To differentiate smoking asthmatics with irreversible airway obstruction from the matched COPD patients by comparing their airway wall biopsies (Old dilemma: asthma with irreversible airway obstruction or COPD. Virchows Arch. 2015 Nov;467(5):583-93).

3. To investigate the effect of atopy on respiratory symptoms in COPD patients and to compare the effect of inhaled corticosteroid between atopic and nonatopic COPD patients (Atopy is a risk factor for respiratory symptoms in COPD patients: Results from the EUROSCOP study. Respir Res. 2013 Jan 28;14:10).

4. To investigate the effect of atopy and inhaled corticosteroid use on IL-17 expression in the airways of asthma patients (Atopy and Inhaled Corticosteroid Use Associate with Fewer IL-17+ Cells in Asthmatic Airways. PLoS One. 2016 Aug 23;11(8):e0161433).

5. To investigate the effect of inhaled corticosteroid on production of neutrophil and Th17 cell attracting chemokine CCL20 in airway epithelium (Glucocorticoids induce the production of the chemoattractant CCL20 in airway epithelium. Eur Respir J. 2014 Aug;44(2):361-70.)

6. To investigate the effect of smoking and inhaled corticosteroid on epithelial HDAC-2 expression in the epithelial cells of asthma patients (Smoking and corticosteroid use independently associate with higher epithelial HDAC-2 expression in asthma).

7. To investigate the effect of primary lysis/necrosis of eosinophils on clinical control of asthma (Authors' response to Persson C: primary lysis/necrosis of eosinophils and clinical control of asthma. Thorax. 2013 Mar;68(3):295-6.).

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Chapter 2

Smoking and non-smoking asthma: differences in clinical outcome and pathogenesis.

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Abstract

Cigarette smoking in asthma is frequently present and is associated with worsening of symptoms, accelerated lung-function decline, a higher frequency of hospital admissions, a higher degree of asthma severity, poorer asthma control and reduced responsiveness to corticosteroids. Furthermore, it is associated with reduced numbers of eosinophils and higher numbers of mast cells in the submucosa of the airway wall. Airway remodeling is increased as evidenced by increased epithelial thickness and goblet cell hyperplasia in smoking asthmatics. The pathogenesis responsible for smoking-induced changes in airway inflammation and remodeling in asthma is complex and largely unknown. The underlying mechanism of reduced corticosteroid responsiveness is also unknown. This article discusses differences between smoking and nonsmoking asthmatics regarding the clinical expression of asthma, lung function, response to corticosteroids, airway inflammation and remodeling processes. Possible pathogenetic mechanisms that may explain the links between cigarette smoking and changes in the clinical expression of asthma will be discussed, as well as the beneficial effects of smoking cessation.

Introduction

Active cigarette smoking is surprisingly frequent in adult asthma patients, with prevalence rates relatively close to that found in the general population. Approximately 50% of adult asthmatics in Western countries are current or former cigarette smokers [1]. Smoking in asthma patients may not only induce a chronic obstructive pulmonary disease (COPD)-like airway obstruction and airway inflammation, but may also worsen asthma stability [201,202]. Smoking is associated with more severe asthma symptoms, a higher frequency of asthma attacks, more hospital admissions and lower quality of life. The detrimental effects of smoking in asthma may be due to the induction of a relative unresponsiveness to inhaled and systemic corticosteroids, leading to their inability to successfully inhibit the underlying inflammatory processes in the airways [202]. This non-systematic review discusses differences between smoking and nonsmoking asthmatics regarding the clinical expression of asthma, lung function, response to corticosteroids, airway inflammation and remodeling processes. Possible pathogenetic mechanisms that may explain the links between cigarette smoking and changes in the clinical expression of established asthma will be discussed, as well as the beneficial effects of smoking cessation. This article does not study the potential role of active smoking in the induction of asthma, nor the negative effects of environmental tobacco smoke on asthma expression.

Clinical outcome

Current cigarette smoking in asthma is reported to increase both morbidity and mortality from asthma [1]. It is associated with increased presence of asthma symptoms including cough [2,3], wheeze [2–4], shortness of breath [2,3], nocturnal symptoms [2,3] and numbers of life-threatening asthma attacks [5–7]. Consequently, smoking is an independent risk factor for more severe asthma, both in the setting of a general practice and a chest clinic [2,8,9]. In line with this, the composite measure of asthma control is significantly worse in smoking asthmatics as compared with never-smoking asthmatics regarding nighttime awakening, morning symptoms, dyspnea, wheeze, activity limitation and use of reliever inhaler, all independently of the level of forced expiratory volume in 1 s (FEV1) [10]. Smoking has been reported to worsen asthma-specific quality-of-life scores and to increase healthcare utilization in a prospective study in 865 asthmatic patients in the USA [11]. Similarly, worsened quality of life, and increased need for rescue medication and doctor visits were found in smoking asthmatics compared with nonsmoking asthmatics in another study [12]. In line with the previous observations, increased numbers of visits to the emergency department and hospital admissions have been reported in smoking asthmatics, both in the acute as well as chronic state of their disease [13–16]. Above reports show that the frequency of exacerbations, emergency department visits and hospital admissions are higher in asthmatics who smoke [13–16], and yet another study showed that these acute asthma exacerbations for which asthmatics seek help may be of similar severity [17].

Maternal smoking during pregnancy is a well-known risk factor for both maternal and neonatal outcomes, and this may even be worse in asthmatic women who smoke. Indeed, in a recent large prospective study among pregnant women with asthma in the USA, active smoking was associated with increased asthma symptoms and asthma severity of the mother [18]. Fortunately, smoking was not associated with worse lung function, increased asthma exacerbations or more frequent hospitalizations during this period of pregnancy. An explanation might be that the women in this study were young (a mean age of 23.7 years) and had smoked too little to have clinical consequences. Intriguingly, a decreased rate of preeclampsia in smokers with asthma was found, similar to previously reported rates in obstetric populations without asthma [19]. Smoking of the mother also has disadvantageous effects on the neonate and increased risks of lower birth weight and delivery of children who are relatively small for their gestational age. Perinatal death was also reported in smoking compared with nonsmoking asthmatic women [18].

Taken together, the aforementioned studies show that cigarette smoking has several important hazardous effects on the clinical outcomes of asthma as well as on pregnancy outcomes.

Lung function

Only a limited number of cross-sectional and longitudinal studies are available that specifically describe the effects of smoking on lung function in asthma. A recent cross-sectional study in our center showed that 35 smoking asthmatics (median age: 50 years) had a significantly lower FEV1 percent-predicted than 66 never-smoking asthmatics (median age: 47 years) [20]. In this study, the level of FEV1 correlated with the number of pack-years in smoking individuals, however, bronchodilator reversibility did not differ between smokers and nonsmokers. In line with this, McKay et al. found that 31 smoking asthmatics (median age: 38 years) had a significantly lower FEV1 percent-predicted than 35 nonsmoking asthmatics (median age: 38 years) [21]. In contrast to our study, the smoking asthmatics in the latter study demonstrated higher bronchodilator reversibility. In a study in 1492 adolescents with asthma [4], the FEV1: forced vital capacity (FVC) was found to be lower in smokers than nonsmokers, but there was no significant difference in bronchial hyperresponsiveness between the two groups, which is similar to the older subjects in our study. A recent Korean study demonstrated that the proportion of asthmatic patients with fixed airway obstruction was higher in smokers than in nonsmokers (13 vs 10%) [22]. Additionally, a higher emphysema score on high-resolution CT scan was also observed in smoking asthmatics compared with nonsmoking asthmatics (18.8 vs 4.4%). One study in mild-to-moderately severe asthmatics aged between 18 and 45 years demonstrated reduced forced expiratory flow values (25-75%) and signs of lung hyperinflation, suggesting that small airways dysfunction occurs more frequently in asthmatics who smoke [23]. Similarly, signs of emphysema and hyperinflation were more frequently reported in smoking asthma patients [22–25]. In contrast to the aforementioned studies, a recent study showed no difference in FEV1 levels as well as FEV1: FVC ratios between 60 smoking and 74 never-smoking asthmatics [10]. Smoking asthmatics had a high number of pack-years (mean: 31). However, a drawback of this study is that the age of the asthmatics and the duration of asthma differed importantly between smokers and nonsmokers (54 vs 42 years and 12 vs 22 years, respectively), which likely affected the results. It should be considered that these cross-sectional studies included relatively small numbers of patients, which may have led to selection bias.

Several longitudinal studies suggest that cigarette smoking importantly contributes to the accelerated decline of lung function in subjects with asthma [26–30]. However, there are also

two conflicting studies [31,32] that might be explained by the relatively low number of smoking asthmatics included in these studies. A large study over 10 years in the USA found that the combination of asthma and heavy smoking (≥15 cigarettes per day) has a synergistic effect on FEV1 decline [33]. In fact, the additional effect of smoking on the estimated FEV1 decline from 18 to 40 years of age was 9.3%, compared with 4.2% if there was no synergy. These data are corroborated by the well-known Copenhagen study demonstrating that the annual decline in FEV1 measured over 15 years was significantly greater in smoking asthmatics 40-59 years of age than in their nonsmoking counterparts [30]. The average decline in FEV1 in smokers versus nonsmokers was 57 ml/year versus 32 ml/year in men, and 38 ml/year versus 31 ml/year in women. The presence of chronic mucus hypersecretion appeared to be an additional risk factor for the accelerated decline in FEV1 in this study.
Together, most cross-sectional and longitudinal studies suggest that smoking has a detrimental effect on lung function.

Response to corticosteroids

Asthma patients who smoke are less sensitive to the beneficial effects of inhaled corticosteroids with respect to respiratory symptoms and lung function as compared with asthma patients who do not smoke, both in the short and long term. The effect of 3-week treatment with inhaled fluticasone propionate (1000 µg daily) was studied in a randomized placebo-controlled study in 17 smoking and 21 nonsmoking asthmatics who had never been treated with corticosteroids [34]. Active smoking was associated with reduced effectiveness of fluticasone on morning peak expiratory flow, FEV1 and PC20 (provocative concentration causing a 20% fall in FEV1) methacholine and sputum eosinophils. The effect of 8-week treatment with inhaled hydrofluoroalkane (HFA)-beclomethasone dipropionate (400 µg daily) was studied in a placebo-controlled crossover study in 39 light smoking and 44 nonsmoking subjects with mild asthma. Beclomethasone significantly reduced sputum eosinophils and eosinophilic cationic protein both in smokers and nonsmokers, but increased FEV1 only in nonsmokers [35]. The effect of 12-week treatment with inhaled beclomethasone (400 or 2000 µg daily) was studied in a randomized placebo-controlled study in 40 smoking and 55 nonsmoking subjects with mild persistent asthma [36]. Active smoking in this study was associated with reduced effectiveness of 400 µg beclomethasone per day on morning peak expiratory flow values and exacerbation rate. Interestingly, this unresponsiveness was not observed in smoking asthmatics who used 2000 µg beclomethasone per day. The effect of 9-month treatment with inhaled budesonide (400 and 1600 µg/day) was studied using a randomized controlled method in 37 smoking and

47 nonsmoking asthmatics [37]. Lung function, hyperresponsiveness to histamine and eosinophilic inflammation improved with budesonide in a dose-dependent manner, however, this beneficial effect of budesonide was not observed in asthmatics who smoked [37]. The effect of 1-year treatment with escalating doses of inhaled fluticasone (up to 1000 μg daily) or salmeterol/fluticasone (up to 100/1000 μg daily) was studied in 3416 subjects with uncontrolled asthma [38]. The probability to have uncontrolled asthma at the end of the study was 2.7-times higher in current smokers compared with never-smokers [38]. Finally, in a more than 23-year follow-up period of adult patients with moderate-to-severe asthma in our center, inhaled corticosteroids did not decelerate the annual decline in FEV1 in individuals with five or more pack-years smoking [39]. Clearly, noncompliance of patients may have affected the described study results, particularly as smoking has been found to be a risk factor for nonadherence to anti-inflammatory agents in asthma [40].

One might speculate that smoking leads to decreased responsiveness of inhaled corticosteroids owing to reduced bronchial bioavailability. However, this is clearly not the only explanation as a placebo-controlled crossover study showed that smoking reduced the FEV1 response to 2-week treatment with 40 mg oral prednisolone as well [41]. Another interesting observation is made by Livingston et al., who demonstrated in 39 smoking and 36 never-smoking asthmatics that smoking reduced the cutaneous vasoconstrictor response to topical beclomethasone [42]. This finding indicates that smoking-induced corticosteroid unresponsiveness also affects tissue sites other than the airways. Taken together, the aforementioned studies suggest that smoking reduces the overall clinical response to corticosteroid therapy in asthma.

The underlying pathogenetic mechanisms for this smoking-induced corticosteroid unresponsiveness are not clear. First, it has been shown that smoking is associated with higher IL-8 concentration and neutrophils in induced sputum in asthmatics similar to healthy individuals [43]. Neutrophils are inflammatory cells that are generally not responsive to corticosteroids, probably because corticosteroids inhibit instead of promote apoptosis. Interestingly, nonsmoking asthmatics may also have a non-eosinophilic sputum phenotype that is less responsive to corticosteroids [44]. We speculate that these observations may indicate that the smoking-induced changes in inflammatory profile are more important for the induction of corticosteroid unresponsiveness than the direct effects of smoking itself. Other possible mechanisms explaining corticosteroid unresponsiveness in smoking asthmatics may be related to oxidative stress. Cigarette smoke may induce oxidative stress that not only activates the nuclear factor-κB pathway but also alters the histone deacetylase/histone acetyltransferase balance via post-translational modification of histone deacetylases [45]. Another possible

mechanism explaining the observed corticosteroid unresponsiveness might be a reduced glucocorticoid receptor α:β ratio, which is observed in healthy smokers as well as in asthmatic smokers [46].
Only a small number of clinical trials studied whether it is possible to reverse the corticosteroid unresponsiveness in smoking asthma. A randomized study comparing inhaled beclomethasone with low-dose theophylline in addition to inhaled beclomethasone demonstrated that the addition of theophylline improved both lung function and asthma symptoms in 68 smoking asthma patients [47]. A randomized clinical trial suggested that leukotriene antagonists may have a beneficial effect in smokers with mild asthma [35], but replication of this study in smoking asthmatics is lacking so far. A new potentially promising drug is the peroxisome proliferator-activated receptor (PPAR)γ agonist rosiglitazone, which improved the lung function of 23 smoking asthmatics after 4 weeks of treatment, in contrast to 23 smoking asthmatics treated with low-dose beclomethasone (200 µg daily) [48]. Further trials are clearly needed to confirm efficacy of this treatment in steroid-resistant smoking asthmatics. We believe that in the near future the underlying mechanisms of smoking-induced corticosteroid unresponsiveness will be elucidated, which will lead to new treatment options. This is not only important for asthmatic (and COPD) patients who smoke, but also for asthmatics that have 'spontaneous' corticosteroid unresponsiveness.

Biological & pathological changes

Long-term cigarette smoking may be expected to induce inflammatory processes in the asthmatic airways based on previous reports in nonasthmatic smokers. These reports show cigarette smoke induced inflammatory changes in the airways, such as increased numbers of eosinophils and macrophages in induced sputum [49,50], increased neutrophil, macrophage and CD8+ lymphocyte counts in bronchoalveolar lavage (BAL) fluid [51–54], as well as increased neutrophil, eosinophil, macrophage and mast cell counts in the bronchial wall [51,54–56]. The production of numerous proinflammatory mediators from macrophages, most notably IL-8, was also found to be upregulated after acute cigarette smoke exposure [57,58]. However, for asthma, the potential influence of smoking on airway inflammation and the pattern of inflammatory cells and mediators are less acknowledged. In nonsmoking asthmatics, a chronic inflammatory response in the airways includes high numbers of
eosinophils, lymphocytes, Th2 cells and activated mast cells [59]. Cigarette smoking tends to alter this type of airway inflammation and cytokine profile, which has been described for induced sputum and BAL obtained from smoking asthmatics [20,21,23,43,60–62].

Table 1. Inflammatory cells and mediators in smoking versus nonsmoking asthmatics.

Study (year)	Patient characteristics	Inflammatory cells	Mediators/cytokines	Ref.
Sputum				
Broekema *et al.* (2009)	35 smokers (median age: 50 years), atopic: 64% Median of pack-years: 15 66 never-smokers (median age: 47 years), atopic: 74%	↓ Total cells (×10^6/ml) ↓ Macrophages (×10^6/ml) ↓ Neutrophils (×10^6/ml) ↓ Lymphocyte % (×10^6/ml) No significant change in eosinophil % (×10^6/ml)	↓ Neutrophil elastase (μg/ml) ↓ ECP (μg/l) No change in histamine	[20]
Krisiukeniene *et al.* (2009)	19 smokers (age: 54.7 ± 5.1 years), nonatopic: all Pack-years: 17.4 ± 7. 3 26 never-smokers (age: 56.9 ± 1.7 years), nonatopic: all	↓ Eosinophil % ↑ Neutrophil % (positive trend: p = 0.074) No significant change in total cells, macrophages and lymphocytes (%)	↓ IL-5 (pg/ml) ↑ Eotaxin-1 (pg/ml) ↑ Eotaxin-2 (pg/ml)	[61]
Kanazawa *et al.* (2009)	25 smokers (median age: 31 years) 24 nonsmokers (median age: 33 years) Atopy and pack-years data was not available in the study	↑ Neutrophil % No significant change in total cells, macrophages, lymphocytes, eosinophils and epithelial cells (%)		[60]
Rovina *et al.* (2009)	24 smokers (age: 46 ± 7 years), atopic: 42% Pack-years: 31 ± 15 22 nonsmokers (age: 44 ± 11 years), atopic: 59%		↓ IL-18 (pg/ml)	[62]
Livingston *et al.* (2007)	39 smokers (age: 47.4 ± 7.4 years), atopic: 62% Pack-years: 37.7 ± 17.2 36 never-smokers (age: 45.1 ± 10.9 years), atopic: 91%	↓ Bronchial epithelial cell % ↑ Neutrophil % (positive trend: p = 0.07) No significant change in macrophages, lymphocytes and eosinophils (%)		[42]
Lazarus *et al.* (2007)	39 smokers (age: 29 ± 6.5 years) Median of pack-years: 7 44 nonsmokers (age: 28.9 ± 5.9 years) Atopy data was not available in the study	No significant change in epithelial cells, macrophages, eosinophils, neutrophils and lymphocytes (%)		[35]
Boulet *et al.* (2006)	22 smokers (age: 31 ± 8 years), atopic: 82% Pack-years: 14.0 ± 7.6 27 nonsmokers (age: 29 ± 6 years), atopic: 93%	↑ Neutrophil (×10^6/ml) ↑ Bronchial cell (×10^6/ml) No significant change in eosinophils, total cells, macrophages and lymphocytes (%, ×10^6/ml)		[23]
McKay *et al.* (2004)	31 smokers (median age: 38 years), atopic: 65% Median of pack-years: 22 35 nonsmokers (median age: 38 years), atopic: 49%	↑ Neutrophil % ↓ Lymphocyte % ↓ Eosinophil %	↓ IL-18 (pg/ml)	[21]
Chalmers *et al.* (2001)	31 smokers (age: 36.3 ± 10.6 years) Pack-years: 21.0 ± 16.6 36 nonsmokers (age: 36 ± 8.9 years) Atopy data was not available in the study	↑ Total cells (×10^6/ml) ↑ Neutrophil % (×10^6/ml) ↓ Eosinophil % (×10^6/ml)	↑ IL-8 (pg/ml) No change in ECP (μg/l)	[43]

Bronchial biopsy				
Broekema *et al.* (2009)	35 smokers (median age: 50 years), atopic: 64% Median of pack-years: 15 66 never-smokers (median age: 47 years), atopic: 74%	↓ Eosinophils (EPX^+), cells/0.1 mm^2 ↑ Mast cells ($AA1^+$), cells/0.1 mm^2 No significant change in macrophage ($CD68^+$), neutrophil ($NP57^+$) and lymphocyte markers ($CD3^+$; cells/0.1 mm^2)		[20]
St Laurent *et al.* (2008)	12 smokers (age: 32.7 ± 2.3 years), atopic: 58% Pack-years: 16.7 ± 2.2 12 nonsmokers (age: 25.8 ± 2.3 years), atopic: 83%	↑ $Elastase^+$ cells (cells/mm^2) No significant change in $CD3^+$, $CD68^+$, MBP^+ and $tryptase^+$ cells (cells/mm^2)	↑ IL-8 ↑ IFN-γ No change in IL-4, IL-5, TNF and TGF-β	[120]
Tsoumakidou *et al.* (2007)	24 smokers (age: 31 ± 6 years) Median of pack-years: 8 21 never-smokers (age: 32 ± 6 years) Atopy data was not available in the study	↓ B cells ($CD20^+$), cells/mm^2 ↓ Mature dendritic cells ($CD83^+$), cells/mm^2		[64]
BAL				
Krisiukeniene *et al.* (2009)	19 smokers (age: 54.7 ± 5.1 years), Pack-years: 17.4 ± 7.3 nonatopic: all 26 never-smokers (age: 56.9 ± 1.7 years), nonatopic: all	No significant change in neutrophils, total cells, macrophages and lymphocytes (%)	IL-5 (pg/ml; analogous trend with sputum) No significant change in eotaxin-1, eotaxin-2 and eotaxin-3	[61]
Kane *et al.* (2009)	13 smokers (median age: 33.7 years), atopic: 100% Median pack-years: 15.9 19 nonsmokers (median age: 34.2 years), atopic: 95%	↑ Total cells ($\times 10^4$/ml) ↑ Macrophages ($\times 10^4$/ml) No significant change in lymphocytes, eosinophils and neutrophils		[75]
Serum				
Krisiukeniene *et al.* (2009)	19 smokers (age: 54.7 ± 5.1 years), Pack-years: 17.4 ± 7.3 nonatopic: all 26 never-smokers (age: 56.9 ± 1.7 years), nonatopic: all		↓ IL-5 (pg/ml) ↑ Eotaxin-1 (pg/ml; positive trend: p = 0.062) ↑ Eotaxin-2 (pg/ml) No significant change in eotaxin-3	[61]

Data regarding characteristics of patients are shown as mean ± standard deviation (or standard error of the mean). Some data was reported as a median.
↑ and ↓ show a significant difference in smoking compared with never/nonsmoking asthmatics.
BAL: Bronchoalveolar lavage; ECP: Eosinophilic cationic protein; MBP: Major basic protein; TNF: Tumor necrosis factor.

The data from the literature comparing the pattern of inflammatory cells and cytokines in smoking and nonsmoking asthma patients are summarized in Table 1.

Inflammatory cell profile

Cigarette smoking may have a large impact on many aspects of airway inflammation in asthma (see Table 1 and Figure 1). The largest study in this field of research has been performed by our group and included 147 asthma patients: 35 current smokers, 46 ex-smokers and 66 never-smoking asthmatics. This study showed lower neutrophil, macrophage and lymphocyte counts in sputum of smoking asthma subjects compared with never-smokers. By contrast, higher numbers of mast cells were found in bronchial biopsies of smoking asthmatics compared with nonsmoking asthmatics [20], which was in line with reports showing their increase in the lungs

and skin of asymptomatic smokers [63]. Lower lymphocyte percentages in sputum [21] as well as lower B-cell numbers in bronchial biopsies [64] were also found in other studies. Numbers of dendritic cells were not measured in our study, but were also found to be reduced [64]. By contrast, some studies found higher neutrophil counts in sputum of smoking asthmatics [21,23,42,43,61,60]. This discrepancy in inflammatory cell count in various studies can be explained by the heterogeneity of the studied population with respect to the use of inhaled corticosteroids, severity of asthma, being allergic versus nonallergic, sex and age. Another possible explanation could be differences in the number of pack-years smoked and concentration of components in cigarette smoke such as nicotine. For instance, in vitro studies with nicotine have shown dose-dependent suppression of chemotaxis and phagocytosis of neutrophils and enhancement of neutrophil degranulation [65], showing concentrationdependent variable effects of nicotine on neutrophil function. Moreover, we should keep in mind that some studies considered ex-smokers and never-smokers as one group of nonsmokers.

Smoking also affects eosinophil numbers. Chalmers et al. demonstrated that higher sputum neutrophil counts were accompanied by lower eosinophil counts that was associated with a lower lung function (FEV1% predicted) when compared with nonsmokers [43]. Similar findings concerning reduced eosinophils in sputum [61] as well as in bronchial biopsy [20] in smoking asthma subjects were reported in other studies. In peripheral blood, smoking also affects eosinophil numbers, as smoking asthmatic patients were reported to have lower eosinophil numbers [66]. By contrast, studies on normal (nonasthmatic) smokers showed the opposite effect, that is, higher blood eosinophil counts in smokers [67,68]. In the asthma mouse model, inhibitory effects of short-term (3-week) smoke exposure on eosinophilia was also found. In an ovalbumin (OVA)-induced mouse model of allergic airway inflammation, we showed that smoking reduced airway responsiveness to OVA and methacholine in OVA mice and decreased eosinophil numbers in BAL fluid and lung tissue compared to nonsmoking mice [69].

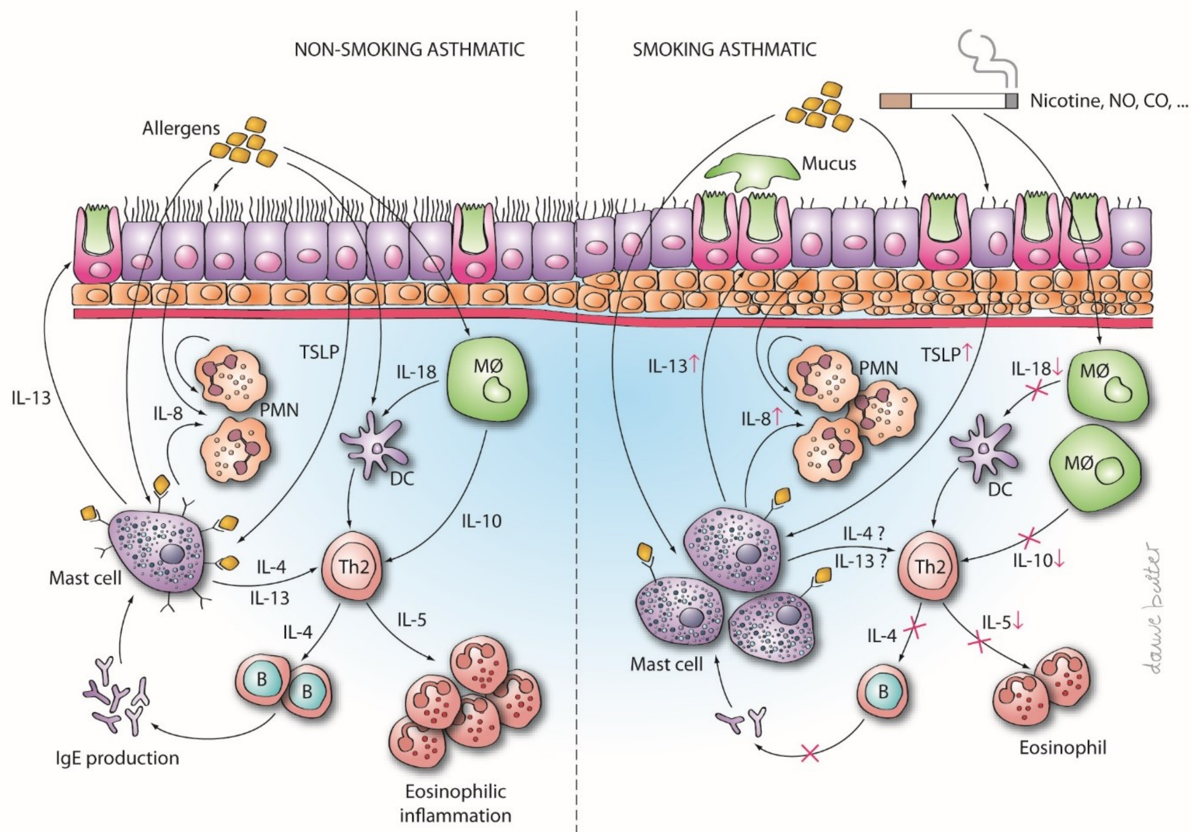

Figure 1. Smoking alters inflammatory pathways in asthma. *An update of the possible underlying mechanisms of smoke-induced changes in asthmatic airway inflammation. As shown in* ***(A)****, DCs play a pivotal role in the induction of the Th2-type lymphocytes in nonsmoking asthma. This may be due to the lack of IL-12 production and upregulation of costimulatory molecules. Th2 cells produce IL-4, a cytokine necessary for IgE class switching in plasma cells, and IL-5, known to be involved in eosinophilic migration and activation. After the first contact with allergen, IgE will bind to its receptor on mast cells, which will crosslink after the second contact with the same allergen and induces degranulation of mast cells. A variety of mediators will be released, one of which is IL-13, which may contribute to development of airway remodeling. In allergic asthma, repeated exposure to allergens leads to TSLP production by epithelial cells, which can directly stimulate mast cell activation and release of its mediators. Repeated allergen exposure also activates MØ to produce IL-18 and IL-10, also important in driving a Th2-type response.* ***(B)*** *Smoking leads to increased exposure to a variety of toxic chemicals, present in cigarette smoke, such as nicotine, NO and CO. Exposure to smoke in asthma increases the number of MØ, which are involved in phagocytosis of smoke particles and apoptotic cells. These MØ produce less IL-18 and IL-10, which leads to less Th2-cell development, lower numbers of B cells and lower amounts of IL-4 and IL-5. This will lead to less activity and presence of eosinophils in smoking asthmatics and lower production of IgE, the latter being demonstrated in animal studies. Alongside this, exposure of epithelial cells to cigarette smoke is known to enhance production of mediators necessary for repair. In smoking asthma, increased levels of TSLP may further stimulate mast cells to release remodeling mediators such as IL-13, independently of Th2 cells. Increased mast cells, together with smoking-induced epithelial cell activation, may increase IL-8 production, contributing to increased infiltration of neutrophils (PMNs).*

↑ and ↓ indicate higher and lower cytokine levels in smoking compared with nonsmoking asthmatics.

CO: Carbon monoxide; DC: Dendritic cell; MØ: Macrophage; NO: Nitric oxide; PMN: Polymorphonuclear neutrophil; TSLP: Thymic stromal lymphopoietin.

Macrophages have not been studied in detail as yet in the context of asthma and their contribution to allergic airway inflammation is therefore unclear [70]. Recent data from our laboratory has shown that the alternatively activated subset may be involved in the pathogenesis of asthma [71], while others have shown that the classically activated subset may be more protective [72,73]. Interestingly, when corticosteroid-sensitive asthmatics were compared with corticosteroid-resistant asthmatics, Goleva et al. found increased expression of markers of classical activation in the corticosteroid-resistant patients [74]. This coincided with increased levels of lipopolysaccharide in the lungs of those patients. This could imply that classical activation is not beneficial to disease progression in already established disease and that it induces corticosteroid resistance.

Even more unknown are the effects of smoking on macrophages during allergic inflammation. The few studies published in humans and animal models generally observed an increase in macrophage numbers in smoking asthmatics as compared with nonsmoking asthmatics [21,69,75–80], similar to data found for non-asthmatic subjects [81]. None of these reports, however, studied macrophage phenotypes. In nonasthmatic subjects, smoking was found to reduce the ability of macrophages to phagocytose and kill pathogens [75,82–88] and to induce their capacity for tissue remodeling [89–91]. This points at reprogramming of macrophages from a classically activated phenotype towards a more alternatively activated phenotype [91–93]. As we found alternatively activated macrophages to be involved in asthma pathogenesis in a mouse model [71], this may give us new leads in explaining why smoking predisposes to the development of asthma. What smoking does to macrophages of patients already suffering from asthma remains a question that needs further study.

With regards to smoke-induced altered macrophage function, the question arises about the effect of smoking on the innate immune system regarding, for instance, Toll-like receptor regulation. In healthy smokers, it has been described that cigarette smoking suppresses Th1-mediated immune responses to Gram-negative bacterial infections by interfering with MyD88/IRAK signaling, thereby reducing lipopolysaccharide-induced TLR-4 expression [94]. This can explain the much higher rate of respiratory infections in smokers [95–97]. As a high percentage of smokers have respiratory infections, it would be interesting to know whether the observed type of inflammation in smoking asthmatics is due to the airways microbiome composition.

The aforementioned studies show that smoking has an immune-modulating effect and may play a different immunological role in asthmatic patients compared with nonasthmatic patients. Most strikingly though is that smoking in asthma seems to reduce eosinophilic inflammation,

which is a major characteristic of asthma. Mechanisms related to the suppression of eosinophils in smoking asthmatic patients have not been clarified, but we can speculate about the underlying mechanisms. As nitric oxide (NO) has been shown to increase apoptosis of activated eosinophils, this molecule, present in cigarette smoke, may be involved in eosinophil downregulation [98]. However, endogenous NO production was significantly less in mild steroid-naive asthma patients who smoked compared with nonsmoking asthmatics [99]. Another, already mentioned, candidate is nicotine, which has been described to suppress both innate and adaptive immune responses [100] and was shown to inhibit cytokine synthesis from macrophages using the cholinergic anti-inflammatory pathway [101]. Interestingly, Mishra et al. found in rats treated with nicotine and challenged with allergens, a reduction in inflammatory parameters in the lung including eosinophilic migration, expression of the Th2 cytokines and eotaxin, as well as total and specific IgE. This result was obtained even after multiple allergen challenges were given. It suggested that nicotine modulates asthma/allergy primarily by suppressing eosinophil trafficking and suppressing Th2 cytokine responses without reducing goblet cell metaplasia or mucous production. It may explain the lower risk of allergic diseases in smokers [102]. Carbon monoxide (CO) in cigarette smoke is another putative factor that is known to have cytoprotective effects and inhibit allergic airway inflammation [103,104]. CO is present in cigarette smoke, but could also be produced by the enzyme heme oxygenase-1 (HO-1). HO-1 is involved in the breakdown of heme to equimolar amounts of biliverdin, free iron and CO, and is rapidly upregulated with oxidative stress [105]. However, the exact mechanisms behind the protective effects of HO-1 are still poorly understood.

Cytokine & mediator profiles

Smoking asthmatics have higher concentrations of IL-8 in sputum than nonsmoking asthmatics, as shown by Yamamoto et al. [106]. This was confirmed by a study in mild asthmatics (sufficiently mild for symptoms to be controlled with inhaled bronchodilators without additional inhaled steroids), in which the authors also found increased neutrophilic airway inflammation in smoking asthmatics. Neutrophilic airway inflammation was positively correlated with the IL-8 content in sputum and smoking history in pack-years. In addition, a negative correlation was found between sputum IL-8 and lung function, expressed as FEV1 percent-predicted in smoking asthmatics [43]. These findings suggest an indirect association between cigarette smoking, airway inflammation and lung function level in smoking asthma patients. Moreover, they found sputum eosinophilia in mild non-smoking asthma patients

compared with smoking asthma subjects. They concluded that the neutrophilic inflammation related to smoking may be additive to the underlying asthmatic airway inflammation [43].
IL-18 is also a proinflammatory cytokine that has an important role in the development of a Th1-type response and could have a regulatory role in asthma by inhibiting Th2-type responses. In the study by McKay et al., it has been shown that the IL-18 levels in sputum were lower in smoking compared with nonsmoking asthmatics [21]. Likewise, data from another recent study showed that cigarette smoking reduced IL-18 levels in induced sputum in both healthy and smoking asthmatics, which was correlated with higher airway obstruction and airway hyperresponsiveness in smoking asthmatic patients. It was therefore suggested that IL-18 is implicated in airway hyperresponsiveness characterizing bronchial asthma [62].
Regarding other mediators, smoking was also described to stimulate production of eotaxins (1 and 2) in sputum as well as in BAL and serum of asthmatic patients, which was related to numbers of eosinophils and neutrophils in all the studied tissue compartments [61]. This is in agreement with the role of eotaxin-1 as an important eosinophil chemoattractant, promoting both local eosinophil recruitment to the lung and, in cooperation with IL-5, rapid mobilization of eosinophils and their progenitors from the bone marrow. In contrast to this, levels of IL-5 were lower in sputum in these smoking asthmatics. Analogous trends were noted in the experimental studies in Brown Norway rats [102] and guinea pigs [107]. Low expression of IL-5 could be an explanation for the observed low eosinophil numbers in smoking asthmatics, also in the aforementioned study, as IL-5 is thought to be a major cytokine in stimulating differentiation, activation, migration, regulation and in situ survival of eosinophils [108].
According to the aforementioned data, it can be concluded that the pattern of airway inflammation is different in smoking asthma subjects when compared with nonsmoking asthma subjects. It seems likely that cigarette smoking in allergic asthma changes the typical Th2-type eosinophilic airway inflammation into a more COPD-like neutrophilic inflammatory pattern.

Airway remodeling

In addition to published effects of smoking on inflammatory responses in healthy smokers, we know that long-term cigarette smoking induces changes in the structure of the airways, referred to as remodeling. Remodeling in healthy smokers involves epithelial cell regeneration, as a reaction for restoring the bronchial epithelium after smoke-induced damage [102], induction of epithelial layer thickness and squamous or mucous cell metaplasia [109,110], remodeling of the basement membrane due to deposition of tenascin and laminin under the basement membrane [51,55,111], and induction of intraepithelial macrophages and CD8+ T cells in the peripheral

airways [1]. The mechanisms involved in the epithelial changes could be secondary to epithelial migration or proliferation and differentiation or both [112], and epithelial layer thickness is likely to be caused by all three factors resulting in epithelial cell hyperplasia, hypertrophy and mucous metaplasia [109]. Goblet cell hyperplasia and increased mucus production could arise from smoke exposure itself, or indirectly via the activation of mucin gene transcription by inflammatory cell mediators [109]. Regarding the smoking effect in asthmatic individuals, it has been suggested that airway remodeling is more severe in smoking asthmatics than nonsmoking asthmatics [1]. However, the number of studies in this field is very limited. The large study on effects of smoking in asthmatics from our group showed more goblet cells, mucus-positive epithelium and increased epithelial thickness in bronchial biopsies from smokers than never- and ex-smoking asthmatics. Smoking subjects also had more proliferation of intact and basal epithelium than never- and ex-smoking asthmatics [20]. As we found a correlation between particular remodeling markers and symptoms such as self-reported shortness of breath and phlegm production, it could be suggested that increased epithelial thickness may underlie increased respiratory symptoms in smoking asthmatics. To explain epithelial cell changes due to smoking, the epithelial cell-derived cytokine thymic stromal lymphopoietin (TSLP) could be implicated. In an OVA model of asthma in which mice were exposed to cigarette smoke extract (CSE), CSE was shown to induce expression of TSLP [113]. TSLP exerts a profound effect on the polarization of dendritic cells to drive Th2 cytokine production [114], but can also activate mast cells to produce IL-13 in a T-cell-independent manner [115]. This is of interest since mast cells, highly present in smoking asthmatics, are good candidates to mediate epithelial changes by releasing mediators such as tryptase, IL-13 and TNF [116]. A role for IL-13 in this context is supported by a study in children who were exposed to parental smoking, in which augmented production of IL-13 was found by nasopharyngeal aspiration [117].

Interestingly, in ex-smoking asthmatics, the number of goblet cells and mucus-positive epithelial cells was similar to those in never-smoking asthmatics. Ex-smoking asthmatics also had similar epithelial thickness, epithelial proliferation rate and mast cell numbers as never-smokers. These findings suggest that the remodeling changes in the airways caused by smoking in asthma patients are reversible by smoking cessation. However, these are cross-sectional studies that may have been confounded by other factors. Longitudinal studies are needed to confirm this.

Regarding other pathways related to remodeling, the effect of smoking on the arginine pathway was reported in the study by Bergeron et al. on 24 steroid-naive subjects with mild asthma.

They found expression of arginase I and ornithine decarboxylase in 12 smoking asthmatics compared with 12 nonsmoking asthmatics, while no difference in expression of inducible NO synthase was found. Moreover, significantly higher arginase I and ornithine decarboxylase expression was found in airway epithelial cells and fibroblasts after stimulation with nicotine in vitro [118]. Smoking also seems to affect the number of elastic fibers necessary for lung elasticity. In a prospective study on specimens obtained from asthmatic patients dying of nonrespiratory causes, the network of elastic fibers was greater in smoking asthmatics than nonsmoking asthmatics. These fibers form discrete longitudinal bundles in the submucosa of small and large airways and the increased size and altered composition of longitudinal bundles in asthma may influence airway function by altering the mechanical properties of the airway wall or by changing the folding behavior of the airway mucosa [119]. A bronchial biopsy study by St Laurent et al. included 12 smoking and 12 nonsmoking subjects with mild asthma who all were steroid naive. Squamous cell metaplasia and goblet cell hyperplasia were more frequently present in the bronchial epithelium from smokers than nonsmokers: zero out of 12 versus three out of nine and two out of ten versus five out of seven, respectively. The same pattern was seen in subepithelial mucosa morphology (smooth muscle bundles, subepithelial glands, connective tissue and thickness of the reticular basement membrane) in both groups. However, there was not a significant change in intact, partially or completely shedded epithelium between the two groups [120].

In summary, smoking in asthma has been shown to increase airway remodeling.

Smoking cessation

As already discussed earlier, it is clear that tobacco smoking has important hazardous effects on the clinical and pathological outcomes of asthma. Smoking cessation should therefore be considered as smoking is the most important preventable health issue in asthma patients, as it is in many other diseases. However, quitting smoking closely depends on the beliefs and habits of the patients. In an interview-based study of smoking asthmatics, the observed smoking cessation rate was poor and the median time until smoking cessation was 17 years [121]. This poor cessation rate was associated with lower educational status. Likewise, another study using a multivariate analysis showed that social status and education was independently associated with smoking cessation in adults with a history of asthma [122]. Interestingly, in a large survey in South Australia it was found that more than 40% of smoking asthmatics did not believe that smoking worsened their health condition, and two-thirds of them did not think that future health problems would be serious [123]. These wrong beliefs among smoking asthmatics are

very alarming and therefore health professionals above all should try to increase patients' knowledge on the effects of smoking by educational programs, as well as encouraging them to quit smoking. Despite the importance of smoking cessation, there are not many reports available on whether this has an effect on symptoms, lung function and corticosteroid responsiveness in asthma. The available studies show significant improvements in symptoms and lung function in those asthmatics who quit smoking successfully [3,124–126]. For instance, a cross-sectional study in Japan reported that ex-smoking asthmatics had less cough, sputum production, wheeze and night symptoms compared with current smoking asthmatics [3]. By contrast, in a study by Chaudhuri et al., similar asthma symptom scores between currently smoking and ex-smoking asthmatics were reported, yet ex-smokers showed a trend towards a greater response to oral corticosteroids compared with current smokers [41]. With respect to the effects of smoking cessation on lung function, a small longitudinal study of 14 asthmatic patients showed improved airway obstruction and bronchial responsiveness to histamine already after 7 days of smoking cessation [127]. In accordance, a study in 32 smoking asthma patients showed a significant difference in FEV1 obtained after 6 weeks of smoking cessation in 21 quitting individuals compared with those who continued smoking (11 subjects) [125]. The mean improvement in FEV1 in the quitting group was 356 ml at 1 week, 390 ml at 3 weeks and 450 ml at 6 weeks of smoking cessation while there was no difference in the FEV1 levels in the smoking group. The proportion of sputum neutrophils also reduced significantly in quitters compared with persistent smokers. Moreover, the cutaneous vasoconstrictor response score to topical beclomethasone improved after smoking cessation in quitters compared with smokers [125]. Additionally, improvements in asthma-specific quality-of-life scores, reductions in respiratory symptoms, bronchial hyperresponsiveness, rescue β2-agonists and in doses of inhaled corticosteroids were observed in quitters. Smaller improvements occurred for smoking reducers in nocturnal use of rescue β2-agonists, doses of inhaled corticosteroids and bronchial hyperresponsiveness [125]. A telephone survey in Canada describing suboptimal asthma control in 10,428 patients with asthma found that ex-smokers were more likely to have well-controlled asthma compared with current smokers [124]. These findings highlight the importance of smoking cessation in improving outcomes of asthma. Therefore, the current clinical practice guidelines recommend that all smokers should be counseled to quit by their physician. Since a high proportion of asthmatic patients are not likely to quit smoking, ongoing research is required to identify therapies that improve symptom control and restore corticosteroid unresponsiveness in asthmatics who continue to smoke.

Expert commentary

Cigarette smoking worsens the clinical outcomes of asthma, importantly as reflected by lower levels of asthma control, higher levels of asthma severity, more frequent asthma exacerbations and subsequent hospital admissions, as well as accelerated lung function decline. The detrimental effects of smoking in asthma are probably due to a switch in the pathological phenotype of asthma. Smoking asthmatics compared with nonsmoking asthmatics have a higher presence of neutrophils, macrophages and lymphocytes in the bronchial lumen, demonstrate COPD-like changes in the epithelium of the bronchial wall, and their airway wall contains higher numbers of mast cells and lower numbers of eosinophils in the submucosa. However, there are some conflicting results in the literature on the underlying pathology of smoking asthma that may be due to differences with respect to age of study populations, use of inhaled corticosteroid, number of pack-years smoking, and the severity and duration of asthma. Additionally, asthmatics with a different underlying etiology or phenotype may have been studied. The fact that results have been derived from different compartments (sputum, BAL, blood and airway wall) may lead to seemingly conflicting findings that need further study. Moreover, the crosssectional studies that compared induced sputum results from smoking and nonsmoking asthmatics may differ importantly regarding the temporal relationship between the last cigarette smoked and the initiation of the experimental procedures. Interestingly, the smoking-induced clinical and pathological changes are associated with reduced responsiveness to inhaled and oral corticosteroid treatment. Possible mechanisms explaining this unresponsiveness are the noneosinophilic airway inflammation in asthma, as well as effects on oxidative stress that activates the nuclear factor-κB pathway and alters histone deacetylase activity. Impaired glucocorticosteroid receptor function may contribute as well.

Five-year view

The best way to improve asthma control in smoking asthmatics is to encourage smoking cessation. For those patients who are not able to quit smoking, some treatment strategies have been suggested, for example, leukotriene receptor antagonists, combination of theophylline and corticosteroids, and new drugs such as PPARγ agonist. However, the number of available clinical studies is currently too small to recommend an 'evidence-based' drug regime for these patients. High-quality, randomized clinical trials are required to identify therapies that restore smoking-induced as well as spontaneous corticosteroid unresponsiveness in asthma. In animal

models for asthma, contradictory findings were reported regarding effects of smoking on sensitization and eosinophilic inflammation. This could be related to variation in genetic background of the mouse strain and the allergic model that was used. Currently, we aim for valid experimental in vitro models, using epithelial brushes from smoking asthmatic patients that can elucidate the underlying mechanisms of smoking-induced corticosteroid unresponsiveness.

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Key issues

- Clinically, cigarette smoking in asthma is associated with worsening of symptoms, accelerated lung-function decline, a higher frequency of hospital admissions, a higher degree of asthma severity, poorer asthma control and reduced responsiveness to corticosteroids.
- Pathologically, cigarette smoking in asthma is associated with reduced numbers of eosinophils and higher numbers of mast cells in the submucosa of the airway wall. Airway remodeling is increased as evidenced by increased epithelial thickness and goblet cell hyperplasia in smoking asthmatics. The pathogenesis responsible for smoking-induced changes in airway inflammation and remodeling in asthma is complex and largely unknown.
- In the interpretation of the role of smoking in the inflammatory profile and phenotype of asthma, it is important to take corticosteroid use, number of pack-years smoking, having allergic or nonallergic asthma and severity of asthma into account.
- Smoking cessation improves asthma control and reverses airway pathological changes.
- Fundamental research may unravel the complex underlying mechanisms of the link between smoking and asthma. This may lead to the discovery of new therapies for smoking-induced corticosteroid unresponsiveness.

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Chapter 3

Old dilemma: asthma with irreversible airway obstruction or COPD

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Abstract

Older asthmatic patients may develop fixed airway obstruction and clinical signs of chronic obstructive pulmonary disease (COPD). We investigated the added value of pathological evaluation of bronchial biopsies to help differentiate asthma from COPD, taking into account smoking, age, and inhaled corticosteroid (ICS) use. Asthma and COPD patients (24 of each category) were matched for ICS use, age, FEV1, and smoking habits. Five pulmonary and five general pathologists examined bronchial biopsies using an interactive website, without knowing patient information. They were asked to diagnose asthma or COPD on biopsy findings in both a pairwise and randomly mixed order of cases during four different phases, with intervals of 4–6 weeks, covering a maximal period of 36 weeks. Clinically concordant diagnoses of asthma or COPD varied between 63 %-73 %, without important differences between pairwise vs randomly mixed examination or between general vs pulmonary pathologists. The highest percentage of concordant diagnoses was in young asthmatic patients without ICS use and in COPD patients with ICS use. In non ICS users with fixed airway obstruction, a COPD diagnosis was favored if abnormal presence of glands, squamous metaplasia, and submucosal infiltrate was present and an asthma diagnosis in case of abnormal presence of goblet cells. In ICS users with fixed airway obstruction, abnormal presence of submucosal infiltrates, basement membrane thickening, eosinophils, and glands was associated with asthma. Histological characteristics in bronchial biopsies are reproducibly recognized by pathologists, yet the differentiation by histopathology between asthma and COPD is difficult without information about ICS use.

Introduction

Asthma and chronic obstructive pulmonary disease (COPD) are heterogeneous chronic lung diseases, characterized by the presence of airway obstruction and airway inflammation [1, 2]. In asthma, airway obstruction is typically completely or nearly completely reversible [2], while irreversible airway obstruction is typical for COPD [1]. Although both diseases have overlapping clinical features, acknowledged in guidelines [3], they are generally regarded as different disorders; each requiring their own diagnostic and management strategies. For example, for the pharmacological management of asthma, inhaled corticosteroids (ICSs) are the most efficacious drugs currently available [2]. In COPD, inhaled bronchodilators are fundamental for treatment, whereas addition of ICS is only recommended for COPD patients suffering from severe disease (FEV1 < 50 % predicted) and/or a history of recurrent

exacerbations [1]. In order to determine appropriate treatment, prognosis and follow-up, international guidelines have emphasized the importance of differentiating asthma from COPD. In general, careful history taking, physical examination, and lung function testing often lead to a clear diagnosis [4]. However, it is frequently difficult if not impossible to achieve an accurate diagnosis of either asthma or COPD in older patients [5–8]. Although asthma generally affects children and young adults, it is not uncommon that asthma starts later in life [9]. In approximately 4 to 8 % of asthmatic cases, the first asthma symptoms are present in late adulthood (late-onset asthma) or even after 65 years of age. With increasing age, a proportion of patients with asthma may develop persistent irreversible airflow limitation, particularly in the presence of risk factors such as smoking [5], blood eosinophilia, chronic mucus hypersecretion, and a low level of FEV1 [10]. This type of asthma is clinically indistinguishable from COPD, which the guidelines called ACOS or asthma-COPD overlap syndrome [3], and medical history, physical examination, and lung function tests may become insufficient to distinguish asthma from COPD to allow the most adequate therapy. The lack of a diagnostic standard to identify asthma at older age, together with poor perception of symptoms such as dyspnea, may further hamper the recognition of asthma in the elderly [11].

In case of doubt, clinicians may attempt to achieve a best possible diagnosis of asthma or COPD by taking bronchial biopsies for histopathological examination, although this is not a common practice. It has been suggested that pathological examination of bronchial tissue, taking features such as denudation of the epithelium in asthma and epithelial hyperplasia in COPD into account, might contribute to resolving the diagnostic difficulty. Despite clear morphological differences between asthma and COPD, some morphological characteristics can be found in both diseases (in particular in chronic or severe cases), which impairs their diagnostic value in an individual case [12]. Bourdin et al. demonstrated that the diagnostic value of histological examination of endobronchial biopsies from subjects with asthma or COPD is limited, sensitivity and specificity ranging between 36–48 and 56–79 % respectively [13]. However, the latter study included mostly young, never-smoking, non-steroid-using asthma patients with normal lung function and high bronchodilator reversibility. Furthermore, asthma and COPD patients were not matched for age, airway obstruction, ICS use, and smoking habits. Since these factors modulate histological features of airway inflammation and remodeling, they therefore may have confounded the results.

In the current study, we aimed to identify the most important histopathological features to differentiate between asthma and COPD in bronchial biopsies. We hypothesized that the accuracy of the pathological diagnosis would improve when taking into account modulating

factors such as smoking, age, and ICS use.

Materials and methods

Patients and matching of biopsies

Biopsies from 24 asthma and 24 matched (see below) COPD patients were included. Subjects were only included when there was no uncertainty in the diagnosis and when subjects met all criteria for either asthma or COPD according to international guidelines [1, 2]. Subjects were selected from several asthma and COPD cohort studies performed in our institute [14–17]. We created three groups (A–C) of asthma patients (n = 8 each group), carefully matched with three groups of COPD patients (n = 8 each group).

A. The first asthma and COPD groups included subjects who did not use ICS, were >45 years old, had a postbronchodilator (BD) FEV1/FVC <70 %, and had smoked >10 pack-years.

B. The second asthma and COPD groups included subjects with the same criteria, but subjects had used ICS during the last 30 months.

C. The third group included asthma patients without ICS use, and with post-BD FEV1 > 90 % predicted, age < 45 years, 0 pack-years smoking, and atopy (Phadiatop > 1.0). This was contrasted with COPD patients without ICS use, with post-BD FEV1 < 50 % predicted, age > 45 years, current smoking with >10 packyears, and without atopy.

Groups A and B included patients with a clinically difficult differential diagnosis between asthma and COPD, whereas control group C included so-called classical cases easy to differentiate.

Table 1 shows the group characteristics (A–C) and Table S1 individual characteristics. Details of the selection process are depicted in the Fig. S1.

Medical records, lung function data, and paraffin-embedded endobronchial biopsies (EBB) were available from all selected patients.

EBB staining, virtual microscopy, and interactive website

EBB slides were stained with hematoxylin and eosin (H&E). An experienced pulmonary pathologist (WT) checked the quality of the slides for each patient and selected the best specimen based on size, intactness, and presence of mucosal and submucosal layers. This quality check was performed without knowing the diagnosis or paying attention to the possible diagnosis. Entire biopsies were scanned using Aperio ScanScope Digital Slide Scanner at ×40 magnification. Afterwards, the images were uploaded to a specially designed interactive website, allowing to view the slides at different magnifications and to navigate into different

areas of the bronchial biopsy like with a normal microscope (Fig. 1). Quality of our web-based virtual microscopy was checked by an experienced independent pulmonary pathologist who compared all 48 slide images with series of biopsies from the original glass slides by microscope.

Table 1. Characteristics of patients with asthma and COPD

	Group A (ICS−)		Group B (ICS+)		Group C (Classical, ICS−)	
Characteristics	**Asthma**	**COPD**	**Asthma**	**COPD**	**Asthma**	**COPD**
Age, years	53 (50–64)	56 (47–63)	61 (54–68)	61.5 (56–72)	29.5 (25–44)	63 (53–64)
Sex, M/F	6M, 2F	5M, 3F	5M, 3F	8M, 0F	4M, 4F	6M, 2F
FEV_1/FVC,%	64 (48–69)	62 (48–70)	53 (40–66)	53 (36–69)	81 (75–98)	41 (30–47)
FEV_1, %pred	83 (60–108)	82 (70–106)	83 (43–99)	71 (46–90)	105 (95–122)	45 (41–50)
Pack-years	31 (10–44)	29 (15–43)	20 (12–64)	38 (19–51)	0.0 (0.0–0.0)	32 (21–56)
Current smoking, ex smoking,	8 current, 0 ex	8 current, 0 ex	6 current, 2 ex	6 current, 2 ex	0 current, 0 ex	8 current, 0 ex

Data presented as median (minimum-maximum) except for sex (M male, F female) and smoking (current, ex smoking). Group A: asthma and COPD patients without ICS use, age > 45 years, post bronchodilator (BD) FEV_1/FVC <70 %, and >10 pack-years smoking. Group B: asthma and COPD patients with the same criteria, but subjects had to use ICS during last 30 months. Group C: "classical" asthma patients without ICS use, and with post BD FEV_1 > 90 % predicted, age < 45 years, 0 pack-years smoking, and atopy. Classical asthma was contrasted with classical COPD: no ICS use, post BD FEV_1 < 50 % predicted, age > 45 years, current smoking with >10 pack-years, and no atopy

Table 2 Design of the study

	Time (weeks)	**Examination of slides**	**Description**
Phase 1	2	Pairwise (2 × 24 slides)	Matched asthma and COPD slides were offered pairwise. The pathologists were informed
Interval	4–6		
Phase 2	4	Randomly mixed (48 slides)	The 48 slides were randomly mixed. The pathologists were informed about this and had to opt for asthma or COPD per slide. Once chosen, the pathologists were not able to change their choice or go back to slides shown earlier.
Interval	4–6		
Phase 3b	2	Randomly mixed + criterion list (24 slides)	Conform phase 3a. This phase aimed to test repeatability.
Interval	4–6		
Phase 4	2	Pairwise (2 × 24 slides)	Conform phase 1. This phase aimed to test repeatability (and/or potential learning effects).
	Totally 28-36 weeks		

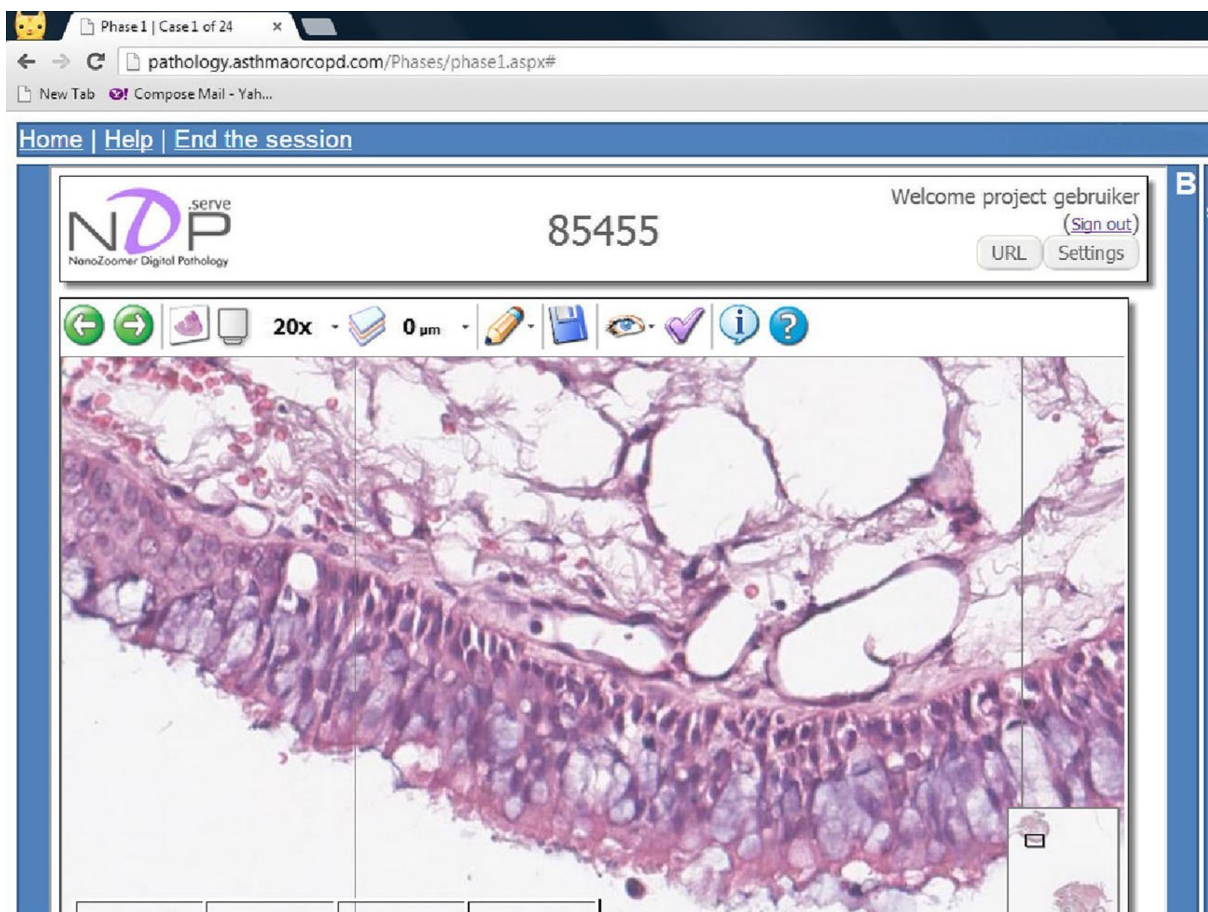

Fig. 1 Example slide on interactive website. *Screenshot of the interactive website, showing a slide with a representative bronchial biopsy at ×20 magnification. The small image in the lower part of the picture is the overview window, showing the current position and size of the large window. The website allowed to view the slides at different magnifications and to navigate into different areas of the bronchial biopsy like a normal microscope*

Study protocol

Ten pathologists from different countries (Netherlands, Brazil, Canada, Austria, Switzerland), five of them specialized in pulmonary pathology, participated in the study and used our interactive website following a strict protocol (Table 2). The pathologists were informed about the design of the study but had no clinical information (age, sex, ICS use, smoking, lung function). In phases 1 and 4, the pathologists were offered 48 slides, i.e., 24 pairs of matched asthma and COPD patients, and were asked to indicate per pair which one was asthma and which one COPD. In phases 2 and 3, all 48 slides were offered in a randomly mixed order and the pathologists were asked to choose for either asthma or COPD. In phase 3, the pathologists were additionally asked to indicate the presence or absence of a pathological criterion using the criteria list of Bourdin's study [13], with small modifications (Box 1). In addition, they were asked how sure they felt about their diagnosis using a 0–10 visual analog scale (VAS) score (0

not sure, 10 very sure) and to rank what they considered the three most relevant features (Fig. 2).

Box 1

Questionnaire for pathologists

Indicate per criterion if it is abnormally (disease-related) present (yes/no)

Epithelium
- Denudation (loss of epithelium, including loss of super-basal epithelium)
- Squamous metaplasia
- Hyperplasia (=thickened epithelium)
- Goblet cells

Basement membrane
- Thickening

Submucosa
- Inflammatory infiltrate (lymphocytes, macrophages or neutrophils)
- Eosinophils
- Smooth muscle
- Glands
- Sub-mucosal fibrosis

Diagnosis:
- Asthma or COPD?
- How sure are you about the diagnosis (score 1-10): ....
- Which criterion was most important for your diagnosis?
  First: ....
  Second:................
  Third:..................

Statistical analyses

Data from the interactive website were automatically and anonymously saved to an Excel file (MS Excel 2010) and transferred to SPSS software (version 19.0; SPSS Inc., Chicago, IL). The individual data was computed to a concordant or discordant diagnosis of asthma or COPD (i.e., concordant between pathological and clinical diagnosis), and results were expressed as the mean percentage of the concordant diagnosis. In phase 3, the reported presence of each pathological criterion was compared between slides of asthma and COPD using the Mann-Whitney test. Sensitivity, specificity, and accuracy of each pathological criterion for pathologists making a concordant diagnosis of asthma were calculated. A high sensitivity for asthma indicated automatically a high specificity for COPD and vice versa. Pathological criteria with a p value <0.2 in the univariate analysis were entered in a logistic regression

analysis on the presence of asthma. Pathological criteria which contributed independently to a concordant diagnosis of asthma or COPD were combined to find higher accuracy rates for the concordant diagnosis. Selection of combined criteria was based on the highest Wald value in the regression analyses.

Analyses were performed in the total group of patients, within patient groups (A–C), and between pathologists (general, specialized). Intra- (between phases 3a and 3b, see Box 1) and inter-observer (within phase 3a) agreements were assessed with Cohen's kappa test and Fleiss' kappa, respectively. The significance level was set at 0.05.

Results

Concordant diagnoses (pathology concordant with clinical diagnosis)

Table 3 shows the percentage of concordant diagnoses per phase, for each disease group (A–C) per pathologist group. The percentages of concordant diagnoses, per pathologist, per phase, are shown in Fig. S2. The percentage of concordant diagnoses of asthma or COPD, per pathologist group, in phase 3a is shown in Table S2. Overall, the highest number of concordant diagnoses was observed in phase 4, particularly by pulmonary pathologists. The highest score for asthma was observed in phase 3a, in the classical asthma/COPD group (C), by pulmonary pathologists, 91.4 %. The highest score for COPD was also observed in phase 3a, in the non-classical asthma/COPD group of ICS users (B), with no difference between pulmonary and general pathologists. Feeling sure about the diagnosis of asthma (in asthma and COPD cases together) was rated on a VAS scale from 1 to 10. In groups A–C, the mean (SD) VAS score for asthma was 5.6 (2.5), 5.2 (2.0), and 6.1 (2.4), respectively. For COPD, this was 5.9 (2.4), 5.8 (2.1), and 5.0 (2.6).

Pathological criteria in phase 3

Table 4 shows the reported presence of pathological criteria in the airway wall biopsies in asthma or COPD per disease group (4A–C). Criteria that differed significantly between asthma and COPD were not comparable between the three groups. Eosinophilia was significantly more frequently reported in asthma in groups B + C but not in A (subjects with asthma and COPD in B used ICS, in contrast to subjects in A + C). Submucosal inflammation was significantly more frequently reported in asthma in groups B + C than in group A, yet more frequently in COPD in group A. Table S3 and Box 2 show which pathological criteria significantly contribute to a concordant diagnosis of asthma or COPD in a multiple regression model. Significant criteria present in all groups were goblet cells, inflammatory infiltrate, and glands. Significant criteria

present in two groups were eosinophils (group B + C), and in one group squamous metaplasia (group A), BM thickening (group B), hyperplasia (group C), smooth muscle (group C), and submucosal fibrosis (group C). Significant ($p < 0.05$) Wald values of these models are also shown in Table 4.

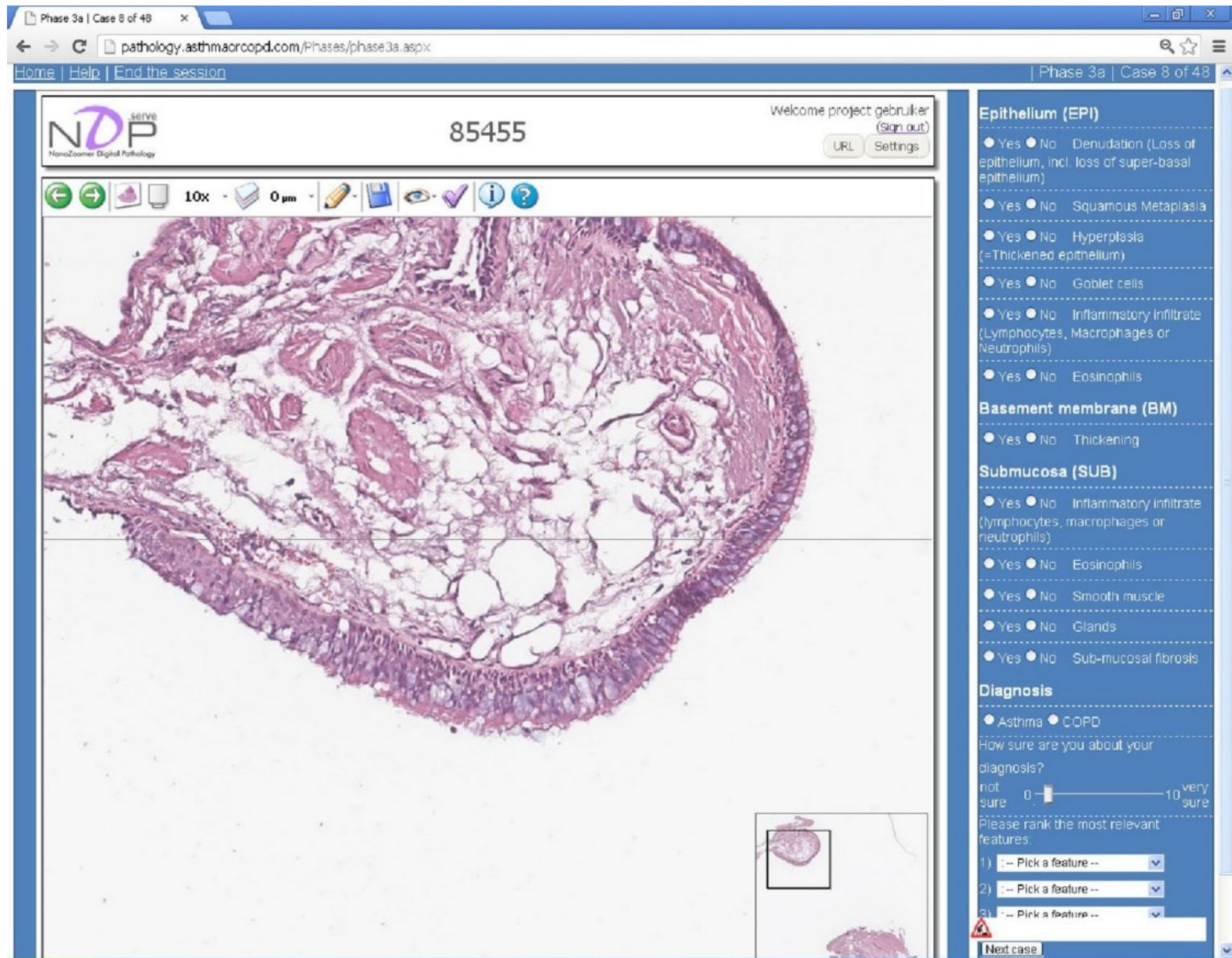

Fig. 2 Example slide on interactive website including the list with pathological criteria.
Screenshot of the interactive website, showing a slide (phase 3a) with a representative bronchial biopsy at ×10 magnification. The small image in the lower part of the picture is the overview window, showing the current position and size of the large window. At the right side of the slide the pathologists may record the observed abnormal presence of 12 pathological criteria (yes/no), the diagnosis of asthma or COPD, how sure they feel about the diagnosis, and rank the 3 most relevant features

Sensitivity, specificity, and accuracy of criteria for a concordant diagnosis

Table 4 shows the sensitivity, specificity, and accuracy for asthma/COPD diagnosis for groups A–C. Pathological criteria with high accuracy differed importantly between groups A–C. In group A (no ICS use), glands, goblet cells, squamous metaplasia, and submucosal infiltrate showed the highest accuracy. In group B (with ICS use), the highest accuracy was provided by submucosal inflammation, basement membrane thickening, eosinophilia, and glands. In group

C (classical asthma and COPD), the highest accuracy was shown by submucosal fibrosis, epithelial hyperplasia, eosinophilia, and glands. Combinations of relevant pathological criteria did improve sensitivity or specificity, but in general not accuracy (Table S4).

Agreement between and repeatability within pathologists

Agreement between pathologists for a concordant diagnosis of asthma or COPD in groups A–C was 0.40, 0.45, and 0.48, respectively. This was 0.33, 0.45, and 0.72 for asthma and 0.60, 0.57, and 0.27 for COPD, respectively. Repeatability within pathologists for a concordant diagnosis in groups A– C was 0.75, 0.78, and 0.80 respectively. This was 0.75, 0.70,
and 0.80 for asthma and 0.70, 0.85, and 0.80 for COPD, respectively. Agreement between and repeatability within pathologists for the reported presence of pathological criteria in groups A–C is presented in Table 4. Overall agreement in groups A–C varied between 0.61 and 0.92. Overall repeatability in groups A–C varied between 0.68 and 0.95.

Discussion

Asthma and COPD are obstructive airway diseases with clear differences in etiology and pathophysiology, yet at older age, they frequently are difficult to discriminate, the currently so-called asthma-COPD overlap syndrome (ACOS) [3]. In the current study, there was no discussion about the original underlying disease, as we had historical data from well-characterized cohort studies. Therefore, we had the opportunity to carefully match bronchial biopsies from 24 asthma and 24 COPD patients taking age, FEV1, ICS use, and smoking habits into account. Ten pathologists, not informed about the individual clinical background of the patients, but knowing the study design, were asked to diagnose asthma or COPD. The important outcome of this study is that histological examination of bronchial biopsies alone does not allow differentiating between asthma and COPD. However, as recognition of the specific histological criteria was good, diagnostic value can be expected to improve when selected pathological criteria are applied and adequate clinical information is provided, in which in particular, knowledge about the use of ICS is essential.

In this study, we aimed for high-quality matching of asthma and COPD patients, considering age, smoking, ICS use, and airway obstruction as important modulators of airway inflammation and remodeling. This matching is important because it fits with the original question of the study: whether it is possible for pathologists to discriminate between older asthma patients with fixed airway obstruction and COPD patients, using bronchial biopsies. We designed our study into four phases, gradually increasing the level of difficulty and anticipated that a head-to-head

comparison of two paired slides, one from an asthma patient and one from a COPD patient, would help to discriminate between the two diseases. Interestingly, this was not the case. Success rates of scoring of the paired slides were not higher than those of randomly mixed slides. Furthermore, we anticipated that systematic scoring of textbook pathological criteria for asthma or COPD would help to set the correct (=concordant) diagnosis; however, this was not the case.

The overall percentages of a diagnosis of asthma or COPD concordant with the clinical diagnosis were low, taking into account a 50 % concordance by chance. We expected the highest percentage of concordant diagnoses in young asthmatic patients without ICS use and with a normal lung function, assuming that the recognition of the underlying disease is easier when inflammation is not treated with ICS or changed by age- and smoking-related remodeling processes. Indeed, the highest percentage of a concordant diagnosis for asthma in the randomly mixed slides was observed in the group of classical cases (group C, without ICS) and the lowest in the group of asthma subjects who used ICS clinically which are difficult to diagnose (group B). Interestingly, this contrasted with the findings for COPD, where the highest percentage of concordant diagnoses occurred in the group of ICS users. It is not clear at first sight why ICS use reduced the percentage of concordant diagnosis for asthma, yet improved this for COPD. This may well be because corticosteroids reduce eosinophil survival but prolong neutrophil survival; hence, ICS use may have reduced eosinophilia in asthma and increased neutrophilia in COPD [18], eosinophilia being considered a hallmark for asthma and neutrophilia for COPD. This contrasting effect of ICS use on giving a concordant diagnosis of asthma or COPD is an important finding of our study, which—it goes without saying—emphasizes the need for effective transfer of adequate clinical data from clinicians to pathologists.

The reported presence of pathological criteria was significantly different between slides from asthma and COPD patients, in striking contrast with the low percentage of concordant diagnoses of asthma and COPD based on biopsies alone. Additionally, the agreement between the 10 pathologists (interobserver variability) and repeatability within pathologists (intra-observer variability) was good to excellent for many pathological criteria, independent of a concordant diagnosis. Thus, all expertise to allow a concordant diagnosis of asthma or COPD was present, yet the pathologists appeared to need additional information to be able to apply this expertise successfully, considering the relatively low percentage of concordant diagnoses. A priory, one might expect that the diagnosis of pathologists based upon histology only might be more accurate because it allows integration of all relevant information from all parts of a

biopsy. Apparently, this is a difficult task, which is in particular complicated when clinical characteristics like age, smoking, ICS use, and airway obstruction, that affect accuracy and significance of potentially useful pathological criteria, are not known, as shown in the present study.

Table 3. Percentage of concordant diagnoses in different phases of the study

		Phase 1	Phase 2		Phase 3a		Phase 4
		Pairwise	Asthma	COPD	Asthma	COPD	Pairwise
All pathologists	*All*	68.7	65.6	62.5	63.5	62.5	72.6
	A (ICS−)	65.0	*67.5*	62.5	*56.3*	*65.0*	63.8
	B (ICS+)	73.8	*52.5*	66.2	*52.5*	*72.5*	76.3
	C (classical)	67.1	*78.6*	58.7	*84.3*	*50.0*	78.6
Lung pathologists	*All*	71.3	66.1	60.0	70.4	61.7	76.5
	A (ICS−)	62.5	*67.5*	57.5	*67.5*	*57.5*	*62.5*
	B (ICS+)	80.0	*52.5*	65.0	*55.0*	*72.5*	*85.0*
	C (classical)	71.4	*80.0*	57.5	*91.4*	*55.0*	*82.9*
General pathologists	*All*	66.1	65.2	65.0	56.5	63.3	68.7
	A (ICS−)	67.5	67.5	67.5	*45.0*	*72.5*	65.0
	B (ICS+)	67.5	52.5	67.5	*50.0*	*72.5*	67.5
	C (classical)	62.9	77.1	60.0	*77.1*	*45.0*	74.3

Values are percentage of concordant diagnoses. Italic values: $p < 0.05$ between A, B, and C within 3a phase. Group A: asthma and COPD patients without ICS use, age > 45 years, post bronchodilator (BD) FEV_1/FVC <70 %, and >10 pack-years smoking. Group B: asthma and COPD patients with the same criteria, but subjects had to use ICS during last 30 months. Group C: "classical" asthma patients without ICS use, and with post BD FEV_1 > 90 % predicted, age < 45 years, 0 pack-years smoking, and atopy. Classical asthma was contrasted with classical COPD: no ICS use, post BD FEV_1 < 50 % predicted, age > 45 years, current smoking with >10 packyears, and no atopy

Next, we tried to identify pathologists with a higher percentage of concordant diagnoses in order to learn and copy their strategy. Beforehand, we expected that pulmonary pathologists would do better than general pathologists. Indeed, at a group level, the mean scores of the pulmonary pathologist group tended to be higher in every phase of the study, and the pathologists with the highest percentages of concordant diagnoses were almost always the pulmonary pathologists. For example, one of the pulmonary pathologists (pathologist B in Table S2) had high accuracy rates, but importantly, in the subgroup of patients who did use ICS (group B), this pathologist had poor results, again demonstrating the importance of knowledge of the clinical background of patients. The pathological criteria that were most frequently ranked as important for the diagnosis by the best scoring pathologists were squamous metaplasia in group A (without ICS use), basement membrane thickening in group B (with ICS

use), and submucosal fibrosis in group C (with “classical” asthma and COPD). This is not unexpected as these pathological criteria demonstrated also high accuracy rates at a group level. Finally, we tried to improve accuracy rates by combining pathological criteria that independently associated with the clinical diagnosis of asthma or COPD. Whereas it was not difficult to find combinations with a very high sensitivity or specificity rates, the accuracy rates of these combinations were generally not better than the individual pathological criteria.

We hypothesize that pathologists may improve their ability to differentiate asthma from COPD if they use selected pathological criteria, i.e., those with a high accuracy rate for concordance, provided that the relevant clinical information is known. In non ICS users with fixed airway obstruction the abnormal presence of goblet cells directs towards an asthma diagnosis, whereas glands, squamous metaplasia, and submucosal infiltrate direct towards COPD (Box 2). In ICS users with fixed airway obstruction, the abnormal presence of submucosal infiltrate, basement membrane thickening, eosinophils, and glands direct towards asthma diagnosis. In classical cases, the diagnosis is already known on the basis of clinical characteristics. Nevertheless, the clinical diagnosis can be confirmed by the observation of submucosal infiltrate and eosinophils indicating asthma, whereas submucosal fibrosis, hyperplasia, and glands indicate COPD.

Box 2

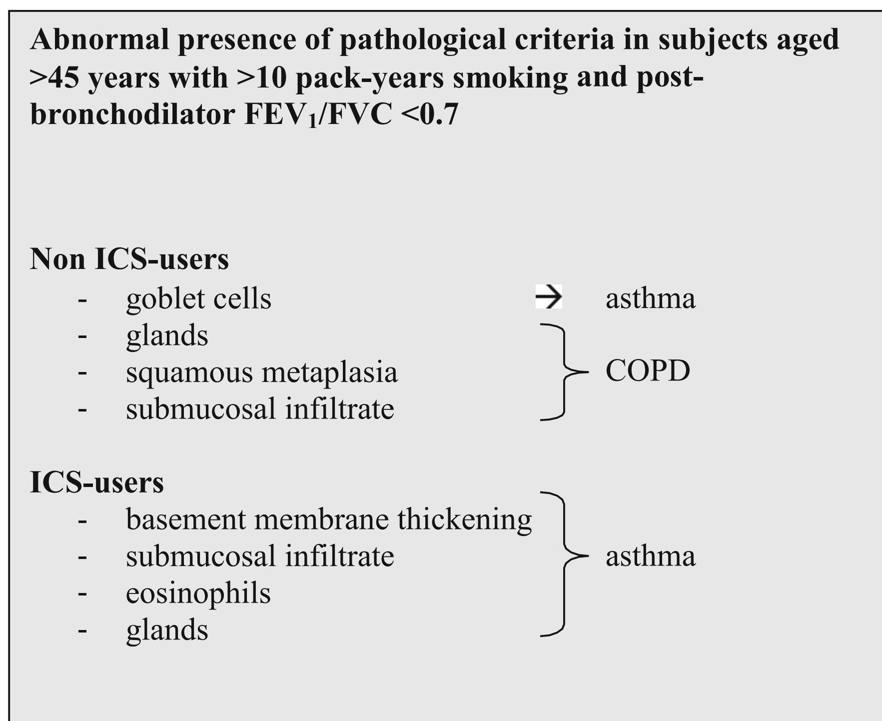

Table 4. Characteristics of the pathological criteria for the diagnosis of asthma or COPD in group A (non-ICS users), group B (ICS users), and group C (classical group)

Criteria	Reported (%) in Asthma	Reported (%) in COPD	OR (asthma) (95%CI)	Wald value	Sensitivity (%)	Specificity (%)	Accuracy (%)	Agreement* between pathologists	Repeatability* within pathologist
Group A (non ICS users)									
Denudation	85.0	83.8	1.1 (0.47–2.58)		85.0	16.3	50.6	0.76 (0.77; 0.76)	0.90 (0.95; 0.85)
Squamous Metaplasia	22.5	53.8	0.25 (0.13–0.49)	*7.74*	77.5	53.8	65.6	0.72 (0.77; 0.68)	0.80 (0.90; 0.70)
Hyperplasia	52.5	55.0	0.90 (0.49–1.69)		47.5	55.0	51.2	0.63 (0.73; 0.54)	0.73 (0.75; 0.70)
Goblet cells	60.0	32.5	3.11 (1.63–5.95)	*5.54*	60.0	67.5	63.7	0.85 (0.87; 0.84)	0.85 (0.90; 0.80)
BM thickening	73.8	67.5	1.35 (0.68–2.68)		73.8	32.5	53.1	0.67 (0.69; 0.64)	0.85 (0.85; 0.85)
Inflammatory infiltrate	53.8	72.5	0.44 (0.23–0.85)	*11.84*	46.3	72.5	59.4	0.61 (0.53; 0.69)	0.78 (0.60; 0.95)
Eosinophils	22.5	12.5	2.03 (0.87–4.73)		22.5	87.5	55.0	0.72 (0.64; 0.79)	0.85 (0.75; 0.95)
Smooth muscle	60.0	81.3	0.35 (0.17–0.71)		40.0	81.3	60.6	0.76 (0.78; 0.74)	0.85 (0.80; 0.90)
Glands	23.8	72.5	0.12 (0.06–0.24)	*21.62*	76.3	72.5	74.4	0.85 (0.93; 0.76)	0.90 (1:00; 0.80)
Sub mucosal fibrosis	67.5	58.8	1.46 (0.76–2.78)		32.5	58.8	45.6	0.63 (0.66; 0.60)	0.75 (0.85; 0.65)
Group B (ICS users)									
Denudation	71.3	58.8	1.74 (0.90–3.36)		71.3	41.3	56.2	0.62 (0.61; 0.63)	0.73 (0.65; 0.80)
Squamous metaplasia	33.8	35.0	0.95 (0.49–1.82)		66.3	35.0	50.6	0.80 (0.80; 0.81)	0.85 (0.70; 1.00)
Hyperplasia	51.3	53.8	0.90 (0.49–1.68)		48.8	53.8	51.2	0.67 (0.71; 0.63)	0.90 (0.85; 0.95)
Goblet cells	72.5	60.0	1.76 (0.90–3.41)		72.5	40.0	56.2	0.72 (0.68; 0.76)	0.60 (0.60; 0.60)
BM thickening	57.5	25.0	4.06 (2.07–7.95)	*18.24*	57.5	75.0	66.2	0.71 (0.78; 0.64)	0.75 (0.80; 0.70)
Inflammatory infiltrate	63.8	23.8	5.65 (2.84–11.23)	*19.86*	63.8	76.3	70.0	0.71 (0.73; 0.70)	0.83 (0.80; 0.85)
Eosinophils	30.0	3.8	11.0 (3.16–38.34)	*4.27*	30.0	96.3	63.1	0.85 (0.76; 0.93)	0.83 (0.70; 0.95)
Smooth muscle	66.3	61.3	1.24 (0.65–2.37)		66.3	38.8	52.5	0.77 (0.86; 0.68)	0.80 (0.80; 0.80)
Glands	48.8	33.8	1.87 (0.99–3.53)	*8.88*	48.8	66.3	57.5	0.81 (0.79; 0.83)	0.90 (0.90; 0.90)
Sub mucosal fibrosis	66.3	67.5	0.94 (0.49–1.83)		33.8	67.5	50.6	0.65 (0.64; 0.66)	0.78 (0.85; 0.70)
Group C (classical group)									
Denudation	91.4	80.0	2.67 (0.98–7.25)		91.4	20.0	53.3	0.76 (0.86; 0.68)	0.75 (0.80; 0.70)
Squamous metaplasia	0.0	17.5	0.000 (0.000-)		100.0	17.5	56.0	0.92 (1.00; 0.86)	0.95 (1.00; 0.90)
Hyperplasia	10.0	61.3	0.07 (0.03–0.17)	*16.95*	90.0	61.3	74.7	0.80 (0.84; 0.77)	0.88 (0.95; 0.80)
Goblet cells	54.3	43.8	1.53 (0.80–2.91)		54.3	56.3	55.3	0.74 (0.73; 0.75)	0.68 (0.70; 0.65)
BM thickening	74.3	71.3	1.17 (0.57–2.40)		74.3	28.8	50.0	0.71 (0.78; 0.66)	0.78 (0.80; 0.75)
Inflammatory infiltrate	91.4	65.0	5.74 (2.21–14.92)	*8.88*	91.4	35.0	61.3	0.72 (0.84; 0.62)	0.73 (0.80; 0.65)
Eosinophils	47.1	20.0	3.57 (1.73–7.34)	*3.97*	47.1	80.0	64.7	0.71 (0.58; 0.83)	0.80 (0.60; 1.00)
Smooth muscle	87.1	77.5	1.97 (0.82–4.72)	*6.34*	87.1	22.5	52.7	0.77 (0.83; 0.71)	0.93 (0.85; 1.00)
Glands	10.0	43.8	0.14 (0.06–0.35)	*8.66*	90.0	43.8	65.3	0.89 (0.89; 0.89)	0.93 (1.00; 0.85)
Sub mucosal fibrosis	14.3	78.8	0.04 (0.02–0.11)	*19.98*	85.7	78.8	82.0	0.72 (0.77; 0.68)	0.78 (0.65; 0.90)

**Repeatability and agreement data presented for asthma and COPD together and separately (asthma; COPD). ORs > 1 indicate a positive association with the presence of asthma; OR < 1 indicate a positive association with the presence of COPD. Sensitivity and specificity for asthma are calculated for the reported presence of variables with OR > 1. Sensitivity and specificity for COPD are calculated for the reported presence of variables with OR < 1. Significant (p < 0.05)*

Wald values were derived from logistic regression analyses on the concordant diagnosis of asthma and COPD (Table S4)

This study has a few limitations. First, one can argue whether or not a physician's diagnosis of asthma or COPD is an adequate gold standard. Importantly, patient charts of many years back showed that even patients with asthma and fixed airway obstruction at current investigation had reversible airway obstruction at a younger age. After accepting that our physician's diagnosis is reliable, one might still argue that the selected cases are not the ones that normally would need a pathological examination, because the clinical diagnosis was already established with certainty. Unfortunately, we have no biopsies available to compare cases with certain versus uncertain clinical diagnosis. Our aim however was to establish clues at the microscopical level with strong relation with either asthma or COPD, to be used as clues in difficult biopsies to direct towards a likely diagnosis. Secondly, one could argue that biopsies were not examined by microscope but on screen, which reflects but is not identical to the real-life situation in daily diagnostic practice. This indeed should be validated as is indicated by a recent guideline [19]. Nevertheless, in our approach, all pathologists were exposed to identical images, which improved standardization and allowed pathologists to scroll easily through complete high-resolution slides and varying magnification as in classical microscopy. Third, we did not include slides from healthy controls or from subjects with another lung disease. Consequently, the terms sensitivity and specificity only refer to asthma and COPD, compatible with our study question whether a pathologist is able to give a concordant diagnosis of asthma or COPD. We therefore consider our study design appropriate.

In conclusion, we show that the differentiation between asthma and COPD, based on histopathological examination only of a bronchial biopsy, without adequate clinical information, is difficult. This contrasts with the high percentage of concordant diagnosis observed for a number of reported pathological criteria. We postulate that the diagnostic value is likely to improve when selected pathological criteria (Box 2) are applied and adequate information is provided with respect to the use of ICS. Prospective studies incorporating medical decision techniques may validate algorithms that take these issues into account.

Ethical approval: The study protocol involving human samples was approved by the Medical Ethics Review Committee (METc) of the University Medical Center Groningen. All procedures conformed to the ethical standards of the responsible committee on human sample experimentation and performed according to the appropriate guidelines. Informed consent was obtained from all patients during the original studies.

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Supplement data
Old dilemma: Asthma with irreversible airway obstruction or COPD

Table S1. Individual data from asthma patients and pair-matched COPD subjects (group A-C).

	Asthma patients					COPD patients				
	Age	Sex	FEV_1/FVC (%)	FEV_1 (%pred)	Pack-years	Age	Sex	FEV_1/FVC (%)	FEV_1 %pred	Pack-years
Group A (ICS-)	53	M	59.0	92.0	31	54	M	58.5	92.3	30.4
	53	F	66.3	107.8	10	55	F	65.0	105.7	28
	50	M	63.3	73.2	25	48	F	69.5	75.5	22
	52	M	65.3	66.5	37	57	M	53.5	74.0	29.8
	57	F	63.9	93.7	15	59	F	65.4	87.7	15.3
	64	M	68.8	107.8	39	47	M	67.4	102.2	28.1
	57	M	47.6	59.8	44	63	M	47.8	69.6	42.8
	52	M	60.8	67.2	30	57	M	53.5	74.0	29.8
Group B (ICS+)	68	M	39.9	56.2	12	66	M	36.3	57.8	19.3
	66	M	43.2	64.3	13	72	M	41.7	65.4	20.5
	61	F	66.1	91.1	64	57	M	59.9	90.3	41.0
	61	M	50.2	42.5	34	63	M	49.7	46.2	51.0
	57	M	50.1	85.1	18	56	M	50.2	70.4	28.3
	54	F	65.6	91.3	13	56	M	61.8	78.8	42.0
	59	M	55.0	81.3	34	63	M	54.8	71.5	47.0
	64	F	64.5	99.3	21	60	M	68.9	79.5	35.9
Group C (Classical)	44	M	75.4	101.5	0	59	F	30.1	47.3	36.0
	38	M	81.6	107.1	0	53	M	45.1	41.1	31.2
	30	F	78.9	94.8	0	64	F	47.4	49.7	32.6
	27	M	89.6	122.0	0	63	M	45.5	44.4	28.7
	25	F	97.5	107.1	0	56	M	32.7	47.7	56.2
	29	F	97.7	102.1	0	63	M	41.5	46.1	30.8
	38	M	78.7	98.8	0	63	M	35.	40.8	55.6
	27	F	81.2	113.3	0	63	M	39.7	43.1	21.2

Values are the percentage concordant diagnoses for asthma and COPD per pathologist. Note that some pathologists scored very high for one disease and at the same time very low for the other, showing they favored one diagnosis. Group A: asthma and COPD patients who did not use ICS, were aged >45 years, had a post bronchodilator (BD) FEV1/FVC <70%, and had smoked more than 10 pack-years. Group B: asthma and COPD patients with the same criteria, but subjects had to use ICS during last 30 months. Group C: "classical" asthma patients without ICS use, and with post BD FEV1 >90% predicted, age <45 years, zero pack-years smoking, and atopy. Classical asthma was contrasted with "classical" COPD: no ICS use, post BD FEV1 <50% predicted, age >45 years, current smoking with >10 pack- years, and no atopy.

Table S2. Percentage concordant diagnoses per pathologist in phase 3a

		Total		Group A		Group B		Group C	
		Asthma	COPD	Asthma	COPD	Asthma	COPD	Asthma	COPD
Lung pathologists	A	74	54	75	38	50	75	100	50
	B	74	63	100	88	50	25	71	75
	C	70	67	50	63	63	100	100	38
	D	74	54	75	38	50	88	100	38
	E	61	71	38	63	63	75	86	75
	average	*70*	*62*	*68*	*58*	*55*	*73*	*91*	*55*
General pathologists									
	F	48	67	25	88	50	75	71	38
	G	65	58	38	88	63	75	100	38
	H	52	46	38	38	50	50	71	25
	I	61	75	63	75	38	88	86	63
	J	57	71	63	75	50	75	57	63
	average	*56*	*63*	*45*	*73*	*50*	*73*	*77*	*45*

Values are the percentage concordant diagnoses for asthma and COPD per pathologist. Note that some pathologists scored very high for one disease and at the same time very low for the other, showing they favored one diagnosis. Group A: asthma and COPD patients who did not use ICS, were aged >45 years, had a post bronchodilator (BD) FEV_1/FVC <70%, and had smoked more than 10 pack-years. Group B: asthma and COPD patients with the same criteria, but subjects had to use ICS during last 30 months. Group C: "classical" asthma patients without ICS use, and with post BD FEV_1 >90% predicted, age <45 years, zero pack-years smoking, and atopy. Classical asthma was contrasted with "classical" COPD: no ICS use, post BD FEV_1 <50% predicted, age >45 years, current smoking with >10 pack-years, and no atopy.

Table S3. Multiple logistic regression analysis of reported pathological criteria on the diagnosis of asthma or COPD

Group	Criteria	B	Wald Value	P-value	Exp (B)=OR	Lower (95%CI)	Upper (95%CI)
Group A (non-ICS use)	Squamous metaplasia	-1.251	7.735	**0.005**	0.286	0.118	0.691
	Goblet cells	1.018	5.535	**0.019**	2.767	1.185	6.462
	Inflammatory infiltrate	-1.602	11.843	**0.001**	0.201	0.081	0.502
	Eosinophils	0.936	2.852	0.091	2.550	0.860	7.556
	Smooth muscle	-0.849	3.291	0.070	0.428	0.171	1.071
	Glands	-1.960	21.617	**0.000**	0.141	0.062	0.322
Group B (ICS use)	Denudation	0.664	2.312	0.128	1.942	0.826	4.567
	Goblet cells	0.847	3.827	**0.05**	2.331	0.998	5.445
	BM thickening	1.988	18.241	**0.000**	7.304	2.933	18.189
	Inflammatory infiltrate	2.112	19.858	**0.000**	8.265	3.265	10.927
	Eosinophils	1.573	4.267	**0.039**	4.819	1.084	21.433
	Glands	1.340	8.878	**0.003**	3.820	1.582	9.223
Group C (Classical group)	Denudation	1.78	3.47	0.062	5.94	0.91	38.73
	Hyperplasia	-3.84	16.95	**0.000**	0.02	0.003	0.13
	Goblet cells	1.58	5.43	**0.020**	4.85	1.28	18.29
	Inflammatory infiltrate	2.72	8.88	**0.003**	15.29	2.54	91.92
	Eosinophils	1.31	3.97	**0.046**	3.699	1.02	13.39
	Smooth muscle	2.21	6.34	**0.012**	9.10	1.63	50.75
	Glands	-2.63	8.65	**0.003**	0.07	0.01	0.42
	Sub mucosal fibrosis	-3.12	19.98	**0.000**	0.04	0.01	0.17

Dependent variable: clinical diagnosis for asthma or COPD (+B value: asthma, -B value: COPD).

Table S4. Examples of combinations of pathological criteria

Group	Combinations of pathological criteria presence (+) or absence (-)	Sensitivity (%)	Specificity (%)	Accuracy (%)
Group A (non-ICS use)	Goblet cells +	60	68	64
	Glands –	76	73	74
	Inflammatory infiltrate –	46	73	59
	Goblet cells + and/or glands -	46 / 90	89 / 51	68 / 71
	Goblet cells + and/or inflammatory infiltrate -	21 / 85	95 / 45	58 / 65
	Inflammatory infiltrate – and/or glands -	33 / 90	93 / 53	63 / 71
Group B (ICS use)	BM thickening +	58	75	66
	Inflammatory infiltrate +	64	76	70
	Eosinophils +	30	97	63
	BM thickening + and/or inflammatory infiltrate +	31 / 90	96 / 55	64 / 73
	BM thickening + and/or eosinophils +	19 / 69	100 / 71	59 / 70
	Inflammatory infiltrate + and/or eosinophils +	27 / 66	96 / 76	62 / 71
Group C (Classical group)	Inflammatory infiltrate +	91	35	61
	Submucosal fibrosis –	86	79	82
	Hyperplasia –	90	61	75
	Inflammatory infiltrate + and/or submucosal fibrosis -	77 / 100	85 / 29	81 / 62
	Inflammatory infiltrate + and/or hyperplasia -	83 / 99	79 / 18	81 / 82
	Submucosal fibrosis – and/or hyperplasia-	76 / 100	88 / 53	82 / 74

Values of sensitivity, specificity, and accuracy are presented for the combination of two pathological criteria; the first value in each cell represents a condition that both criteria are present (the "and" function), the second value represents the condition that at least one of thetwo criteria is present (the "or" function). Combinations were selected on basis of high Wald values. This table shows that combining two pathological criteria may improve sensitivity or specificity, but in general not accuracy.

Figure S1. Flowchart of selected biopsies

This flow chart shows how biopsies were selected on basis of availability of biopsies, age, pack-years, and inhaled corticosteroid (ICS) use. Matching of selected asthma and COPD biopsies into group A, B, and C is described in the methods

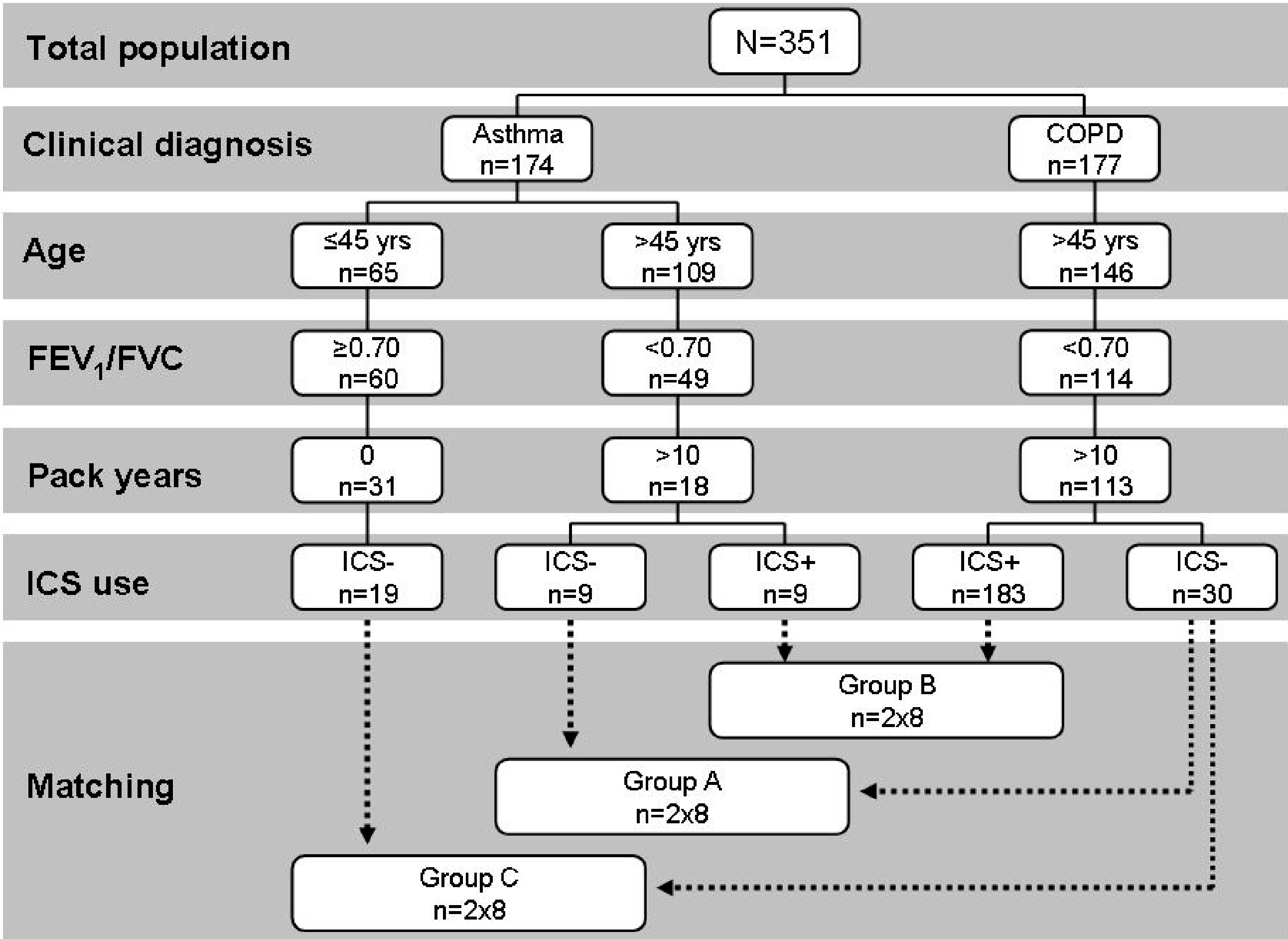
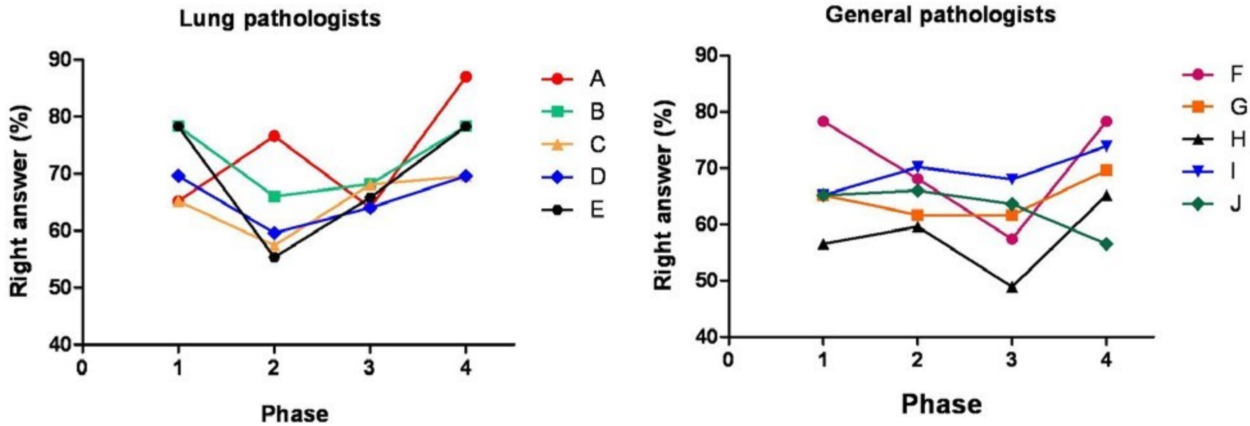


Figure S2. Concordant answers in different phases



Percentage of concordant answers in the four different phases for the lung pathologists (left panel) and general pathologists (right panel). A-J represent the individual pathologists (see also table S2). In phase 1 matched asthma and COPD slides were offered pairwise. In phase 2 the slides were randomly mixed. In phase 3 the pathologists were asked per slide to score for the presence or absence of a criterion that drove their diagnosis. In phase 4 matched asthma and COPD slides were offered pairwise again (like phase 1).

Chapter 4

Atopy is a risk factor for respiratory symptoms in COPD patients: results from the EUROSCOP study.

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Abstract

Background: The pathogenesis of COPD is complex and remains poorly understood. The European Respiratory Society Study on Chronic Obstructive Pulmonary Disease (EUROSCOP) investigated long-term effects of budesonide; 18% of the COPD participants were atopic. So far effects of atopy on the long-term course of COPD have not been elucidated.

Methods: Factors related to the presence of atopy (positive phadiatop) in 1277 mild-to-moderate COPD patients participating in EUROSCOP were analysed using regression analysis. Incidence and remission of respiratory symptoms during 3-year follow-up were analysed using generalised estimating equations models, and association of atopy with lung function decline using linear mixed effects models.

Results: Independent predisposing factors associated with the presence of atopy were: male gender (OR: 2.21; 95% CI: 1.47–3.34), overweight/obese (OR: 1.41; 95% CI: 1.04–1.92) and lower age (OR: 0.98; 95% CI: 0.96–0.99). Atopy was associated with a higher prevalence of cough (OR: 1.71; 95% CI: 1.26–2.34) and phlegm (OR: 1.50; 95% CI: 1.10–2.03), but not with lung function levels or FEV1 decline. Atopic COPD patients not treated with budesonide had an increased incidence of cough over time (OR: 1.79, 95% CI: 1.03–3.08, p = 0.038), while those treated with budesonide had increased remission of cough (OR: 1.93, 95% CI: 1.11–3.37, p = 0.02) compared to non-atopic COPD patients.

Conclusions: Atopic COPD patients are more likely male, have overweight/obesity and are younger as compared with non-atopic COPD patients. Atopy in COPD is associated with an increased incidence and prevalence of respiratory symptoms. If atopic COPD patients are treated with budesonide, they more often show remission of symptoms compared to non-atopic COPD patients who are treated with budesonide. We recommend including atopy in the diagnostic work-up and management of COPD.

Introduction

Atopy, coming from the Greek atopos, meaning “out of place”, refers to the hereditary predisposition to produce Immunoglobulin E (IgE) antibodies against common environmental allergens. This may lead to clinical expression of atopic diseases such as allergic rhinitis, asthma and atopic eczema [1]. The prevalence of atopic disorders has increased over recent decades [2] and a decrease in microbial exposure and changes in lifestyles (e.g. dietary habits, obesity, less physical activity) have been suggested to be causative factors [3].

In asthma, most patients have an atopic phenotype [1], a feature that is associated with less severe disease and better lung function [4]. Furthermore, it is well known that atopic asthmatics respond better to corticosteroids as they have an eosinophilic inflammatory pattern in the airway wall compared to non-atopic asthmatics [5,6]. Therefore, recommendations for the treatment of allergy are also included in the treatment guidelines of asthma. In contrast, in chronic obstructive pulmonary disease (COPD), a common and disabling smoking-related disease responsible for considerable morbidity and mortality worldwide [7], the international diagnostic and treatment guidelines do not incorporate recommendation for the treatment of allergy. This lack of recommendations is largely due to insufficient knowledge on the role of atopy in the pathogenesis and outcome of COPD. Nevertheless, it has been reported that around 18% of COPD patients are atopic [8,9] and that atopy is a possible risk factor for developing COPD [10-13]. Therefore, there has been a growing interest in finding the link between atopy and COPD and its consequence on the disease outcome. However, the effect of atopy on respiratory symptoms or lung function in COPD patients has not been studied yet. Understanding this issue is of clinical importance as it may help to know the prognosis and to apply appropriate medical interventions for atopic and non-atopic COPD patients. The European Respiratory Society Study on Chronic Obstructive Pulmonary Disease (EUROSCOP) has measured the atopic status in its study population. EUROSCOP is a large multicenter study performed in 39 centers in 9 European countries and has been designed to assess the effect of 3-year treatment with inhaled budesonide on lung function decline in smoking COPD patients. The results showed a small improvement in lung function after 6 months in the treated group but no differences in long-term lung function decline [8]. So far, the effect of atopy on lung function and respiratory symptoms was not evaluated in this large longitudinal study. Therefore, we assessed factors associated with the presence of atopy in this COPD population and investigated whether there is a difference between atopic and non-atopic COPD patients regarding prevalence, incidence and remission of respiratory symptoms as well as lung function decline over the 3-year follow-up of the study.

Methods

Subjects

We analysed data from the EUROSCOP study [8,9,14], included 1277 COPD patients (from nine European countries) aged 30–65 years who had failed to quit smoking during a 3-month smoking-cessation program. They were currently smoking ≥ 5 cigarettes/day, had smoked for ≥10 years or had a smoking history of ≥5 packyears. Post-bronchodilator FEV1 was between 50 and 100% of the predicted value, and the ratio of prebronchodilator FEV1 to slow vital capacity (VC) was <70%. Subjects with a history of asthma, reversible airflow limitation, any atopic diseases like allergic rhinitis, or allergic eczema and those who had used oral glucocorticoids for ≥4 weeks during the prior six months were excluded. The participants were allocated to two treatment groups in a randomised, double-blind parallel-controlled way, either receiving twice daily 400 µg budesonide (Pulmicort, Astra, Stockholm, Sweden) or placebo from a dry-powder inhaler (Turbuhaler, Astra) for a period of 3 years. Approval from regulatory and ethics committees was obtained at all centers. All subjects gave written informed consent.

Measurements

At baseline height, weight, and smoking habits were assessed. BMI (weight/height2) was divided into 3 categories: underweight (<18.5 kg/m2), normal weight (18.5–24.9 kg/m2), and overweight/obesity (≥25 kg/m2) [15]. Atopy was determined in 1163 patients by measuring specific IgE, using the Phadiatop test (Pharmacia & Upjohn, Uppsala, Sweden). Total serum IgE level was measured in 678 patients [8]. Information on respiratory symptoms was assessed at baseline and annually thereafter [8]. The symptoms analysed in the present study were: 1) cough in the morning, during the day or at night in winter, 2) phlegm in the morning, during the day or at night in winter, 3) wheezing/whistling in the chest at any time, 4) attacks of shortness of breath after activity, 5) ever trouble with breathing and 6) woken with a feeling of tightness in the chest [9].

Spirometry, using the criteria of the American Thoracic Society [16], was performed at baseline and at 3-monthly intervals using a dry rolling-seal spirometer. Post-bronchodilator FEV1 was obtained 15 min after inhalation of 1 mg terbutaline [8]. Reference values of the European Respiratory Society [17] were used to calculate FEV1% predicted.

Statistical analyses

1) Possible predictors for the presence of atopy (positive Phadiatop) were analyzed using univariate analyses at baseline. Given the fact that the prevalence of atopy differs between males and females [18-20], we stratified for gender. Differences between atopics and non-atopics were assessed in the gender strata using 2-sample Student's t test or rank-sum test (where appropriate) for continuous variables (age and packyears) and $\chi 2$ test or Fisher's exact test for categorical variables (sex and BMI). Subsequently a multivariate (unstratified) model adjusted for gender was performed using logistic regression, including all variables with a p value <0.30 in either the male or female univariate analysis. As the number of subjects with underweight was very low (Table 1), we combined underweight with normal weight in the multivariate regression analyses.

2) Differences in the prevalence of respiratory symptoms at baseline between atopics and non-atopics were analyzed using $\chi 2$ test. Multiple logistic regression adjusted for sex, age, BMI and packyears was performed for each symptom separately to investigate the association between atopy and respiratory symptoms at baseline. This analysis was performed for the total population, stratified for gender, and interactions between atopy and gender were investigated.

3) Association between atopy and changes in the presence of respiratory symptoms during the study period were analysed using generalised estimating equation (GEE) models as described by Watson et al. [9]. In brief, pairs of observations were formed between the baseline and the first 12-monthly visit (0–12 months), and between 12–24 months and 24–36 months. In the analysis on symptom incidence, only paired observations where the symptom under study was not present at the first observation of the pair were included. The symptom status at the second observation of the pair was taken as the outcome variable. For the analyses on symptom remission only paired observations where the symptom under study was present at the first observation of the pair were included. Each person could contribute one to three paired observations. For the incidence and remission of each symptom, Odds ratios (ORs) for atopy were calculated. These analyses were performed for the two treatment groups separately as ICS treatment may modify the association between atopy and symptom incidence/remission. In addition, this effect modification by treatment was investigated by entering an interaction term between atopy and treatment in the unstratified models. The analyses were further stratified by gender and were adjusted for age, BMI, atopy, packyears, and FEV1 % predicted, all measured at baseline.

4) To investigate the association between atopy and FEV1 decline over time, linear

mixed effects models were used. Since the FEV1 decline in EUROSCOP is not linear over time, two separate periods (0–6 and 6–36 months) were investigated [8]. The models were stratified for gender and treatment group and adjusted for age, packyears and height.

Table 1 Characteristics of atopic and non-atopic COPD patients in the EUROSCOP study stratified by gender

Baseline variables	Males (843 patients)			Females (320 patients)		
	Atopic	Non-atopic	p. value	Atopic	Non-atopic	p. value
	n = 181	n = 662		n = 32	n = 288	
Age, yr	53.0 (48.0–58.0)	54.0 (48.0–59.0)	0.164	51.0 (46.0–58.7)	52.0 (47.0–58.0)	0.391
Height, cm	176 (172–181)	176 (171–180)	0.403	165.5 (160.5–169.7)	165 (160–169)	0.494
Weight, kg	80.0 (72.0–88.0)	78.0 (70.0–85.0)	0.017	64.0 (58.5–73.7)	62.0 (55.0–70.0)	0.206
BMI, kg/m^2	25.3 (23.5–27.5)	24.8 (22.6–27.1)	0.016	23.8 (21.8–25.9)	22.9 (21.0–25.4)	0.205
Underweight (<18.5)	2 (1.1%)	7 (1.1%)		0 (0.0%)	17 (5.9%)	
Normal weight (18.5–24.9)	77 (42.5%)	342 (51.7%)	0.102[#]	20 (62.5%)	186 (64.6%)	0.277[#]
Overweight/Obese (≥25)	101 (55.8%)	312 (47.1%)		12 (37.5%)	84 (29.2%)	
Packyears of smoking	40.0 (29.2–55.5)	38.7 (28.5–50.0)	0.288	32.5 (26.7–36.0)	29.9 (21.3–39.0)	0.462
FEV_1, liter*	2.7 (2.4–3.3)	2.8 (2.3–3.2)	0.680	2.1 (1.6–2.4)	2.0 (1.7–2.4)	0.904
FEV_1 % pred.*	78.7 (69.4–87.0)	79.3 (68.1–89.1)	0.685	79.4 (63.6–89.2)	80.8 (70.5–88.5)	0.666
FEV_1%FVC*	63.9 (56.7–68.5)	64.4 (58.1–68.7)	0.443	66.4 (61.4–70.0)	65.5 (60.9–70.4)	0.823
Reversibility % pred.	2.9 (0.8–5.2)	2.8 (0.0–5.4)	0.932	3.2 (0.3–7.6)	2.9 (0.0–5.5)	0.323
Total IgE, kU/l**	248.5 (84.0–617.2)	37.0 (15.0–82.0)	<0.0001	161.0 (25.5–1373.0)	28.0 (13.0–75.0)	0.002

Data are presented as median (interquartile range) or number (%). Bold p-values lower than 0.05 indicate significant differences between atopic and non-atopic patients within males or females.
** Respiratory function tests were performed after inhalation of 1 mg terbutaline.*
*** Available in 678 patients.*
 # P.value refers to Chi-square analysis between classes of BMI and atopy.

Results

Baseline characteristics

The baseline characteristics of the atopic and nonatopic males and females are shown in Table 1. In total, 213 (18.3%) patients were atopic. Atopy was more prevalent in males than females [21.5% and 10% respectively, $p < 0.001$]. Atopic males had a higher weight ($p = 0.017$) and BMI ($p = 0.016$) than non-atopic males. Atopic patients had significantly higher total serum IgE (kU/l) levels than non-atopic patients ($p < 0.0001$ in males and $p = 0.002$ in females). There were no significant differences in age and lung function parameters at baseline between atopic and non-atopic patients.

Factors associated with the presence of atopy

Multiple logistic regression analysis showed that male gender (OR: 2.21; 95% CI: 1.47–3.34), overweight/obese (OR: 1.41; 95% CI: 1.04–1.92) and lower age (OR: 0.98; 95% CI: 0.96–0.99) were independently associated with the presence of atopy. There was no significant association between the number of packyears (OR: 1.007; 95% CI: 0.99–1.01) and the presence of atopy.

Atopy and respiratory symptoms

Atopic patients had a higher prevalence of cough ($p = 0.02$) and phlegm ($p = 0.08$) than non-atopic patients (Table 2). After stratifying by gender, a higher prevalence of cough ($p < 0.0001$) and phlegm ($p = 0.008$) was found in atopic males than non-atopic males, without a significant difference in females (Table 2). Woken with chest tightness was more prevalent in atopic females than non-atopic females ($p = 0.042$) (Table 2). In the multiple logistic regression model adjusted for confounders, atopy was associated with a higher prevalence of cough (OR: 1.71; 95% CI: 1.26–2.34) and phlegm production (OR: 1.50; 95% CI: 1.10–2.03) in the total population, and with woken with chest tightness in females only (OR females: 2.69; 95% CI: 1.11–6.55, OR male: 0.84; 95% CI: 0.47–1.49, female vs male: OR: 3.21; 95% CI: 1.12–9.25).

Atopy and incidence and remission of symptoms

The association between atopy and incidence and remission of symptoms during the 3 years of the study, stratified by treatment group and gender is shown in Tables 3 and 4 respectively. In the placebo group, atopy was significantly associated with an increased incidence of cough (OR: 1.79, 95% CI: 1.03–3.08, $p = 0.038$). Atopy was not significantly associated with the incidence of the other symptoms.

Analyses on remission of symptoms showed that remission of cough was higher in atopic than non-atopic patients receiving budesonide (OR: 1.93, 95% CI: 1.11–3.37, $p = 0.02$). After stratifying by gender, remission of cough was higher in atopic males than non-atopic males receiving budesonide (OR: 1.94, 95% CI: 1.05–3.57, $p = 0.034$) as was trouble with breathing (OR: 2.76, 95% CI: 1.45–5.26, $p = 0.002$), but differences were not present in female subjects receiving budesonide. In contrast, atopic females receiving placebo, had increased remission of woken with chest tightness (OR: 5.76, 95% CI: 1.67–19.86, $p = 0.006$) than non-atopic females receiving budesonide.

The incidence and remission of cough and phlegm production in two treatment groups are

shown in Figure 1.

Atopy and lung function decline

There was no significant difference in changes of postbronchodilator FEV1 between atopic and non-atopic patients neither in males nor in females, a finding that was true for both placebo and budesonide treated groups during month 0 to 6 (Figure 2). From 6 to 36 months, atopic females who received placebo showed a smaller decline in FEV1 compared to the non-atopic females in the placebo group (p = 0.008, Figure 2).

Discussion

Our study shows that male gender, overweight/obesity and lower age are independently associated with the presence of atopy in COPD. Moreover, atopic patients showed a higher prevalence of respiratory symptoms than non-atopics. Interestingly, atopic patients without ICS treatment more frequently developed respiratory symptoms than non-atopics, while atopic patients on treatment with ICS showed increased remission of respiratory symptoms compared to non-atopic patients.

We found that the prevalence of atopy is two times higher in males than females both in univariate and multivariate analyses. This confirms previous findings in the literature. Sears et al. found that boys (age of 13 years) had a higher prevalence of positive skin tests and a higher response to house dust mite and cat than girls with the same age [21]. With increasing age, a significant decrease in male/female ratio of sensitization was described after the age of 8 years although a male predominance persists [18] also in older men [19,20]. This can be explained by a population study in adults which showed that atopy significantly decreased after menopause in both asthmatic and nonasthmatic women, suggesting that the pathophysiology of atopy changes over the lifespan depends on the hormonal pattern [19]. We corroborate these findings by showing a male preponderance of atopy in COPD.

Younger age was also associated with the presence of atopy in our COPD patients. This finding is in line with results from studies in the general population showing that allergen sensitivity and the incidence of atopic disorders decreases with age [22-25].

Another interesting finding in our study was that overweight/obesity was associated with the presence of atopy in COPD patients. The previous studies in the general population also showed a significant association between overweight/obesity and atopy in adolescents [26] and in adults [24]. In asthma, it has been suggested that the systemic inflammatory effects of obesity itself may enhance eosinophilic airway inflammation [27]. We do not know whether

this is also true for COPD and the atopy-overweight/obesity relationship in COPD has to be fur ther explored.

Table 2 Respiratory symptoms in atopic and non-atopic COPD patients at baseline stratified by gender

Respiratory symptoms	Total population		Males		Females	
	1163 patients		843 patients		320 patients	
	Atopic	Non-atopic	Atopic	Non-atopic	Atopic	Non-atopic
	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
Subjects number	213 (18.3)	950 (81.7)	181 (21.5)	662 (78.5)	32 (10)	288 (90)
Wheezing at anytime	111 (52.1)	527 (55.5)	95 (52.5)	353 (53.3)	16 (50)	174 (60.4)
Cough day/night or a.m.	155 (72.8)*	609 (64.1)	110 (60.8)*	291 (44.0)	18 (56.2)	170 (59.0)
Phlegm day/night or a.m.	127 (59.6)#	498 (52.4)	87 (48.1)*	244 (36.9)	12 (37.5)	101 (35.1)
Trouble with breathing	104 (48.8)	443 (46.7)	85 (47.0)	287 (43.4)	19 (59.4)	156 (54.2)
Woken with chest tightness	24 (11.3)	102 (10.7)	16 (8.8)	68 (10.3)	8 (25.0)*	34 (11.8)
Attack of dyspnea after activity	82 (38.5)	350 (36.8)	68 (48.1)	234 (35.3)	14 (43.8)	116 (40.3)

**Significant difference between atopic and non-atopic patients at $p < 0.05$. #: $p = 0.08$.*

Table 3 Association between atopy and incidence of respiratory symptoms stratified by the treatment group and gender

Respiratory symptoms	Total population		Males		Females	
	OR (95% CI) of atopy		OR (95% CI) of atopy		OR (95% CI) of atopy	
	Placebo	Budesonide	Placebo	Budesonide	Placebo	Budesonide
Wheezing at any time	1.15 (0.71–1.87)	0.79 (0.45–1.43)	1.24 (0.74–2.08)	0.92 (0.48–1.75)	0.64 (0.15–2.78)	0.43 (0.10–1.85)
Cough day/night or a.m.	1.79 (1.03–3.08)*	0.83 (0.42–1.62)	1.69 (0.93–3.08)#	0.84 (0.38–1.84)	2.52 (0.64–9.86)	0.60 (0.17–2.08)
Phlegm day/night or a.m.	1.50 (0.84–2.69)	0.91 (0.54–1.53)	1.55 (0.82–2.93)	0.85 (0.46–1.59)	1.30 (0.36–4.67)	0.92 (0.38–2.27)
Trouble with breathing	1.12 (0.66–1.89)	0.68 (0.37–1.24)	1.09 (0.59–1.99)	0.79 (0.42–1.51)	1.23 (0.44–3.44)	0.22 (0.4–1.13)#
Woken with chest tightness	1.33 (0.74–2.38)	1.07 (0.57–1.99)	1.35 (0.69–2.65)	1.08 (0.55–2.12)	1.08 (0.27–4.34)	1.11 (0.23–5.39)
Attack of dyspnea after activity	0.98 (0.59–1.62)	1.49 (0.88–2.51)	0.76 (0.43–1.33)	1.55 (0.88–2.73)$	2.79 (0.94–8.31)#	0.89 (0.22–3.62)

** OR is significant at $p < 0.05$. Each model was adjusted for sex, age, BMI, pack years of smoking, number of cigarettes and FEV_1 % pred. #Trend (0.05 < p. value < 0.1).*
$Interaction between Phadiatop and treatment group has a p.value < 0.05.

With respect to respiratory symptoms, our study revealed a higher prevalence of cough and phlegm in atopic COPD patients compared to non-atopic COPD patients indicating that atopy (i.e. positive phadiatop) contributes importantly to symptoms in COPD. The association

between atopy and a higher prevalence of respiratory symptoms was also found in the general population, as various respiratory symptoms have been associated with positive skin test reactivity [10,28] and eosinophilia [28,29]. But in COPD, according to our knowledge, there is no published paper showing an association between atopy and respiratory outcomes. One recent ATS abstract [30] is in line with our findings, investigating 1424 COPD patients from "The National Health and Nutrition Examination Survey (NHANES)" III (1988–1994). The investigators defined allergic/atopic COPD subjects (n = 346) as the presence of any one of the following criteria: at least one positive skin prick test, self-reported doctor diagnosed hay fever, or symptoms induced by house dust, animals or pollen. They found that individuals with indications of allergic disease more likely reported having episodes of sinusitis, and an additional trend towards more frequent reporting of cough and wheeze [30] compared to non-allergic individuals. Our study defined atopy objectively by specific IgE positivity and excluded subjects with a history of asthma, allergic rhinitis, or allergic eczema. As we excluded subjects with allergic diseases, we believe our data more closely reflects the effect of atopy on COPD-related cough. Regarding the importance of cough and phlegm, it should be noted that these symptoms are highly prevalent in COPD patients and have been reported to predict disease progression, exacerbations and hospitalizations [31]. It has been argued that these symptoms can constitute a sign of inflammation and may identify patients at higher risk of clinical worsening [31]. Thus, our finding that atopy associates with this clinical phenotype may have important consequences for future studies on intervention in this phenotype with an important clinical impact on COPD, as shown in our study.

Table 4 Association between atopy and remission of respiratory symptoms classified by the treatment group and gender

Respiratory symptom	Total population		Males		Females	
	OR (95% CI) of phadiatop		OR (95% CI) of phadiatop		OR (95% CI) of phadiatop	
	Placebo	Budesonide	Placebo	Budesonide	Placebo	Budesonide
Wheezing at any time	0.99 (0.59–1.67)	1.18 (0.70–1.99)	0.88 (0.48–1.60)	1.26 (0.70–2.26)	2.06 (0.55–7.78)	0.51 (0.15–1.71)
Cough day/night or in a.m.	0.85 (0.53–1.36)	1.93 (1.11–3.37)*,$	0.87 (0.52–1.45)	1.94 (1.05–3.57)*	0.79 (0.26–2.41)	1.84 (0.47–7.30)
Phlegm in day/night or in a.m.	1.21 (0.72–2.03)	1.67 (0.99–2.82)#	1.14 (0.65–2.00)	1.63 (0.93–2.87)#	1.48 (0.34–6.45)	1.53 (0.28–8.26)
Trouble with breathing	0.84 (0.48–1.47)	1.76 (0.99–3.11)#	0.96 (0.50–1.82)	2.76 (1.45–5.26)*,$	0.47 (0.18–1.21)	0.43 (0.17–1.12)#
Woken with chest tightness	2.18 (0.97–4.90)#	2.32 (0.60–8.91)	2.17 (0.67–6.99)	1.33 (0.22–7.85)	5.76 (1.67–19.86)*	8.82 (0.63–123.66)
Attack of dyspnea after activity	1.01 (0.52–1.95)	1.11 (0.63–1.96)	1.04 (0.49–2.20)	0.87 (0.46–1.64)	1.03 (0.29–3.63)	3.67 (0.68–19.70)

**OR is significant at $p < 0.05$. Each model was adjusted for sex, age, BMI, pack years of smoking, number of cigarette and FEV_1 % pred. #Trend (0.05 < p. value < 0.1). $Interaction between Phadiatop and treatment group has a p.value < 0.05.*

Our study did not show a significant difference in lung function parameters between atopic and non-atopic patients, with the exception of FEV1 decline. Of interest, atopic female COPD patients not using ICS treatment demonstrated a slower decline in lung function than non-atopic females. Additionally, if atopic females used ICS this protective effect of atopy was no longer present. In established COPD, to our knowledge, such an effect of atopy has never been investigated. We do not have a clear explanation for the latter finding, but as this observation is not present in male subjects, we speculate that hormonal-related effects on the immune system play a role. However, the number of atopic females in our study was low (n = 32); so firm conclusions cannot be d It has been shown that atopy is associated with a lower level of lung function [32,33] and FEV1 decline [34] in the general population and also FEV1 decline in healthy former and current smokers [35]. We conclude that, unlike in healthy subjects, atopy is not associated with accelerated decline in lung function in established COPD. It may well be that the effects of atopy are overshadowed by the effects of smoking in our COPD population.

Our study showed that in atopic COPD patients the use of budesonide is associated with higher remission rates of cough and phlegm, whereas placebo is associated with higher incidence rates. This is an important finding as cough and phlegm predict disease progression, exacerbations and hospitalizations [31]. Although this beneficial effect of budesonide may not be specific for atopic COPD and may be present in every atopic subject, the question rises whether we should treat all atopic COPD patients with an ICS (as EUROSCOP included only steroid-naïve patients). Indeed, already in 1978, Sahn suggested that atopic COPD patients are the ones who benefit most from corticosteroid treatment [36]. If we accept that atopic COPD patients from now on should be treated with ICS, this would widen the present indications for ICS as defined by GOLD (Global Initiative for Chronic Obstructive Lung Disease) [37]. At this moment GOLD recommends ICS use for symptomatic patients with an FEV1 < 50% predicted (stage III, severe COPD, and stage IV, very severe COPD) and repeated exacerbations [37,38]. However, before considering to add atopic status as a guideline for ICS treatment in COPD, more studies are needed confirming that atopy is a risk factor for worse COPD outcome.

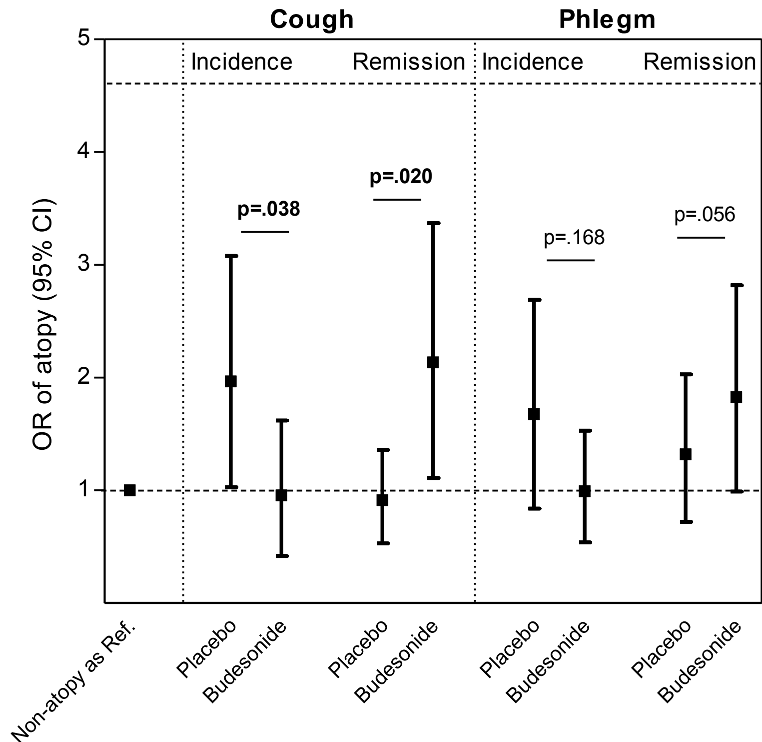

Figure 1 Effect of atopy on incidence and remission of cough and phlegm in the treatment groups. *Logistic regression with adjustment for age, BMI, packyears, and FEV1 % predicted.*

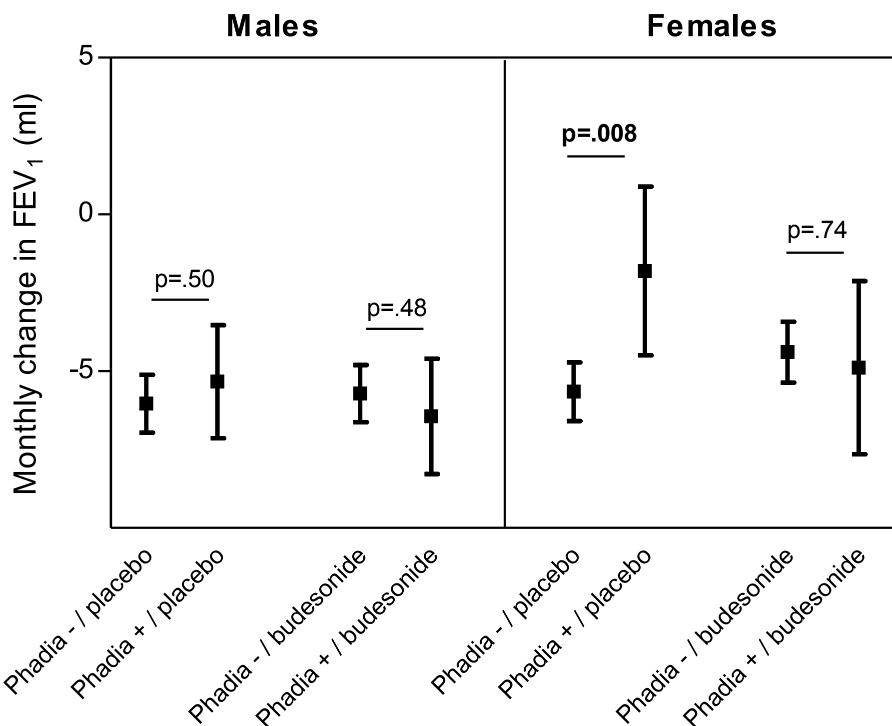

Figure 2 Monthly change of FEV1 from 6 months to 36 months after the start of the study medication. *Linear mixed effect models with adjustment for age, BMI, atopy, packyears, and FEV1 % predicted.*

Conclusion

We conclude that atopy is present in COPD patients and that the prevalence of atopy is higher in males, subjects with overweight/obesity and younger patients. Importantly, atopy in COPD is associated with a higher prevalence and incidence of respiratory symptoms, while when being treated with ICS, the patients have higher remission rates. However, atopy in COPD is not associated with accelerated but rather decelerated FEV1 decline in females. Our results clearly indicate that the atopic status should not be forgotten in the routine work-up of COPD. However, whether every atopic COPD patient should be treated with an ICS needs to be confirmed in future studies.

Authors' contributions

JMV had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis and contributed to the revision of the manuscript. FF: contributed to the data analysis, interpreting of the results and manuscript writing. NHTtH: contributed to the data analysis, interpreting of the results and manuscript writing. C-GL: contributed to the original study design and conception, acquisition of the data, and revision of the manuscript. MNH and WT: contributed to interpreting of the results and revision of the manuscript.

DSP: contributed to the original study design and conception, acquisition of the data, and revision of the manuscript. Other contributions: The data analysed in this study were collected in the EUROSCOP-study. We thank all principle investigators and participants of the EUROSCOP study. All authors read and approved the final manuscript.

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Chapter 5

Atopy and Inhaled Corticosteroid Use Associate with Fewer IL-17+ Cells in Asthmatic Airways.

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Abstract

Background: Interleukin (IL)-17 plays a critical role in numerous immune and inflammatory responses and was recently suggested to contribute to the pathogenesis of nonatopic (non-eosinophil/ neutrophil-dominant) asthma. We aimed to compare expression of IL-17 in bronchial airways between atopic and nonatopic asthmatics, with/without inhaled corticosteroid (ICS) use and to identify its major cellular source.

Methods: Bronchial biopsies from 114 patients with mild-to-moderate asthma were investigated: 33 nonatopic, 63 non-corticosteroid users, 90 nonsmokers. IL-17 expression was correlated with atopy and inflammatory cell counts (EPX, NP57, CD3, CD4, CD8, CD20, CD68), taking ICS use and smoking into account. Multiple linear regression analyses were used to determine the independent factors as well as the most relevant inflammatory cells contributing to IL-17 expression. Double immunostainings were performed to confirm the major cellular source of IL-17.

Results: In non-ICS users, nonatopic asthmatics had more IL-17+ cells in the airway wall than atopic asthmatics. In both atopic and nonatopic asthmatics, ICS use was associated with lower numbers of IL-17+ cells, independent of smoking. The number of IL-17+ cells was associated with the number of neutrophils (B: 0.26, 95% CI: 0.17–0.35) and eosinophils (B: 0.18, 95% CI: 0.07–0.29). The majority of IL-17+ cells were neutrophils, as confirmed by double immunostaining.

Conclusions: We show for the first time that atopy and ICS use are associated with lower numbers of IL17+ cells in asthmatic airways. Importantly, IL-17+ cells were mostly neutrophils which conflicts with the paradigm that lymphocytes (Th17) are the main source of IL-17.

Introduction

Asthma is a chronic inflammatory disease of the airways, characterized by reversible airway obstruction and bronchial hyperresponsiveness (BHR) [1]. One of the oldest ways to discern asthmatic patients is based on the presence or absence of atopy [2]. Not surprisingly, the underlying airway pathology of atopic versus nonatopic asthma is different, showing high numbers of eosinophils, T lymphocytes and Th2 cytokines (interleukin (IL)4 and IL-5) in atopic asthma versus high numbers of neutrophils and non-Th2 cytokines (IL-8) in nonatopic asthma [3]. One of the cytokines that was recently suggested to contribute to the pathogenesis of nonatopic (non-eosinophil/neutrophil-dominant) asthma is IL-17 [4].

IL-17, also called IL-17A, is a proinflammatory cytokine, implicated in the development of autoimmunity, tumorigenesis and host defenses against bacterial and fungal infections [5]. In the lung, increased levels of IL-17 have been demonstrated in inflammatory disorders like asthma and chronic obstructive pulmonary disease (COPD) [6–10]. IL-17 was first shown to be produced by activated CD4+ memory T cells [11]. Thereafter, a specific subset of Th cells, namely the Th17 cells, has been put forward as its main producer [12, 13]. Th17 cells have been shown to mediate airway inflammation and hyperresponsiveness associated with non-eosinophilic asthma in mice, and importantly do not respond well to glucocorticoid treatment [14]. In humans, Th17 cells have also been suggested to play a role in regulating a neutrophil and macrophage dominant type of inflammation in the lung, particularly in severe, steroid-insensitive asthma and COPD [6]. In line with this, IL-17 levels were found to correlate positively with sputum neutrophilia in severe asthma [7, 15].

On the other hand, IL-17 has also been implicated in Th2 responses. In mouse models of asthma, Th17 cells were shown to home to the lung and enhance not only neutrophilic airway inflammation but also Th2 cell-mediated eosinophilic airway inflammation [16]. And in patients with allergic asthma increased levels of IL-17 were demonstrated after a challenge with house dust mite [17].

Although there has been substantial interest in elucidating the role of IL-17 in neutrophil-dominant/nonatopic asthma in humans [4, 18], our understanding regarding this phenotype of asthma is still very limited. Although recent studies suggest that a higher level of IL-17 expression is associated with severe asthma, the atopic status was not included in their analysis [19–22]. In fact, there is no data comparing IL-17 expression between atopic and nonatopic asthma patients. We therefore investigated the expression of IL-17 in bronchial biopsies from a large cohort of well characterized atopic and nonatopic asthmatic patients, also taking into account the effect of inhaled corticosteroid (ICS) and smoking.

Additionally, we identified the major cellular source of IL-17 in the airway walls of these asthma patients.

Materials and Methods

Subjects

We investigated 114 stable, mild-to-moderate subjects with current asthma from our large asthma cohorts that were recruited previously by our research group in the University Medical Center Groningen [23]. Atopic and nonatopic patients, with or without ICS use, aged between 19–71 years were included (Table 1). All patients had a doctor's diagnosis of asthma and demonstrated reversibility and BHR to histamine and/or adenosine 5'-monophosphate (AMP) [23]. All patients also had alveolar and bronchial exhaled nitric oxide (NO) values on the Aerocrine NO system (Niox; Aerocrine AB, Stockholm, Sweden) measured in accordance with international guidelines as described in an earlier study [23]. Atopic status was determined by Phadiatop for all 114 patients using the ImmunoCap system (Phadia AB, Uppsala, Sweden), and expressed as ratios (fluorescence of the serum of interest divided by the fluorescence of a control serum). A positive Phadiatop was defined as patient serum/control serum >1. The Medical Ethics Committee of the University Medical Center Groningen approved the study protocol and all subjects gave written informed consent.

Immunohistochemical staining and cellular quantification of bronchial biopsies

Paraffin embedded bronchial biopsies were cut into 3-μm-thick sections. Sections were deparaffinized and, after antigen retrieval, incubated with appropriate polyclonal antibodies against IL-17 (R&D Systems, polyclonal Goat anti-Human, AF-317-NA), using the DAKO autostainer in three consecutive runs. The slides were included in random fashion in each run to avoid group wise staining [19]. The number of positive cells was counted by a blinded observer in the submucosal area 100 μm under the basement membrane in the biopsy sample (19) using Aperio Image Scope software. The same techniques had been already applied for immunohistochemical staining and cellular quantification of other inflammatory cells including: neutrophils (NP57, DAKO, Glostrup, Denmark), eosinophils (eosinophil peroxidase; EPX, laboratories of NA Lee and JJ Lee, Mayo Clinic, Scottsdale, AZ), macrophages (CD68, DAKO, Glostrup, Denmark), mast cells (AA1, DAKO, Glostrup, Denmark) and T-cells (CD3, CD4, CD8, DAKO, Glostrup, Denmark)[23].

Double immunostainings were performed to elucidate whether granulocytes are a source of IL-17 in bronchial biopsies of asthmatics. Primary neutrophil and eosinophil antibodies suit-

able for double staining with IL-17 were used; a polyclonal Rabbit anti-Human Myeloperoxidase (MPO) antibody (DAKO, Glostrup, Denmark) was used to identify neutrophils and a Mouse anti-Human EPX antibody (Mayo Clinic, Scottsdale, AZ, USA) to identify eosinophils. After deparaffinizing the slides, a heat-induced antigen (epitope) retrieval protocol was used and blocking for endogenous peroxidase was applied. As secondary antibodies, peroxidase conjugated Swine anti-Rabbit IgG Antibody (DAKO, Glostrup, Denmark) was used for detecting MPO stained cells, biotinylated labeled Rabbit anti-Mouse antibody (DAKO, Glostrup, Denmark) for detecting EPX stained cells and Alkaline Phosphatase conjugated Donkey anti-Goat IgG antibody (SouthernBiotech, USA) for detecting IL-17 stained cells. Double immunostaining with lymphocytes was unnecessary because the vast majority of the IL-17+ cells showed the morphology of granulocytes. This was confirmed by the MPO/IL17 and EPX/IL17 double immunostainings.

Statistics

All analyses were performed using SPSS software (version 19.0; SPSS Inc., Chicago, IL). Normality of distributions was assessed using histograms and/or p-p plots.

For quantitative variables analysis, one-way ANOVA followed by Tukey post-hoc test was performed for multiple comparisons and t tests or Mann-Whitney U tests was used for two samples comparison.

Chi-square tests were used to compare groups for dichotomous variables. Correlations were evaluated by Pearson (for normally distributed data) or Spearman (for non-normally distributed data) tests. Multiple linear regression analysis was used to assess the independent contribution of ICS use (yes/no), smoking (smoking vs. nonsmoking) and Phadiatop (atopic vs. nonatopic) to IL-17 expression (dependent variable). To find the most relevant inflammatory cells contributing to IL-17 expression, additional linear regressions were performed on inflammatory markers (neutrophil, eosinophil, T-cell, macrophage, mast cell) as independent variables (separately for each one or in combination with other inflammatory cells) and IL-17 as dependent variable, adjusting for atopy, smoking status and ICS use. For all statistical analyses, p values <0.05 were considered statistically significant.

Table 1. Patient characteristics

	ICS user		Non-ICS user	
	Atopic (n = 39)	*Nonatopic (n = 12)*	*Atopic (n = 42)*	*Nonatopic (n = 21)*
Sex, males/females	19/20	6/6	26/16	10/11
Age, Years	50 (22–68)	50 (30–71)	48 (22–70)	50 (21–65)
BMI, Kg/m²	25.6 (19–39)	26.3 (19.5–44.2)	27.2 (19.3–40.4)	27.2 (21.4–42.4)
Smoking, yes/no	6/33	1/11	12/30	5/16
Age of asthma onset	8 (1–55)	21 (1–40)	7 (1–48)	14.5 (3–22)
Asthma duration	44 (3–58)	22 (5–44)	42 (4–60)	35.5 (1–57)
ICS dose, μg/day	800 (28–2000)	1000 (400–2000)	NA	NA
High doses of ICS (>1000μg/day)	9 (23.1%)	5 (41.7%)	NA	NA
FEV_1, % pred	95.6 (42.5–121.3)	104.5 (52.1–135.3)	97.9 (59.8–122.0)	105.4 (84.0–122.7)$^{\#}$
FEV_1/VC, %	71.5 (39.9–96.7)	73.3 (39.4–94.1)	71.7 (47.6–97.7)	80.0 (67.0–93.6)*
AMP PC20, mg/ml	51.5 (0.0–640)	640 (0.01–640)	83.9 (0.02–640)	640 (0.08–640)$^{\#}$
Reversibility, % pred	8.7 (-0.8–38.4)	7.3 (0.73–20.0)	9.1 (-0.24–28.7)	6.2 (-1.4–17.4)
Total IgE (IU/ml)	146 (5–1668)	78 (17–359)	94 (9–1302)	36.5 (1–295)*
Specific IgE (PAU/l)	24.4 (1.7–128)	0.21 (0.15–0.92)*	22.5 (1.2–106)	0.26 (0.06–0.76)*
NO Bronchial (nl/s)	0.89 (0.2–10.4)	0.3 (0.09–2.5)*	0.8 (0.06–3.2)	0.5 (0.09–1.1)
NO Alveolar (ppb)	6.1 (1.5–51.7)	4.3 (1.9–11.9)	5.9 (2.08–18.3)	3.8 (0.9–8.2)*

Definition of abbreviations: ICS: inhaled corticosteroid (beclomethasone equivalent); FEV_1: forced expiratory volume in one second, measured after inhalation of 800 μg albuterol; VC: vital capacity; MEF_{50}: maximum expiratory flow rate at 50% of vital capacity; AMP PC_{20}: provocative concentration of adenosine 5'-monophosphate causing a 20% fall in FEV_1, NA: not applicable; All values was obtained 15 min after inhalation of 1 mg terbutaline. Reversibility: change in FEV_1, expressed as increase in percentage predicted normal value after 400 μg of albuterol. Normal reversibility was defined a greater than 12% and 200 ml increase in FEV_1 following inhalation of the bronchodilator (Global Initiative for Asthma. Global Strategy for Asthma Management and Prevention. 2014.
Available from: http://www.ginaasthma.com). PAU/l: Phadia Arbitrary Units per litre; NO: nitric oxide Values are number (no.) or medians with minimum-maximum ranges in parentheses.
**p<0.05 vs atopic (in ICS or non-ICS user);*
#trend: 0.05<p<0.10 vs atopic (in ICS or non-ICS user).

Results

Inflammatory cells count in blood, sputum and bronchial biopsies

In the group of asthma patients who did not use ICS, there were trends towards lower blood eosinophil counts (p = 0.08) and lower percentage of sputum eosinophils (p = 0.06) in nonatopic asthma patients compared to atopic patients (Table 2). In addition, nonatopic patients had more neutrophils in the bronchial submucosa than atopic patients, whereas the atopic asthmatics had more eosinophils (Table 2). In the group of patients who did use ICS, the nonatopic individuals had more CD8+ cells in the bronchial submucosa than the atopic ones (Table 2).

Table 2. Inflammation in atopic and nonatopic asthmatics

	ICS user		Non-ICS user	
	Atopic (n = 39)	*Nonatopic (n = 12)*	*Atopic (n = 42)*	*Nonatopic (n = 21)*
Blood				
Eosinophil, $\times 10^9$/L	0.21 (0.04–0.97)	0.15 (0.07–0.78)	0.18 (0.02–0.5)	0.12 (0.03–0.46)$^{\#}$
Sputum				
Total cells, $\times 10^6$/ml	0.5 (0.1–5.2)	0.5 (0.15–1.8)	0.3 (0.3–2.1)	0.4 (0.1–2.2)
Eosinophil, %	1.2 (0–67.1)	0.37 (0–38.6)	1.02 (0–16.7)	0.2 (0–5.2)$^{\#}$
Neutrophil, %	55.8 (16.8–88.8)	53.4 (43.7–88.6)	59.6 (19.8–93.6)	57 (20.7–94.5)
Bronchial biopsies (/0.1mm^2)				
Eosinophils (EPX$^+$)	1.1 (0–32.4)	1.8 (0–23.3)	2.6 (0–40.3)	0.3 (0–19.1)*
Neutrophils (NP57$^+$)	4.8 (0–33.8)	6.2 (0.9–16.2)	5.8 (0–38.2)	10.4 (0–46)*
Mast cells (AA1$^+$)	8 (0–22.2)	8.3 (0–16.7)	8.1 (0–26.3)	8.7 (0–24.4)
Macrophages (CD68$^+$)	11.8 (0–37.1)	19.5 (4.3–57)	11.6 (0.31–30.1)	13.2 (0–36.5)
T lymphocytes (CD3$^+$)	65 (4.2–219)	81.1 (29.7–216)	77.3 (12.5–294)	61 (18–136.7)
T lymphocytes (CD4$^+$)	14.3 (0–101.2)	27.4 (4.3–57.6)	22 (0–67.2)	18.5 (0.85–58.2)
T lymphocytes (CD8$^+$)	17.7 (0.98–205.2)	38.3 (0–78.4)*	21.1 (1.02–112.3)	15.9 (3–139)
B lymphocytes (CD20$^+$)	2.9 (0–37.1)	6.05 (0.9–55.5)$^{\#}$	1.9 (0–98.3)	2.6 (0–20.5)
IL-17$^+$ cells	6.2 (1.2–18.4)	8.5 (4.6–16.9)	11.5 (3.9–29.6)	15.3 (9.7–24.9)*

Values are medians with minimum-maximum ranges in parentheses.
**p<0.05 vs atopic (in ICS or non-ICS user);*
$^{\#}$trend: 0.05<p<0.10 vs atopic (in ICS or non-ICS user).

Lower IL-17 expression in bronchial biopsies associated with atopy and ICS use

In the group of non-ICS users, there were significantly more IL-17+ cells in the bronchial submucosa of nonatopic asthmatics compared to atopic ones (Fig 1). In line with this finding, a negative correlation was found between IL-17+ cells numbers and the Phadiatop score (rs = -0.37, p<0.001) (Fig 2).

Both atopic and nonatopic asthma patients treated with ICS had lower numbers of IL-17+ cells than those without ICS treatment (Fig 1). There were 9 subjects in the atopic group and 5 subjects in the nonatopic group who used high doses of ICS (>1000ug daily) (Table 1). There

was no association between doses of ICS (high doses vs. mild-moderate doses) and cellular infiltrate.

There was a significant negative correlation between IL-17+ cells numbers and reversibility levels in the total population who did not use ICS (both atopic and nonatopic subjects) (rs = -0.33; p = 0.01) (S1A Fig) in line with a negative correlation between neutrophils levels and reversibility levels in the total population who used ICS (both atopic and nonatopic subjects) (rs = -0.27; p = 0.04). There was also a negative correlation between FEV1% predicted and IL17 + cells numbers in the atopic individuals who did not use ICS (rs = -0.39; p = 0.01)(S1B Fig).

There was no association between current smoking and IL-17 levels (S2 Fig) and current smoking had no effect on IL-17 counts in all groups of asthmatics.

Using regression analyses, we demonstrated that the absence of atopy (B: -2.42, 95% CI: -4.16-0.69) and non-ICS use (B: -4.29, 95% CI: -5.85-2.74) most strongly contributed to thenumber of IL-17+ cells. There was no significant contribution of smoking status (defined as smoking and nonsmoking) (B: 0.17, 95% CI: -1.78–2.14).

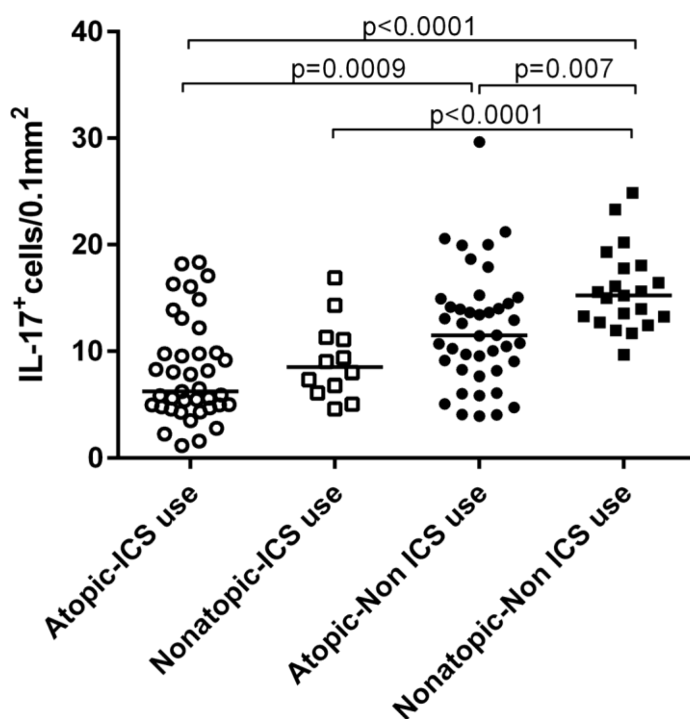


Figure 1. *Number of IL-17$^+$ cells in submucosa in bronchial biopsies from atopic and nonatopic asthmatics who are inhaled corticosteroid (ICS) users or non-ICS users.*

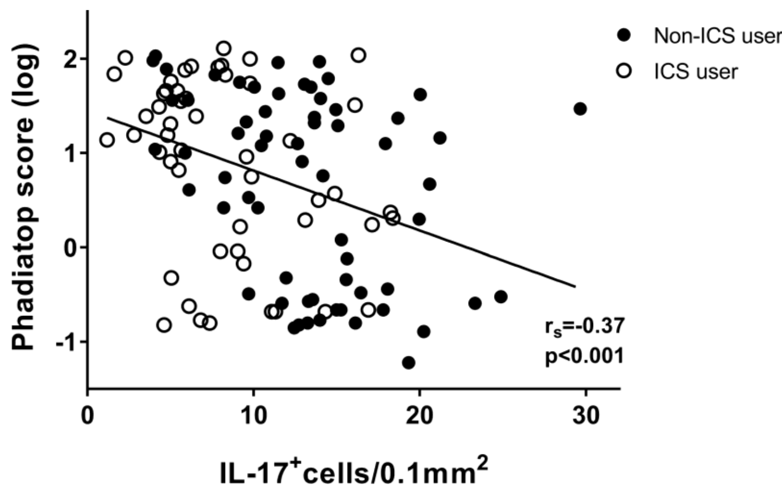


Figure 2. *Negative correlation between the number of IL-17*$^+$ *cells in the submucosa of bronchial biopsies and serum specific IgE (Phadiatop) from asthmatics (rs = -0.37; P<0.001).*

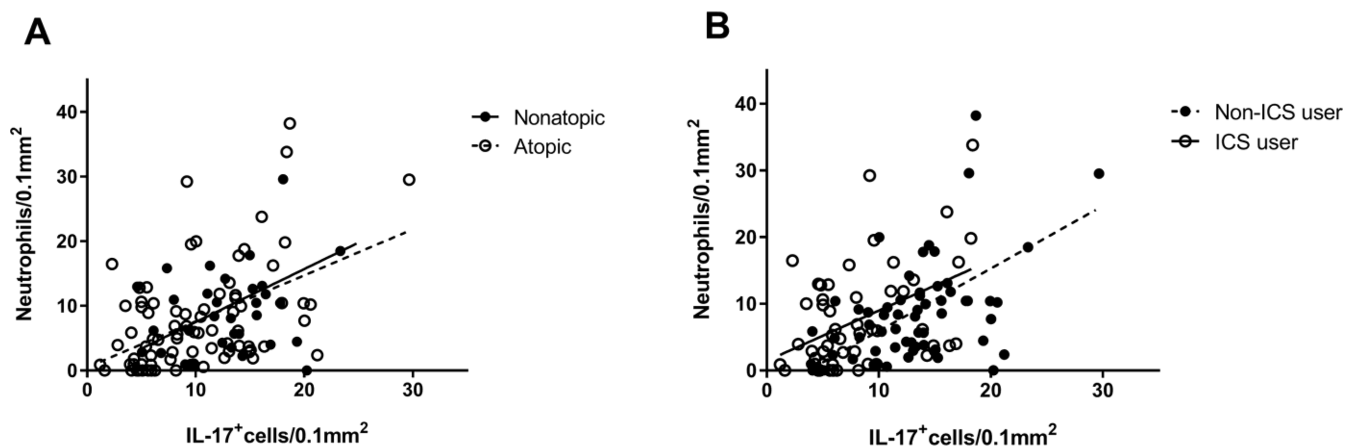


Figure 3. *Positive correlation between the number of IL-17*$^+$ *cells and neutrophils in the submucosa of bronchial biopsies from atopic* ($r_s = 0.44; p<0.001$) *and nonatopic* ($r_s = 0.45$, $p = 0.009$) *asthmatics (A), or from asthmatics who are inhaled corticosteroid (ICS)* ($r_s = 0.35$; $p = 0.01$) *and non-ICS* ($r_s = 0.48; p<0.0001$) *users (B).*

IL-17 expression positively associated with neutrophilic inflammation

The number of IL-17+ cells in airway wall biopsies correlated significantly with the number of neutrophils, both in atopic (rs = 0.44; p<0.001) and nonatopic asthmatics (rs = 0.45; p = 0.009) (Fig 3A), and both in ICS users (rs = 0.35; p = 0.01) and non-ICS users ((rs = 0.48; p<0.0001) (Fig 3B). Additionally, in atopic asthmatics the number of IL-17+ cells correlated significantly with the number of eosinophils (rs = 0.36; p = 0.001), CD4+ cells (rs = 0.33; p = 0.003), CD3+ cells (rs = 0.31; p = 0.005), and CD8+ cells (rs = 0.27; p = 0.015).

Using regression analysis and after adjusting for atopy, ICS use and smoking, we confirmed the contribution of neutrophils (B: 0.26, 95% CI: 0.17–0.35) as well as eosinophils, with lower value (B: 0.18, 95% CI: 0.07–0.29) to the number of IL-17+ cells. Other inflammatory cells did not contribute significantly to IL-17 expression (data not shown).

We found that the majority (~90%) of IL-17+ cells were granulocytes, mostly neutrophils, as indicated by double staining for IL-17 and MPO and nuclear morphology (Fig 4). In addition, we identified a few IL-17+ eosinophils, as indicated by double staining for IL-17 and EPX. Fig 4 shows one representative IHC staining for IL-17 for all 4 studied subgroups (frame A-D) and double staining of IL-17 and MPO (frame E-G) and staining of IL-17 and EPX (frame H-J) from an asthmatic patient with high numbers of neutrophils (frame E-G) or eosinophils (frame H-J).

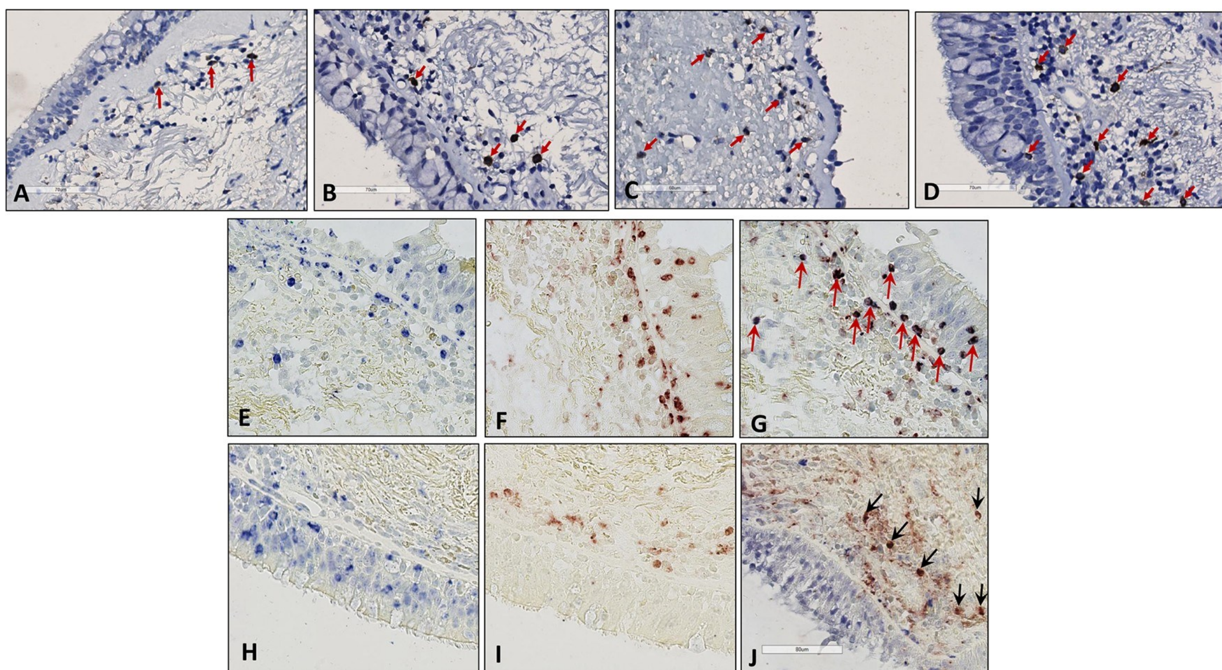


Figure 4. IL-17 expression in the submucosa of bronchial biopsies of 4 groups of studied population. *atopic inhaled corticosteroid (ICS) user (frame A), nonatopic ICS user (frame B), atopic non-ICS user (frame C), nonatopic non-ICS user (frame D). Single staining for IL-17 (frame E; blue) and MPO (frame F; red) and double staining for IL-17 and MPO (frame G; purple) in adjacent sections of a nonatopic non-ICS user asthmatic patient. Single staining for IL-17 (frame H; blue) and EPX (frame I; red) and double staining for IL-17 and EPX (frame J; purple) in adjacent sections of an atopic non-ICS user asthmatic patient.*

Discussion

This is the first study comparing cellular IL-17 expression in well characterized atopic and nonatopic asthma patients. We demonstrate that IL-17 was particularly expressed by neutrophils in the airway biopsies, contrasting with the paradigm that lymphocytes (Th17) are the main source of IL-17. Our results show that in patients who do not use ICS, nonatopic asthmatics have more IL-17 expressing cells in the airway wall than atopic asthmatics. In contrast, ICS use was associated with lower numbers of IL-17 expressing cells in both atopic and nonatopic asthmatics.

A new finding of our study is that IL-17 expressing cells in bronchial biopsies of asthma patients were predominantly granulocytes and not lymphocytes. We confirmed this by double immunostaining with IL-17 and MPO and by demonstrating a strong positive correlation between IL-17 expressing cells and neutrophils. Although, perhaps surprising, neutrophils have been reported as a source of IL-17 in humans [24, 25] as well as in animal studies [25–29]. In vitro investigations also showed production of IL-17 by stimulated neutrophils with immune complex [27]. Eosinophils may be another source of IL-17, as suggested by double immunostaining of IL-17 and EPX, and by the significant correlation between IL-17+ cells and eosinophil numbers in atopic asthma patients. Previous findings in the literature are in line with our finding that IL-17 expressing cells in the airways may be granulocytes. Eustace et al showed that IL17 in bronchial biopsies of COPD patients was expressed by neutrophils, next to mast cells, T cells, and B cells in the subepithelium of the small airways [30]. Molet et al demonstrated in asthma that eosinophils in sputum, brochoalveolar lavage fluid, and peripheral blood express IL-17 [10]. Finally, Tan et al demonstrated in children with cystic fibrosis that neutrophils and γδT cells in the airways produce IL-17, next to Th17 cells [31]. These data together support the reports showing the early sources of IL-17 are the innate immune cells and they have a central role in the initiation of IL-17-dependent immune responses, even before the first CD4+ T cell sees its cognate antigen and initiate the Th17 development program [32].

We found more IL-17 expressing cells in the airway wall of nonatopic than atopic asthmatics, that is those who did not use ICS. It has been suggested that IL-17 may contribute to the pathogenesis of neutrophil-dominant/nonatopic rather than to eosinophil-dominant/ atopic asthma [4]. Presence of fewer eosinophils and more neutrophils in our nonatopic asthmatic subjects and the significant contribution of both cell types to IL-17+ cells in our biopsies support this hypothesis. Interestingly, we found ICS use to be associated with lower IL-17 expression in bronchial biopsies of both atopic and nonatopic asthmatics. This is in line with a

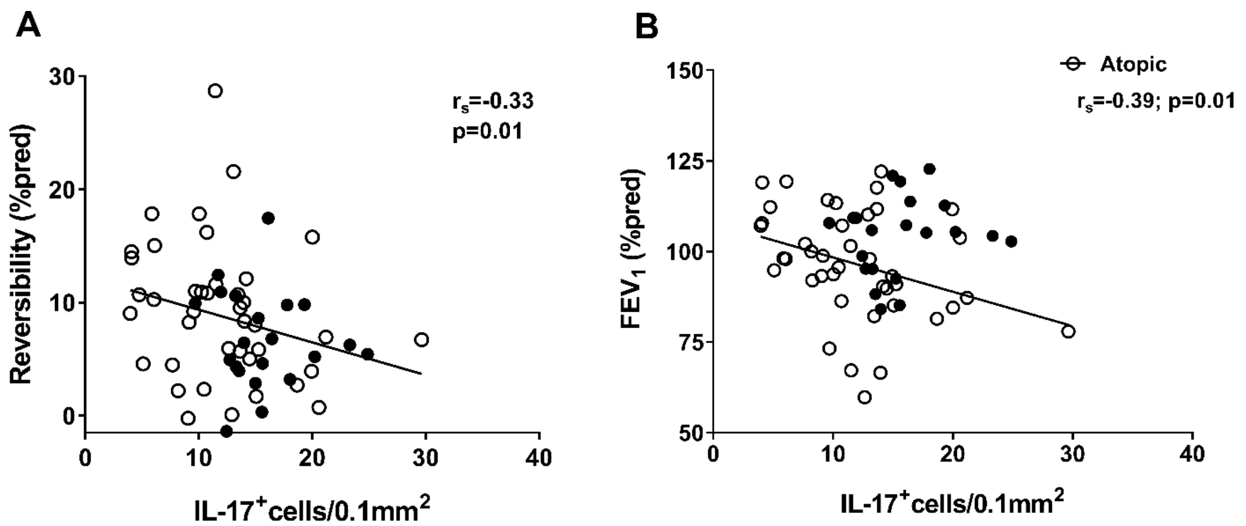
bronchial biopsy study in 10 patients with moderate-to-severe asthma (all atopic) demonstrating a significantly reduction in the number of IL-17+ cells in the airways after a 2-week course of oral corticosteroid treatment [8]. Accordingly, IL-17 levels in sputum of 15 mild-moderate and 15 severe asthmatics decreased after one month of ICS treatment [15]. In vitro data are also in line with these findings as corticosteroids could inhibit IL-17 induction of cytokines in epithelial cells and fibroblasts [10]. We have also shown before that corticosteroids inhibit IL17A-induced IL-8 production of epithelial cells [33].

Regarding the effect of IL-17 levels on the lung function we found a negative correlation between FEV1% predicted and IL-17 levels in the atopic individuals who did not use ICS. In line with our finding, Irvin et al found a negative correlation between FEV1% and IL-17 levels in their asthmatic population [20]. Reduced airway patency due to IL-17 mediated airway inflammation may be responsible for this negative association, but also direct sensitization of airway smooth muscle may play a role, as has been suggested in mouse with house-dust mite-induced allergy [34]. Such a direct role of IL-17 in smooth muscle cell contraction is in accordance with findings of a clinical trial demonstrating clinically meaningful effects of anti–IL17A, especially in a group with high reversibility of FEV1 in response to albuterol [35]. However, our study seems to contradict these results as we found an inverse relationship between IL-17 expression and reversibility of FEV1 to albuterol. A direct comparison between the two studies is unfortunately not possible, as Busse et al didn't measure expression of IL-17 levels in their studied population [35]. Clearly, more research is necessary to understand the "high IL17 phenotype" of asthma and its consequences for personalized medicine.

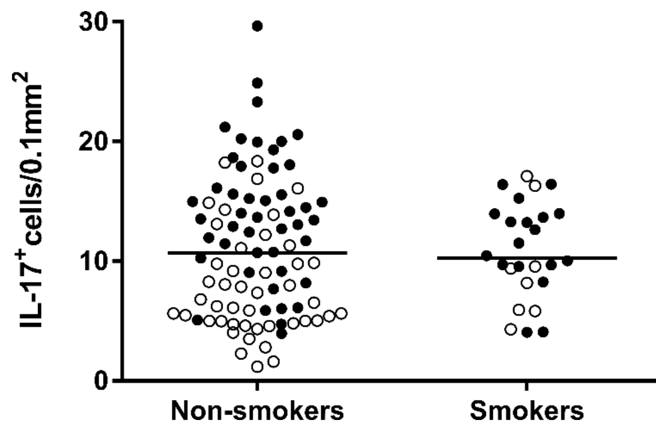
In our study, IL-17 levels was significantly correlated with neutrophilic inflammation but smoking did not contribute to the expression of IL-17. This supports the previous finding by Doe et al where IL-17A and IL-17F expression in the submucosa of the lung tissue was not associated with smoking status in their asthmatics [9]. However, our finding contrasts with a study in healthy smokers and COPD patients, showing that smokers have more IL-17 expressing cells in the submucosa than nonsmokers [36]. We conclude that atopy and ICS use may associate with a lower expression of IL-17 and that there are contradictory findings regarding the contribution of smoking. One of the limitations of our study is that the scarce biopsy material did not allow further investigating a potential explanation for the effect of ICS on IL-17+ cells. A very recent study shows that IL-17A/IL-4 dual producing cells are important in asthma and may provide a potential explanation for ICS use decreasing IL-17A+ cells [20]. Future studies on human biopsy staining are warranted.

Conclusion

In summary, we here show that the IL-17+ cells present in airway wall biopsies of asthmatics are mostly neutrophils and to a smaller extent eosinophils, and not, as the general paradigm assumes lymphocytes (Th17). This is of interest since nonatopic asthmatics who do not use inhaled corticosteroids have higher IL-17 expression in bronchial biopsies than atopic asthmatics, suggesting a potential role of IL-17 in the pathogenesis of nonatopic asthma. ICS use was associated with lower numbers of IL-17+ cells in both atopic and nonatopic asthmatics, suggesting a beneficial effect of ICS in general.



***Supplemental Fig 1**. Negative correlation, in non-inhaled corticosteroid (ICS) users, between the number of $IL17^{+}$ cells in the submucosa of bronchial biopsies and reversibility (%Predicted) in atopic and nonatopic asthmatics (rs = -0.33; p = 0.01) (A), and with FEV1% predicted in atopic asthmatics (rs = -0.39; p = 0.01)(B).*
Solid circles are nonatopic asthmatics, and open circles are atopic asthmatics.



Supplemental Fig 2. *The number of IL-17*$^{+}$ *cells in submucosa in bronchial biopsies from smoking and nonsmoking asthmatics, who are inhaled corticosteroid (ICS) users or non-ICS users. Solid circles are non-ICS users, and open circles are ICS users.*

Acknowledgments

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Author Contributions

Conceived and designed the experiments: FF WT DSP NHTH. Performed the experiments: FF CAB ML MRL. Analyzed the data: FF CAB NHTH. Contributed reagents/materials/analysis tools: WT NHTH MNH ML MRL. Wrote the paper: FF CAB DSP WT MNH NHTH. Contributed to the interpretation of the data: FF CAB ML MRL DSP WT MNH NHTH. Revised the drafts and read and gave final approval of the version to be published: FF CAB ML MRL DSP WT MNH NHTH.

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Chapter 6

Glucocorticoids induce the production of the chemoattractant CCL20 in airway epithelium

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Abstract

Th17-mediated neutrophilic airway inflammation has been implicated in decreased response to glucocorticoids in asthma. We aimed to investigate the effect of glucocorticoids on the airway epithelial release of the neutrophilic and Th17-cell chemoattractant CCL20.

We studied CCL20 and CXCL8 sputum levels in asthmatic subjects using inhaled glucocorticoids or not, and the effect of budesonide on CCL20 and CXCL8 production in primary bronchial epithelial cells. The mechanism behind the effect of budesonide-induced CCL20 production was studied in 16HBE14o-cells using inhibitors for the glucocorticoid receptor, intracellular pathways and metalloproteases.

We observed higher levels of CCL20, but not CXCL8, in the sputum of asthmatics who used inhaled glucocorticoids. CCL20 levels correlated with inhaled glucocorticoid dose and sputum neutrophils. Budesonide increased tumour necrosis factor (TNF)-a-induced CCL20 by primary bronchial epithelium, while CXCL8 was suppressed. In 16HBE14o-cells, similar effects were observed at the CCL20 protein and mRNA levels, indicating transcriptional regulation. Although TNF-a-induced CCL20 release was dependent on the ERK, p38 and STAT3 pathways, the increase by budesonide was not. Inhibition of glucocorticoid receptor or ADAM17 abrogated the budesonide-induced increase in CCL20 levels.

We show that glucocorticoids enhance CCL20 production by bronchial epithelium, which may constitute a novel mechanism in Th17-mediated glucocorticoid-insensitive inflammation in asthma.

Introduction

Asthma is a chronic obstructive airway disease affecting millions of people worldwide, characterised by airway hyperresponsiveness, remodelling and inflammation, the latter predominantly characterised by eosinophils and Th2 cells. Inhaled glucocorticoids are currently the cornerstone of asthma treatment due to their broad anti-inflammatory effects, including a suppressive effect on chemokine production by structural airway cells. Despite this, a subset of asthmatic subjects is relatively insensitive to glucocorticoid treatment. This insensitivity has been associated with a neutrophilic type of airway inflammation [1], which is thought to play a prominent role in acute exacerbations and chronic severe asthma [2].

Recently, neutrophilic airway infiltration has been associated with T-lymphocytes of the Th17 subset [3]. Th17 cells specifically secrete cytokines of the interleukin (IL)-17 family, although they are not the only source of these cytokines, which act on the airway epithelium to induce

the secretion of pro-inflammatory cytokines (e.g. CXCL8, granulocyte–macrophage colony-stimulating factor and CCL20) that recruit neutrophils to the site of inflammation [4–6]. Interestingly, it has recently been demonstrated in mice that passive transfer of Th17 cells and subsequent airway challenge induces glucocorticoid-insensitive neutrophilic airway inflammation and hyperresponsiveness [7]. Despite these novel insights, it is still unknown how Th17-mediated inflammation develops and why glucocorticoids are unable to efficiently suppress Th17-mediated neutrophilic airway inflammation. Neutrophils are relatively insensitive to glucocorticoids; however, the production of their chemoattractants, including CXCL8, by airway epithelium is glucocorticoid sensitive [8].

In addition to CXCL8, chemoattraction of neutrophils as well as Th17 cells can be induced by CCL20 [9]. CCL20 acts on CCR6, which is expressed on memory T-lymphocytes, predominantly of the Th17 subtype, on a subset of neutrophils and on dendritic cells [10]. Airway epithelium is a major producer of CCL20 [11]. Interestingly, increased CCL20 levels have been observed in asthma patients, with a further increase upon allergen challenge [12]. In addition, severe asthma patients displayed higher CCL20 levels in sputum than nonsevere asthma patients, which was associated with higher neutrophil counts [13]. Moreover, increased levels of CCL20 mRNA have been observed in the bronchoalveolar lavage fluid of glucocorticoid-insensitive asthmatic subjects [14]. However, it is still unknown if and how airway epithelial CCL20 production is regulated by glucocorticoids.

In this study, we were interested in assessing whether the epithelial release of CCL20 is sensitive to glucocorticoids. We investigated CCL20 levels in sputum from asthmatics using inhaled glucocorticoids or not, as well as the release of CCL20 by primary bronchial epithelial cells from asthma patients upon treatment with glucocorticoids in vitro. Interestingly, we found that CCL20 levels were higher in the sputum of inhaled glucocorticoid-using subjects, and that glucocorticoids increased the release of CCL20 by primary bronchial epithelial cells rather than inhibiting it. Therefore, we further unravelled the mechanism of CCL20 upregulation by glucocorticoids in the bronchial epithelial cell line 16HBE.

Material and methods

Subjects

Samples from 89 asthmatic individuals were included in a cross-sectional, observational study and classified by the use of inhaled glucocorticoids, rendering a group of 50 subjects using

inhaled glucocorticoids and a group of 39 subjects who did not use glucocorticoids. Table 1 presents the clinical characteristics of these subjects.

All subjects had stable asthma and did not use exacerbation treatment nor was maintenance therapy altered during the 6 months preceding their inclusion in the study.

Primary bronchial epithelial cells were obtained from bronchial brushings from four asthmatic patients and four healthy subjects. All subjects were nonsmokers (<10 pack-years, no smoking in the last year), and between 18 and 65 years. Asthma patients were free of other lung diseases and included on basis of the presence of allergy (either by skin test or phadiatop), forced expiratory volume in 1 s (FEV1) >80% predicted, and documented bronchial hyperresponsiveness defined as either an adenosine monophosphate provocative concentration causing a 20% fall in FEV1 (PC20) <320 mg mL^{-1}, a methacholine PC20 <8 mg/mL^{-1} or a histamine PC20 <8 mg mL^{-1}.

The Medical Ethics Committee of the University Medical Center Groningen (Groningen, the Netherlands) approved the study and signed informed consent was given by participants.

TABLE 1 Subject characteristics

	Not using inhaled GC	Using inhaled GC
Subjects n	39	50
Females	19 (48)	25 (50)
Age years	50 (24–70)	51 (22–71)
Atopy	26 (68)	36 (75)
Current smokers	17 (43)	9 (18)*
Smoking exposure pack-years	8.4 (0–47.3)	0.2 (0–63.8)
Pre-BD FEV_1 L	2.9 (1.6–5.9)	2.7 (1.4–4.5)
Pre-BD FEV_1 % pred	90.3 (53.9–113.7)	86.3 (51.6–128.7)
Pre-BD FEV_1/VC %	69.6 (45.8–89.5)	66.5 (40.3–94.4)
Pre-BD PEF L s^{-1}	7.2 (4.7–14.9)	7.9 (4.0–14.2)
Pre-BD MEF_{50} L s^{-1}	2.4 (1.0–5.6)	2.3 (0.6–5.3)
Reversibility % pred	10.0 (-2.2–33.2)	9.5 (1.5–38.4)
AMP PC_{20} mg mL^{-1}	78.7 (0.02–>640)	51.5 (0.01–>640)
Total IgE IU L^{-1}	45 (0–604)	2 (0–1668)
Blood eosinophils x10^9 per L	0.20 (0.01–0.51)	0.20 (0.06–1.16)
Sputum eosinophils %	1.2 (0–7.3)	0.8 (0–65.8)
Sputum neutrophils %	53.0 (11.8–87.7)	54.0 (15.3–90.0)
Alveolar NO ppb	5.57 (2.13–18.34)	5.63 (1.49–51.72)
Bronchial NO nL s^{-1}	0.64 (0.06–3.17)	0.89 (0.20–10.38)
Control according to GOAL criteria	24 (72)	24 (51)

*Data are presented as n (%) or median (range), unless otherwise stated. GC: glucocorticoid; BD: bronchodilator; FEV1: forced expiratory volume in 1 s; VC: vital capacity; PEF: peak expiratory flow; MEF50: half-maximal expiratory flow; AMP: adenosine monophosphate; PC20: provocative concentration causing a 20% fall in FEV1; GOAL: Gaining Optimal Asthma Control. *: p<0.05 versus not using inhaled GC.*

Sputum induction and processing

Sputum was induced by inhalation of nebulised hypertonic saline (5%) for three consecutive periods of 5 min. Whole sputum samples were processed as described previously [15].

Cell culture and stimulation

Primary bronchial epithelial cells and human bronchial epithelial 16HBE14o-cells (16HBE) (kindly provided by D.C. Gruenert, University of California, San Francisco, CA, USA) were cultured in hormonally supplemented bronchial epithelium growth medium (BEGM, Lonza, Walkersville, MD, USA) containing bovine pituitary extract, epidermal growth factor (EGF), adrenaline, hydrocortisone, retinoic acid and triiodothyronine, or in Eagle's minimal essential median/10% fetal calf serum, in collagen/fibronectin- or collagen-coated flasks respectively, as previously described [16]. Primary cells were used for experimentation in passage 3. Cells were seeded in duplicates at a concentration of 10^5 cells mL^{-1} in 24-well plates, grown to ,90% confluence and hormonally or serum-deprived (16HBE) medium overnight, pre-treated for 2 h with budesonide (AstraZeneca, Lund, Sweden) in concentrations ranging from 10^{-10} to 10^{-7} M, and subsequently stimulated with/without 10 ng mL^{-1} tumour necrosis factor (TNF)-a (Sigma, St Louis, MO, USA) upon 60 min of pre-incubation with/without specific inhibitors for the ERK (extracellular signalregulated kinase) (U0126, 10 mM), p38 (SB203580, 1 mM), STAT3 (signal transducer and activator of transcription) (S3I-201, 100 mM) and phosphoinositol 3-kinase (PI3K) (LY294002, 10 mM) pathways, glucocorticoid receptor inhibitor (RU486, 1 mM), general metalloprotease inhibitor (TAPI-2; Calbiochem, Omnilabo International BV, Breda, The Netherlands; 20 mM), ADAM10/17 (a disintegrin and metalloprotase) inhibitor (GW280264X, 10 mM) or ADAM10-inhibitor (GI254023X, 1 mM) prior to budesonide treatment. GW280264X and GI254023X were kindly provided by GlaxoSmithKline (London, UK). Unless stated otherwise, inhibitors were purchased from Tocris Bioscience (Bristol, UK).

Air–liquid interface culture

Normal human bronchial epithelial (NHBE) cells (Lonza) were grown on semipermeable collagen/ fibronectin-coated membranes in a 1:1 mixture of Dulbecco's modified Eagle's medium (Lonza) and BEGM supplemented with retinoic acid (15 ng mL^{-1}; Sigma) and exposed to an air–liquid interface (ALI) for 4 weeks as described previously [17]. On day 14 of air exposure, cells were placed submerged in growth factor-deprived medium overnight and subsequently treated with 10-8 M budesonide for 24 h. Cell-free supernatants were

harvested from the apical side.

Methods for cytokine measurements and real-time RT-PCR are described in the online supplementary material.

Statistics

We used the t-test for paired observations for differences between conditions within the cell experiments, the Mann–Whitney U-test for differences in continuous data between subject groups and the Chi-squared test for differences in ordinal data between groups. Spearman's rho (rs) test was used for analysis of correlations in patient groups. When analyzing the correlation with glucocorticoid dose, only subjects using glucocorticoids were tested.

Results

Higher sputum levels of CCL20 in asthma patients using inhaled glucocorticoids than in patients who do not

First, we tested CCL20 levels in sputum from asthmatic individuals using inhaled glucocorticoids and those who did not use inhaled glucocorticoids. Both groups had similar disease severity as ascertained by clinical parameters (table 1). Importantly, we observed significantly higher levels of CCL20 in the sputum of asthma patients using inhaled glucocorticoids than the subjects who did not (fig. 1a), while CXCL8 levels were not different (fig. 1b). Within the group of subjects using inhaled glucocorticoids, we observed a significant correlation between the dose of inhaled glucocorticoids and the level of CCL20 in the sputum samples (rs50.28, p50.04; online supplementary fig. S1A). Moreover, CCL20 levels in sputum correlated with the number of neutrophils in sputum (rs50.34, p50.01; online supplementary fig. S1B), although the numbers of sputum neutrophils did not differ between asthma patients using inhaled glucocorticoids and those who did not (table 1). As expected, sputum CXCL8 levels correlated significantly with sputum neutrophils (rs50.24, p50.03; online supplementary fig. S1D).

Glucocorticoids increase CCL20 release in primary bronchial epithelial cells

Next, we examined whether the glucocorticoid budesonide regulates CCL20 secretion by primary bronchial epithelial cells from asthma patients. We used TNF-a as a relevant cytokine to induce a pro-inflammatory response. TNF-a significantly increased CCL20 and CXCL8

secretion (fig. 2a and b). Budesonide significantly inhibited TNF-a-induced CXCL8 secretion (fig. 2b). In striking contrast, the TNF-a-induced secretion of CCL20 was significantly increased upon treatment with BUD (fig. 2a). In addition, we observed that budesonide induced a significant increase of baseline CCL20 levels and significantly enhanced the house dust mite allergen-induced CCL20 secretion (fig. 2c). Budesonide did not significantly decrease levels of CXCL8, probably due to a lack of power (fig. 2d). Similar effects were observed in bronchial epithelial cells from healthy controls (fig. 2e and f), with no significant differences between asthma patients and healthy controls.

To increase the relevance of our findings, we also studied the effect of budesonide on CCL20 secretion in primary human bronchial epithelial cells cultured at ALIs to induce mucociliary differentiation, reflecting the in vivo situation better. Again, treatment with budesonide (10^{-8} M, 24 h) significantly increased CCL20 levels (fig. 2g), while CXCL8 levels were not affected (fig. 2h).

Mechanisms of glucocorticoid-induced CCL20 secretion in 16HBE cells

To further elucidate the underlying mechanisms of glucocorticoid-induced CCL20 upregulation in airway epithelium, we used the human bronchial epithelial cell line 16HBE due to the limited numbers of primary cells. In these cells, TNF-a also induced a significant increase in CCL20 secretion, which was again further upregulated by budesonide (fig. 3a), while CXCL8 secretion was strongly reduced (fig. 3b). Furthermore, budesonide induced a significant increase in baseline levels of CCL20 (data not shown). To determine whether the increased CCL20 secretion induced by budesonide was mediated by glucocorticoid receptor activation, we used the competitive glucocorticoid receptor antagonist mifepristone (RU486) and found that the presence of RU486 completely prevented the budesonide-induced increase in CCL20 secretion (fig. 3c). Next, we studied whether CCL20 was regulated at the transcriptional level and we observed that budesonide was able to increase CCL20 mRNA levels (fig. 3d).

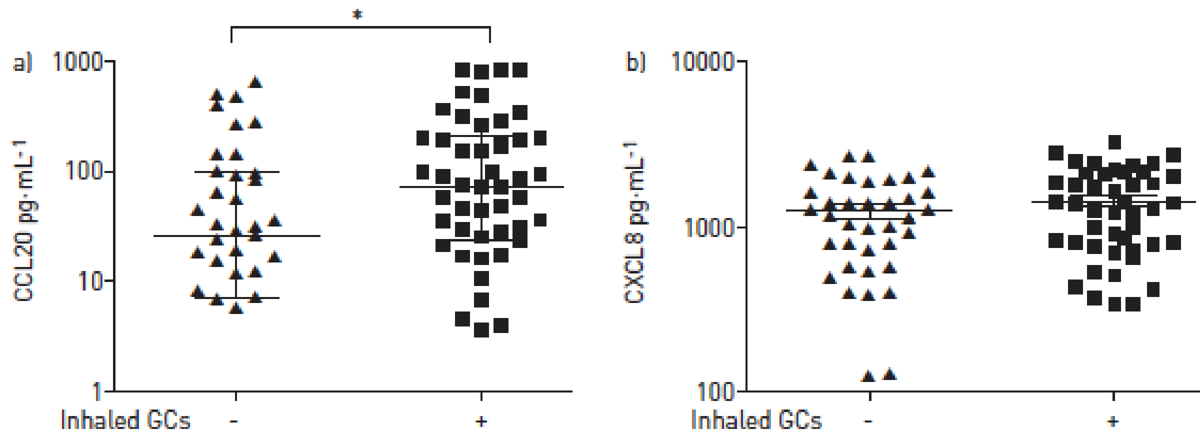

Figure 1. *a) CCL20 levels are significantly higher in sputum of asthmatics using inhaled glucocorticoids (GCs) (n=39) than in those who did not (n=50), while b) CXCL8 levels are similar between groups. Levels of CCL20 and CXCL8 were measured in induced sputum by ELISA. Horizontal lines represent the median and error bars represent the interquartile range. *: p≤0.05*

TNF-a-induced CCL20 production is dependent on ERK, p38 and STAT3

As CCL20 was regulated by budesonide at the transcriptional level, we aimed to further unravel the signal transduction pathways involved in these effects. Since the STAT3, ERK and p38 pathways have been implicated in CCL20 transcription as well as in glucocorticoid-insensitive airway inflammation [18–20], we tested the effect of their specific inhibitors on the TNF-a- and budesonide-induced CCL20 production in 16HBE cells. Pre-incubation with the inhibitors of the ERK (U0126), p38 (SB203580) and STAT3 (S3I-201) pathways significantly reduced the TNF-a-induced CCL20 production, indicating a role for these signalling molecules in CCL20 production (fig. 4a). Inhibition of the PI3K pathway did not affect CCL20 production, although it significantly inhibited IL-8 secretion under the same conditions (online supplementary fig. S2A). Next, we determined whether the budesonide-induced increase was dependent on the aforementioned pathways observed. However, no decrease in the upregulatory effect of budesonide was found upon the use of LY294002, U0126, SB203580 or S3I-201 (fig. 4b), nor did budesonide increase the phosphorylation of p38, ERK or STAT3 (data not shown). Thus, our data indicate that while TNF-a-induced CCL20 production is dependent on the ERK, p38 and STAT3 pathways, the additional upregulatory effect of glucocorticoids is not mediated by these pathways in human bronchial epithelium, suggesting the involvement of additional pathways.

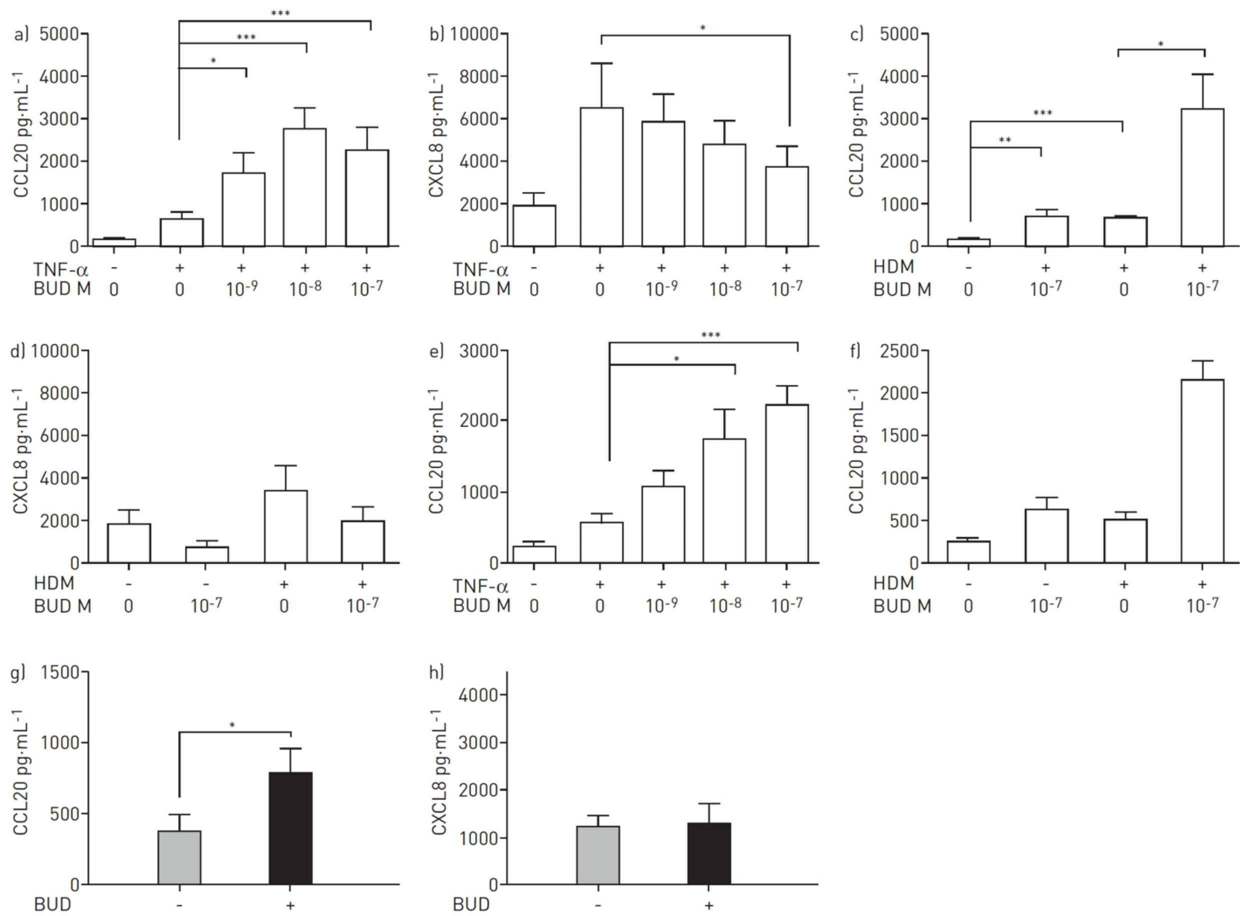

Figure 2. *Budesonide (BUD) enhances the tumour necrosis factor (TNF)-a- and house dust mite (HDM) allergen-induced CCL20 release, but suppresses the TNF-a-induced CXCL8 release in primary bronchial epithelial cells from a–d) asthma patients and e, f) healthy donors. Cells were obtained from four donors per group. Cells were pre-treated for 2 h with or without BUD (10^{-7}–10^{-10} M) and left unstimulated or stimulated for 24 h with a, b, e) 10 ng/mL TNF-a or c, d, f) 50 ug/m HDM. g, h) cells were grown in air–liquid interface culture for 2 weeks. CCL20 and CXCL8 levels were measured in cell-free supernatants from the apical side after treatment with or without BUD (10^{-8} M) for 24 h. Data are presented as mean±SEM (n=4). *: $p \leq 0.05$; **: $p \leq 0.01$; ***: $p \leq 0.001$.*

Glucocorticoid-induced CCL20 secretion is ADAM17 dependent

Previously, KIM et al. [21] have described that ADAM17-dependent EGF receptor (EGFR) stimulation can increase CCL20 production, while glucocorticoids have been reported to increase EGFR activity [22]. Indeed, ADAM17 has been described as a key sheddase of ligands of EGFR [23]. Therefore, we used the broad-spectrum metalloprotease inhibitor TAPI-2, the selective ADAM10/17 inhibitor GW280264X and the selective ADAM10 inhibitor GI254023X, as well as the EGFR inhibitor AG1478 to determine if an ADAM/

EGFR-dependent mechanism could be involved in glucocorticoid-induced CCL20 secretion. TAPI-2 did not significantly inhibit TNF-a-induced CCL20 secretion but completely abrogated the upregulatory effect of budesonide. A similar effect was observed for the selective ADAM10/17 inhibitor GW280264X, while the more ADAM10-specific inhibitor GI254023X did not show a significant effect on the secretion of CCL20 (fig. 5a). Since both GW280264X and TAPI-2 have a higher affinity for ADAM17 than ADAM10, these data suggest that the budesonide-induced increase in CCL20 is dependent on ADAM17 activity. In contrast to the data of KIM et al. [21], we did not observe an effect of the EGFR inhibitor AG1478 on the budesonide-induced increase in CCL20 release (fig. 5a), although it significantly inhibited CXCL8 secretion under the same conditions (online supplementary fig. S2B). This excludes the involvement of EGFR ligand shedding and subsequent EGFR activation in budesonide-induced CCL20 secretion.

Subsequently, we aimed to determine whether the glucocorticoid-induced CCL20 secretion is due ADAM17-mediated shedding of CCL20 itself or is a consequence of downstream signalling induced by the shedding of an ADAM17 substrate other than EGFR ligands. We assessed this by studying the effect of ADAM17 inhibition at the CCL20 mRNA level. Notably, budesonide was no longer able to upregulate CCL20 mRNA when cells were pre-treated with GW280264X (fig. 5b). Thus, our results indicate that the upregulatory effect of glucocorticoids on CCL20 is dependent on ADAM17 activity and downstream signalling of an as yet unknown substrate of ADAM17.

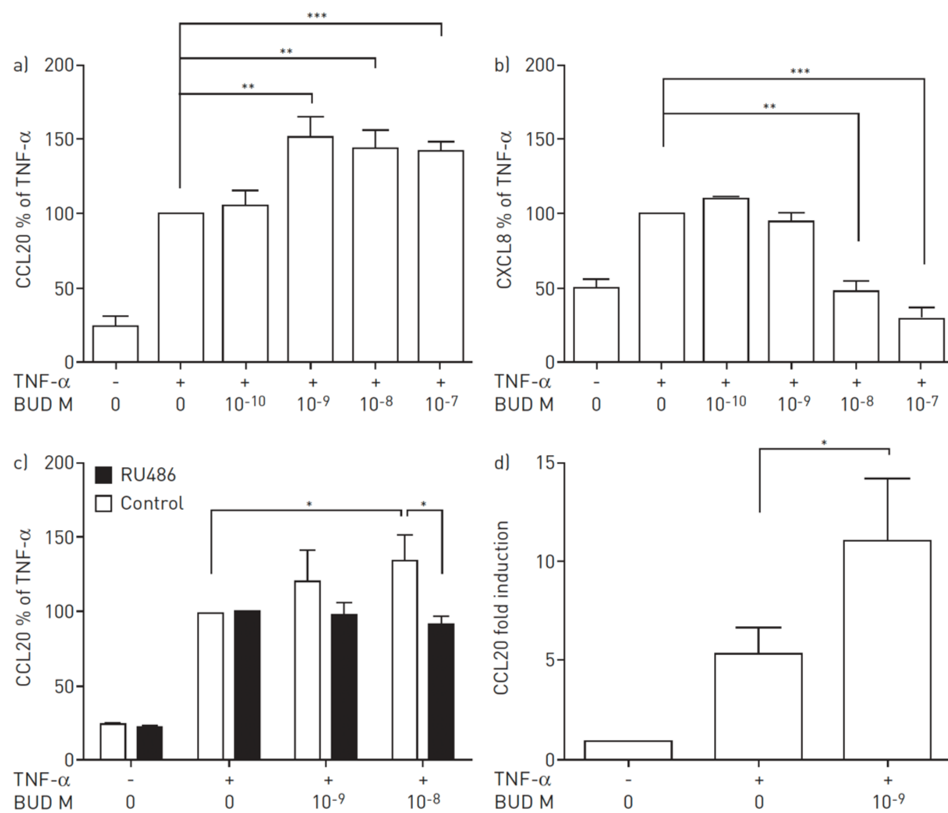

Figure 3. *Budesonide (BUD) enhances tumour necrosis factor (TNF)-a-induced CCL20 release and mRNA expression, which is dependent on glucocorticoid receptor activity, but suppresses TNF-a-induced CXCL8 release in 16HBE cells. Cells were pre-treated with BUD (10^{-7}–10^{-10} M) for 2 h, stimulated with 10 ng/mL^{-1} TNF-a, and mRNA and cell-free supernatants were collected after 6 h and 24 h, respectively. a) CCL20 and b) CXCL8 were measured in cell-free supernatants and expressed as percentage of the TNF-a levels without BUD. c) Prior to BUD, cells were treated for 1 h with 10 mM RU486 and CCL20 levels are expressed as percentage of the TNF-a levels without BUD. d) CCL20 mRNA levels were related to a housekeeping gene and expressed as fold change compared with the unstimulated control (2^{-DDCt}). Data are presented as mean±SEM of four independent experiments. Ct: threshold cycle. *: p≤0.05; **: p≤0.01; ***: p≤0.001.*

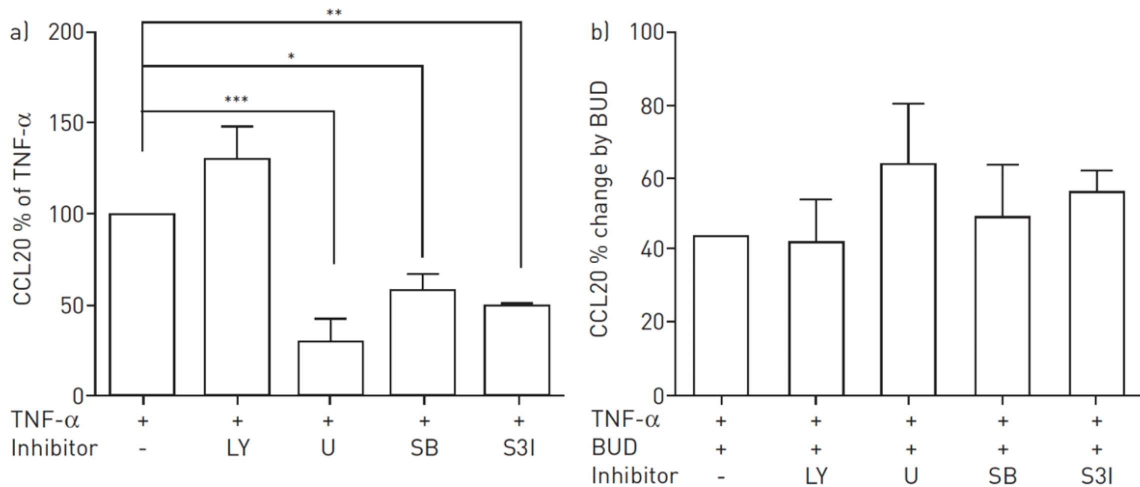

Figure 4. *Inhibition of the ERK (extracellular signal-regulated kinase), p38 and STAT3 (signal transducer and activator of transcription) pathways reduces tumour necrosis factor (TNF)-a-induced CCL20 release, but did not block the upregulatory effect of budesonide (BUD) in 16HBE14o- cells. Cells were treated with LY294002 (LY; 10 mM), U0126 (U; 10 mM), SB203580 (SB; 1 mM) or S3I-201 (S3I; 100 mM) for 30 min prior to pre-treatment with BUD (10^{-8} M) for 2 h and subsequently stimulated with TNF-a (10 ng/mL^{-1}) for 24 h. a) Effect of the inhibitors on TNF-a-induced CCL20 release. CCL20 levels are expressed as percentage of the TNF-a levels without inhibitors. b) Effect of the inhibitors on BUD-induced CCL20 release. CCL20 levels are expressed as percentage increase over the levels with TNF-a alone. Data are presented as mean±SEM of four independent experiments. *: $p \leq 0.05$; **: $p \leq 0.01$; ***: $p \leq 0.001$.*

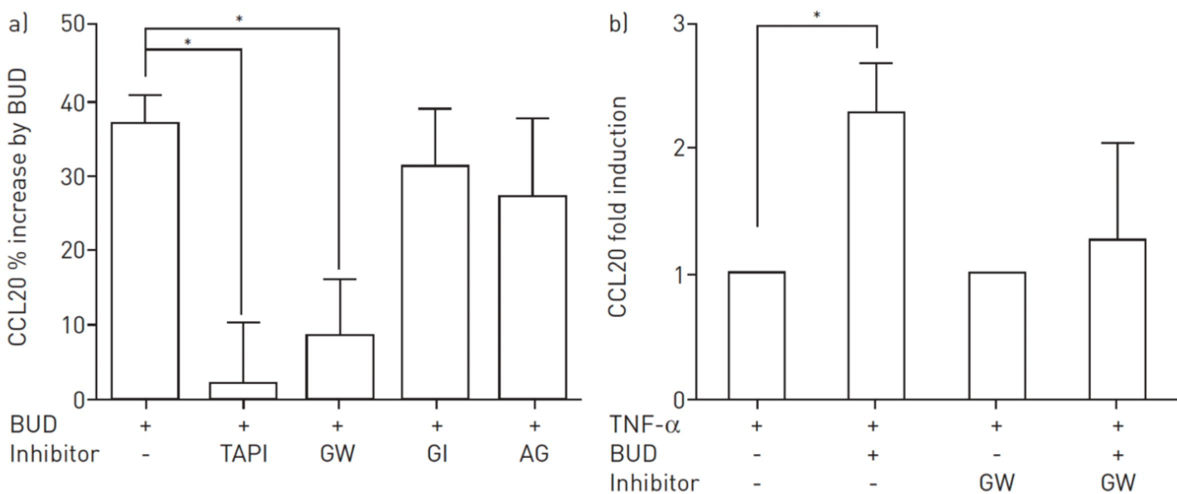

Figure 5. *The effect of budesonide (BUD) is dependent on activity of ADAM17 (a disintegrin and metalloprotease). Cells were pre-treated for 1 h with the broad-spectrum metalloprotease inhibitor TAPI-2 (TAPI; 20 mM), ADAM17 and ADAM10 inhibitor GW280264X (GW; 10 mM), ADAM10 inhibitor GI254023X (GI; 1 mM), or EGFR inhibitor AG1478 (AG; 1 mM), and subsequently incubated with BUD (10^{-9} M) for 24 h (cell-free supernatants) or stimulated with tumour necrosis factor (TNF)-a for 6 h and harvested for mRNA isolation. a) CCL20 was measured in cell-free supernatants and levels are expressed as the percentage increase over the levels with BUD alone. Data are presented as mean±SEM of four independent experiments. b) CCL20 mRNA levels were related to a housekeeping gene and expressed as fold change compared with the levels in the absence of BUD (2^{-DDCt}). Data are presented as mean±SEM of three independent experiments. Ct: threshold cycle. *: $p \leq 0.05$.*

Discussion

The mechanism of glucocorticoid-insensitive Th17-mediated neutrophilic airway inflammation in asthma has remained unclear, and the effect of glucocorticoids on the airway epithelial secretion of the Th17 cell and neutrophil chemoattractant CCL20 has not been studied before. We show for the first time that asthma patients using inhaled glucocorticoids display higher sputum levels of CCL20 than asthmatics who do not use inhaled glucocorticoids, while CXCL8 levels did not differ between the groups. Furthermore, we demonstrate that glucocorticoids upregulate CCL20 secretion in cultured bronchial epithelial cells from asthma patients, whereas CXCL8 is inhibited by glucocorticoids. Our experiments in 16HBE cells further reveal that this effect of glucocorticoids is regulated at the transcriptional level by an ADAM17- and glucocorticoid receptor-dependent mechanism.

Our findings may have important implications for our understanding of the initiation of glucocorticoid-insensitive Th17 and neutrophilic airway inflammation in asthma, as CCL20 has been known to attract both Th17 cells and neutrophils. In addition to allergen-induced airway inflammation, a crucial role for CCL20 has been demonstrated in cigarette smoke-induced airway infiltration of neutrophils, T-lymphocytes and dendritic cells in a mouse model of chronic obstructive pulmonary disease (COPD) [24]. Importantly, both Th17-mediated neutrophilic airway inflammation and cigarette smoking have been related to glucocorticoid insensitivity in asthma [25] and smoking has been shown to induce airway infiltration of both neutrophils and Th17-type cells [26]. Thus, we propose a novel paradigm for the development of glucocorticoid-insensitive airway inflammation in both asthma and COPD, where glucocorticoids enhance CCL20 release, inducing airway infiltration of CCR6+ neutrophils and Th17 cells. In line with this hypothesis, the increased sputum levels of CCL20 in asthma patients using inhaled glucocorticoids were associated with neutrophil counts. Furthermore, COPD patients were found to display higher sputum levels of CCL20 than never-smokers and smokers without COPD. Here, the majority of COPD patients, but none of the control subjects, used inhaled glucocorticoids. It is tempting to speculate that glucocorticoid use contributes to the increased levels of COPD observed in this study, although the comparison of CCL20 levels in COPD patients using and not using inhaled corticosteroids would be required to support this.

We observed that the upregulatory effect of glucocorticoids on CCL20 was mediated at the transcriptional level and involved glucocorticoid receptor activation. Glucocorticoids have been shown to induce gene transcription through binding to a glucocorticoid response element (GRE) in the promoter region. A GRE has been described in an intron downstream of the

transcription start site of the CCL20 gene [27]. The regulatory properties of this GRE have not been extensively studied to our knowledge, but our results suggest that ADAM17 activity is indispensable for the effect of glucocorticoids on CCL20. To our knowledge, GRE binding has not been described to be metalloprotease dependent. As ADAM17 inhibition also abrogated the upregulatory effect of budesonide at the transcriptional level, we anticipate that the upregulatory effect of budesonide is not mediated by ADAM17-dependent shedding of CCL20 itself, but rather involves downstream signalling of an as yet unknown ADAM17 substrate. ADAM17 plays a role in the shedding of many signalling molecules [23], such as Notch [28] and EGFR ligands. The latter has been implicated in many autocrine loops involving pro-inflammatory transcription, including CCL20 [21]. However, our results do not support a role for EGFR activation in glucocorticoid-induced CCL20 production. Indeed, the EGFR-induced CCL20 release described in H292 cells by KIM et al. [21] could not be confirmed in NHBE cells. Further studies will be of interest to elucidate which specific pathways downstream of ADAM17 substrates are affected by budesonide and are involved in the upregulatory effect on CCL20.

Our findings exclude a role for the STAT3, p38 ERK and PI3K pathways in glucocorticoid-induced CCL20 upregulation. We observed that TNF-a-induced CCL20 production was dependent on STAT3, p38 and ERK. In line with this, IL-17-induced CCL20 was shown to be dependent on ERK activity in primary human tracheal cells [18], on both ERK and p38 activity in human gingival fibroblasts [19], and on phosphorylation of STAT3 in na¨ıve T-lymphocytes [29]. ERK and p38 phosphorylation have previously been shown to be inhibited by glucocorticoids [30], while glucocorticoids induce IL-10 in a STAT3- dependent way in B-lymphocytes [31]. In our setting, budesonide did not affect phosphorylation of STAT3 or the p38/ERK pathway, and the use of their inhibitors revealed that these pathways were not involved in the glucocorticoid-mediated enhancement of CCL20 release in human bronchial epithelium.

LANNAN et al. [32] have shown a possible mechanism of co-regulation between TNF-a and glucocorticoids. In their study, the upregulation of serpin A3 required both glucocorticoid activation and the soluble TNF receptor (TNFSR1). TNFSR1 can be shed by ADAM17 [33]. However, we render it unlikely that this mechanism plays a major role in the glucocorticoid-induced increase of CCL20, as the effect occurred regardless of the presence of TNF-a.

Although epithelial cells play an emerging role in the regulation of airway inflammation in asthma [34], we must acknowledge the possibility of other cell types playing a role in the

CCL20-induced chemotaxis of Th17 cells and neutrophils to the inflamed lungs in asthma as well. In particular, macrophages have been shown to produce CCL20 [35]. We cannot exclude that macrophages also contribute to the increased levels of CCL20 in sputum of asthma patients using inhaled glucocorticoids. The same mechanisms could also apply in macrophages as higher levels of CCL20 mRNA have been described in macrophages from glucocorticoid-insensitive subjects compared with glucocorticoid-sensitive subjects [14].

As the data on CCL20 levels in sputum of asthma patients were obtained in a cross-sectional, observational study, we cannot be sure whether glucocorticoid treatment will indeed increase CCL20 levels in the airways. Nevertheless, our sputum data in combination with our in vitro findings strongly suggest that glucocorticoid use in asthma patients leads to increased sputum levels of CCL20 as a consequence of direct effects of the glucocorticoids, inducing CCL20 release by airway epithelium. To confirm this, a future randomised clinical trial on the effect of glucocorticoids on CCL20 and Th17 cells will be required.

In conclusion, we show that levels of CCL20 are higher in asthmatic subjects using inhaled glucocorticoids and that glucocorticoids increase the production of CCL20 in human bronchial epithelium, which is mediated by the glucocorticoid receptor and dependent on ADAM17 activity. Our data may provide new opportunities for therapeutic intervention of glucocorticoid-insensitive asthma.

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Online supplement to:

Glucocorticoids induce the production of the chemo-attractant CCL20 in airway epithelium

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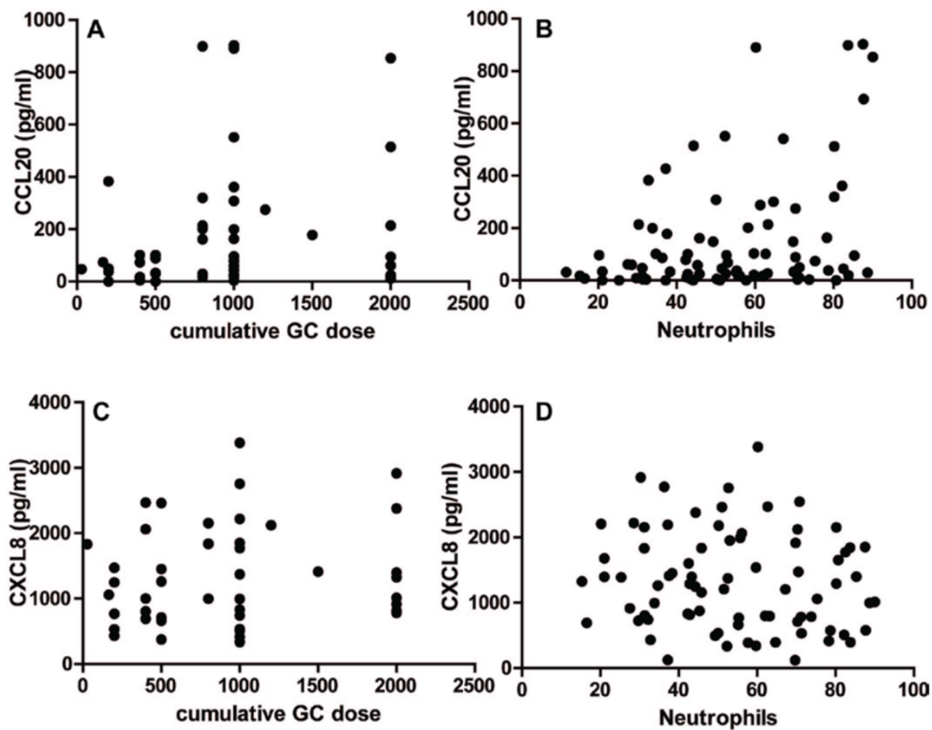
Supplementary methods:

Measurement of cytokine production

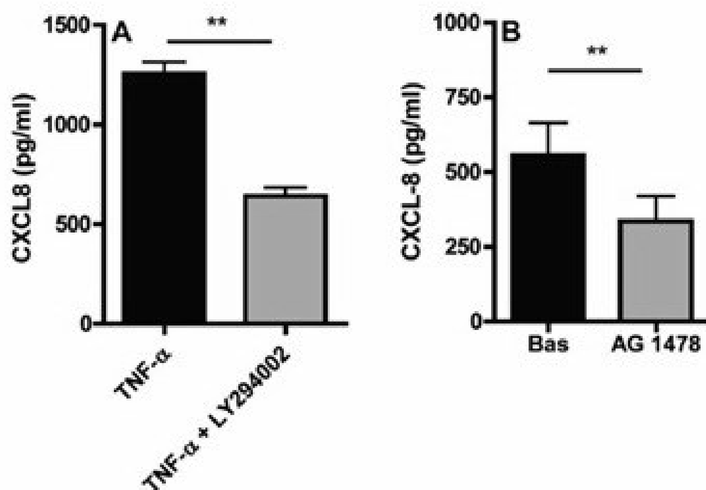
Cell-free supernatants were harvested 24 hour after stimulation. CCL20 and CXCL8 production was measured by ELISA (R&D Systems) according to manufacturer's instructions.

Realtime RT-PCR

RNA was isolated from 16HBE cells 6 hours after stimulation and cDNA was synthesized as previously described [17]. Gene expression was analysed by real-time PCR using the Taqman according to the manufacturer's guidelines (Applied Biosystems, Foster City, CA). Validated probes for CXCL8, CCL20 and the housekeeping genes β2-microglobulin and Peptidylprolyl isomerase A (PPIA) and the TaqMan Master Mix were purchased from Applied Biosystems.



Supplementary figure 1.
CCL20 levels correlate significantly with the dose of inhaled GC (r spearman=0.28, p=0.04, A) and neutrophils (r spearman=0.34, p=0.01, B), while IL-8 levels did not correlate significantly with the dose of inhaled GC (C), but did correlate significantly with sputum neutrophils (r spearman=0.24, p=0.03, D). Levels of CCL20 (ng/ml) and IL-8 (ng/ml) were measured in induced sputum by ELISA in asthma patients using GCs (n=89).



Supplementary figure 2. *CXCL8 production in 16HBE cells is sensitive to the specific PI3K inhibitor LY294002 and EGFR inhibitorAG1478. Cells were treated with LY294002 (10 μM) for 30 min prior to stimulation with TNF-α (10 ng/ml) for 24 hrs (A). Cells were left unstimulated (Bas) or treated with EGFR inhibitor AG1478 (1 μM) for 24 hours (B). CXCL8 was measured in cell-free supernatants (Mean ±SEM, n=4 independent experiments). Black bars are control conditions, grey bars inhibitor treated. **=p<0.01 between the indicated values.*

Chapter 7

Smoking and corticosteroid use independently associate with higher epithelial HDAC-2 expression in asthma.

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Abstract

Cigarette smoke has been described to down-regulate expression of histone deacetylase-2 (HDAC-2) in alveolar macrophages and to impair corticosteroid sensitivity in smoking asthmatics, severe asthmatics and COPD patients. As epithelial cells are the first barrier affected by smoking, we investigated epithelial HDAC-2 expression in smoking and non-smoking asthmatics, and compared effects of inhaled corticosteroids (ICS) use.

Endobronchial biopsies from 96 non-smoking asthmatics and 27 current smoking asthmatics were immunostained for HDAC-2 and percentage of HDAC-2 nucleus positive cells was measured in the intact epithelium.

Our results showed that smoking in asthmatics not treated with ICS was associated with increased HDAC-2 expression in the epithelium. ICS use in nonsmoking asthmatics was also associated with higher HDAC-2 expression, but not in currently smoking asthmatics. Linear regression analysis confirmed that smoking and ICS use independently contributed to HDAC-2 expression (B: 14.47, CI: 8.21–20.73 and B: 10.86, CI 6.12–15.61 respectively). Additionally, smoking interacted negatively with ICS-use.

A new observation in asthma is that smoking increases HDAC-2 expression in epithelial cells, contrasts to findings in alveolar macrophages from healthy smokers. This suggests different effect of smoking on HDAC-2 expression in different compartments. Moreover, the lack of potential beneficial effect of ICS on HDAC-2 in asthmatics who smoke suggests that smoking reduces corticosteroid effectiveness in asthma.

Short report

Corticosteroids, the most effective therapy available for asthma, suppress inflammatory genes by inhibiting histone acetyltransferase and particularly by recruiting histone deacetylase-2 (HDAC-2) to the nuclear factor-κB-activated inflammatory gene complex [1]. The airway epithelium is a major site of inflammatory gene expression and a main localization site for HDAC-2 expression in the airway wall, being therefore an important target for (inhaled) corticosteroids [5]. Indeed, bronchial biopsies from nonsmoking asthmatics treated with budesonide revealed higher HDAC-2 levels than from non-smoking asthmatics untreated with budesonide [5].

Cigarette smoke has been shown to down-regulate expression and activity of HDAC-2 in bronchial biopsies and alveolar macrophages from young healthy smokers [7]. Reduction in HDAC-2 levels/activity caused by cigarette smoke extract (CSE) in human macrophages has

been also shown in vitro studies [9].

Reduced HDAC-2 expression was suggested as one of the underlying mechanisms for reduced corticosteroid responsiveness in COPD, severe asthma and smoking asthmatics [1]. However, HDAC-2 expression data in bronchial epithelial cells in severe asthma did not support this hypothesis as there was no significant difference in its levels between severe asthmatics and mild/moderate asthmatics [2, 4]. Moreover, a recent study unexpectedly demonstrated that bronchial epithelial cells from COPD patients and healthy smokers have higher HDAC-2 expression than those of healthy nonsmokers [8]. So far, the HDAC-2 expression of bronchial epithelial cells has not been investigated in asthmatics who smoke. Because epithelial cells constitute the first barrier against inhaled smoke, we compared epithelial HDAC-2 expression in smoking and nonsmoking asthmatics, and evaluated effects of inhaled corticosteroids (ICS) use.

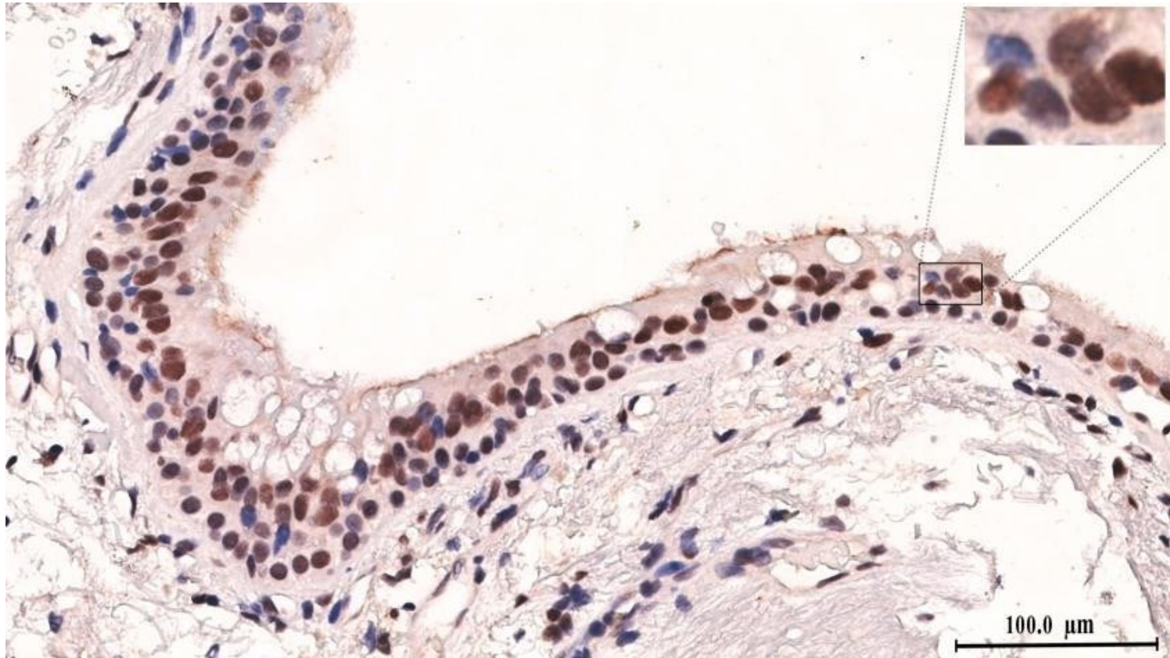
Totally, 123 patients with stable asthma were recruited. Subjects had a doctor's diagnosis of asthma and demonstrated bronchial hyperresponsiveness (BHR) to histamine and/or adenosine 5'-monophosphate (AMP) [3]. All patients originated from cohorts investigated earlier by our research group in University Medical Center Groningen (UMCG) [3]. Patients were classified into two groups based on their smoking history: 96 non-smokers (53 lifelong never-smokers, and 43 ex-smokers) and 27 current smokers. Table-1 shows clinical characteristics of the patients. Endobronchial biopsies were immunostained with a rabit antihuman HDAC-2 (Santa Cruz Biotechnology)[5]. The percentage of HDAC-2 nucleus positive cells was counted in intact epithelium (figure 1a). Totally 500 cells were counted manually in a randomized way by a blinded observer. Data analyses were performed using SPSS (version 18.0; SPSS, Chicago, IL). Mann-Whitney U tests was used to compare the HDAC-2 levels in smoking and non-smoking asthmatics with and without ICS treatment. Interaction between smoking and ICS use was analysed by multiple linear regression. P-values of less than 0.05 were considered statistically significant.

Our results showed that current smoking is associated with higher epithelial HDAC-2 expression in asthmatics who are not treated with ICS (figure-1b). ICS-use is associated with higher HDAC-2 expression in non-smoking asthmatics, but we did not observe this in currently smoking asthmatics. Linear regression analysis confirmed that smoking and ICS-use contribute both independently to the levels of HDAC-2 expression (B: 14.47, 95% CI: 8.21–20.73 and B: 10.86, 95% CI: 6.12–15.61 respectively).

Additionally, smoking interacted negatively with ICS-use (B: -19.62, 95% CI: -30.50– 8.74).

Our study for the first time demonstrates that smoking is associated with higher epithelial HDAC-2 expression in asthmatics. This is compatible with recent findings in epithelial cells from healthy smokers and COPD patients [8], but contrasts to findings in alveolar macrophages from healthy smokers [7] and COPD patients [6]. Together, these observations show that the effect of smoking on HDAC-2 expression varies between compartments. It is intriguing that smoking is associated with higher HDAC-2 expression in epithelial cells, suggesting a possible anti-inflammatory effect in that compartment. Furthermore, we show that ICS use in non-smoking asthmatics is associated with higher HDAC-2 expression in bronchial epithelial cells, in line with previous findings [5]. This potential beneficial effect of ICS on HDAC-2 expression was not found in smoking asthmatics, as smoking interacted negatively with ICS-use. Future studies have to unravel the exact underlying mechanisms; nevertheless our study shows that smoking and ICS use independently associate with higher HDAC-2 expression in bronchial epithelial cells in asthma. Surprisingly the lack of this effect by their combination again suggests that smoking reduces corticosteroid effectiveness in asthma.

A.



B.

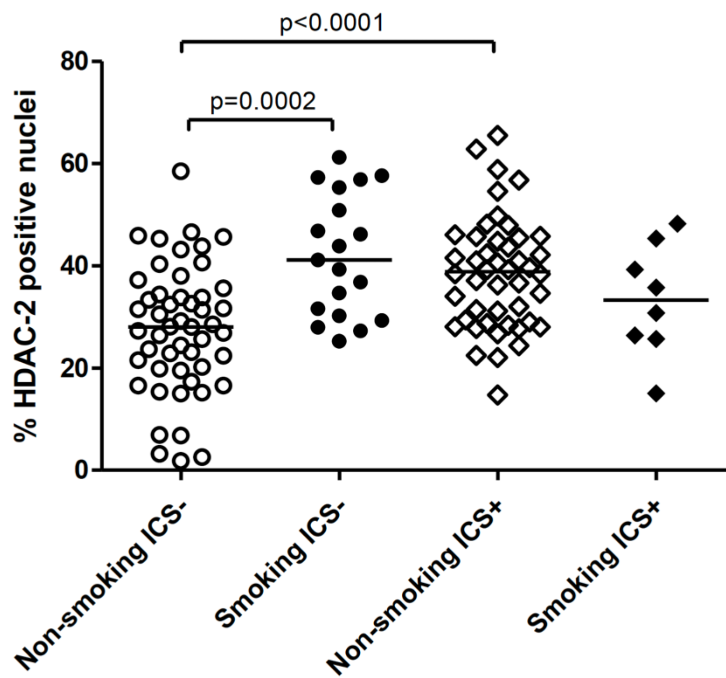


Figure 1: *a) Expression of HDAC-2 in a representative bronchial biopsy from a non-smoking asthmatic on ICS use. b) Percentage of HDAC-2 nucleus positive cells in intact epithelium. Medians and significant differences (MWU) are shown.*

Table 1: Asthmatic patients' characteristics

Characteristics	Non-smoking non-ICS use (50 cases)	Smoking non-ICS use (19 cases)	Non-smoking ICS use (46 cases)	Smoking ICS use (8 cases)
Female sex (%)	26 (52%)	5 (35.7%)	24 (52.2%)	4 (50%)
Age (yr)	47 (25-70)	52 (19-64)	50.5 (19-71)*	36.5 (24-64)
Packyears (yr)	0 (0-44.6)	25.6 (1.4-44)$^{\#,\dagger,\ddagger}$	0.2 (0-63.8)$^{*,\$}$	5.6 (0.4-33.6)$^{\infty}$
Cigarettes/day (n)	--	15 (3-23)	--	10 (3-14)
ICS dose (µg/day) beclomethasone equivalent	--	--	800 (100-2000)	650 (200-1000)
Atopy (%)	33 (66%)	13 (68.4%)	35 (76.1%)	6 (75%)
FEV_1 (% pred)	104 (77.9-127.8)	94.6 (59.8-134.5)	96.3 (42.5-135.5)$^{\$}$	101.4 (81.3-108.6)
FEV_1/VC (%)	78.2 (56.4-97.7)	71.9 (47.6-93.6)	72.5 (39.4-96.7)$^{\$}$	75.9 (54.9-84.4)
Reversibility (% pred)	6.2 (-1.4-28.7)	9.2 (-2.2-17.8)	7.5 (-0.8-38.4)	10.5 (4.6-25.6)
Log PC_{20} AMP	2.8 (-1.7-2.8)	2.3 (-1.7-2.8)‡	2.3 (-2.6-2.8)	0.9 (0.6-1.5)$^{*,\infty}$

Values are medians (ranges) or numbers (proportions).

*ICS= inhaled corticosteroid, Atopy is based on a positive phadiatop, FEV_1= forced expiratory volume in 1 s (FEV_1 was measured after inhalation of 800 µg Albuterol), Reversibility FEV_1= change in FEV_1, expressed as increase in percentage predicted normal value after 400 µg of Albuterol, PC_{20} AMP (mg/ml)= provocative concentration of adenosine 5'-monophosphate causing a 20% fall in FEV_1. *= non-smoking ICS use vs. smoking ICS use $p<0.05$, #= smoking non-ICS use vs. non-smoking non-ICS use $p<0.05$, \$= non-smoking ICS use vs. non-smoking non-ICS use $p<0.05$, †= smoking non-ICS use vs. non-smoking ICS use $p<0.05$, ‡= smoking non- ICS use vs. smoking ICS use $p<0.05$, ∞= smoking ICS use vs. non-smoking non-ICS use $p<0.05$.*

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Chapter 8

Primary lysis/necrosis of eosinophils and clinical control of asthma.
(Authors' response to C. Persson)

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Authors' response

We have read with great interest the comments by Dr Persson [1] on our recent paper in Thorax [2], in which we showed that clinical control of asthma associated significantly with lower numbers of activated eosinophils in the bronchial wall, yet only weakly with sputum eosinophils. As the number of eosinophils in biopsies did not associate with clinical control of asthma, we speculated that activation of eosinophils (measured as eosinophil protein X (EXP)- immunopositive pixels per area) in bronchial biopsies reflects the level of disease control better than the number of eosinophils itself [2]. As lysis of activated eosinophils and degranulation of toxic eosinophil proteins may damage the surrounding tissue [3]. Persson wondered whether EPX immunopositivity in our biopsies associated with epithelial fragility, particularly in uncontrolled asthma.

In line with Persson's hypothesis, the percentage of intact epithelium correlated negatively with EPX immunopositivity (Spearman's $r = -0.30$, $p = 0.016$), whereas there was no significant correlation with the number of eosinophils in bronchial biopsies (Spearman's $r = -0.12$, $p = 0.35$) (figure 1). This was not due to effects of current smoking, which is associated with increased epithelial cell proliferation, goblet cell hyperplasia, as well as with reduced eosinophil numbers in bronchial biopsies in asthma [4]. since we excluded current smokers from our analysis. An additional regression model adjusted for inhaled corticosteroid use and atopy confirmed that loss of epithelial integrity and higher EPX immunopositivity are significantly associated with uncontrolled asthma, yet not with numbers of airway wall eosinophils (data not shown).

Another question from Persson's letter was whether free granules locate in close proximity of denuded epithelium. Unfortunately, this 'geographical' relationship is very difficult to quantify in a reliable way. Moreover, we believe this specific question could be better investigated prospectively using an allergen provocation model; collecting blood, biopsies and sputum at regular time points; similar to what has been done in the past by Aalbers et al. [5] In our existing dataset, the dynamics of transepithelial migration of eosinophils [6] (tissue-lumen correlations) cannot be investigated in a reliable way.

In conclusion, our statistical analysis supports Persson's hypothesis that ongoing lysis of activated eosinophils contributes to uncontrolled asthma. Our previous publication and our current analyses support the notion that loss of epithelial integrity may serve an important role in this respect, since it is independently associated with loss of asthma control.

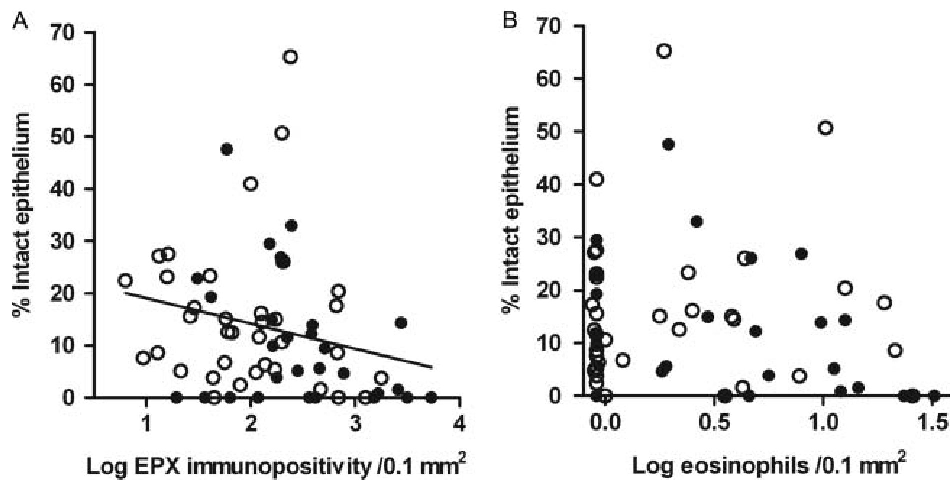

Figure 1 *Correlation between percentage of intact epithelium and eosinophil activation assessed by EPX immunopositivity (panel A) and number of eosinophils (panel B) in bronchial biopsies from non-smoking asthmatics. Open dots: controlled asthma (n=36), solid dots: uncontrolled asthma (n=29). Significant correlation between EPX immunopositivity and % intact epithelium in panel A (Spearman's r=−0.30, p=0.016). Subjects with uncontrolled asthma have less intact epithelium and more EPX immunopositivity (panel A).*

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Chapter 9

Summary, discussion, conclusions and perspectives

Summary, discussion, conclusions and perspectives

The aim of this thesis was to describe, investigate and compare the effects of smoking and atopy in asthma and COPD. How do they change the clinical expression of asthma and COPD and the response to corticosteroids, in relation with underlying changes in pathology and immunology? A part of our research focused on asthmatic patients who have with ageing and smoking a COPD phenotype, resulting in difficulties to discriminate between these two disorders. In chapter 2, we reviewed the literature regarding the differences made by smoking in asthma, both in clinical and pathological aspects. In chapter 3, we focused on this question and addressed whether smoking asthmatics with irreversible airway obstruction could be differentiated from matched COPD patients by comparing their airway wall biopsies. In chapter 4 and 5, atopy was studied as another interesting factor which may change the phenotype of asthma and COPD, resulting in different responses to corticosteroid treatment. Data from a large COPD population (EUROSCOP) was analysed to finding the effect of atopy on incidence/remission of respiratory symptoms in COPD patients after 3-year treatment of inhaled corticosteroids (ICS). In chapter 5, the effect of atopy and the response to ICS were studied in bronchial biopsies from asthma patients. In this population, the expression of the inflammatory markers and IL-17 was compared between ICS and non-ICS users, taking into account the atopy status. In chapter 6, effect of ICS on release of CCL20, as Th17 and neutrophil chemoattractant, from airway epithelial cells was investigated, as well as its implication in a decreased response to glucocorticoids in asthma. In chapter 7, the effect of ICS and smoking on HDAC-2 expression in the bronchial epithelial cells from asthma patients was studied. Finally, in chapter 8, the relationship between lysis of activated eosinophils in the airways of uncontrolled asthma was investigated.

Asthma, smoking and corticosteroid responsiveness

In chapter 2, we reviewed the literature regarding the effects of smoking on asthma outcomes, both in clinical and pathological aspects.

The evidence is now convincing that active smoking is a major factor in modifying the phenotype of asthma and influencing both treatment response and outcomes. Of importance, smoking in asthma patients not only induced a “COPD-like” airway obstruction and airway inflammation, but it also worsened asthma stability [1-3]. Generally, smoking was associated with more severe asthma symptoms, a higher frequency of asthma attacks, more hospital

admissions and lower quality of life [4, 5]. The detrimental effects of smoking in asthma may be due to the induction of a relative unresponsiveness to inhaled and systemic corticosteroids, thereby inducing the inability to inhibit successfully the underlying inflammatory processes in the airways [6, 7]. In our review, we discussed the differences between smoking and nonsmoking asthmatics regarding the clinical expression of asthma, lung function, response to corticosteroids, airway inflammation and remodeling processes. Possible pathogenetic mechanisms that may explain the links between cigarette smoking and changes in the clinical expression of asthma was also discussed, as well as the beneficial effects of smoking cessation. Figure 1 summarizes the possible underlying immunopathological mechanisms explaining the detrimental effects of smoking.

Pathologically, cigarette smoking in asthma is associated with reduced numbers of eosinophils and higher numbers of mast cells in the submucosa of the airway wall. Airway remodeling is increased as evidenced by increased epithelial thickness and goblet cell hyperplasia in smoking asthmatics [8, 9]. The literature clearly describes that the inflammatory profile and phenotype of asthma is affected by corticosteroid use, number of pack-years smoking, having allergic or nonallergic asthma and the severity of asthma [3, 7, 10]. Additionally, smoking cessation has shown to improve asthma control and reverse airway pathological changes [8, 11-13].

Unfortunately, a part of the smoking asthmatics is not able to stop smoking. Future research may perhaps unravel the complex interaction between smoking and asthma, and lead to the discovery of new therapies for smoking-induced corticosteroid unresponsiveness.

Perspective

Asthma is still a major public health problem in most countries; new strategies to better control this disease are necessary especially in the 20% of the asthmatics that smoke (20% being close to the general population) [14]. Any form of tobacco use, especially cigarette smoking, has an important effect in this disease. Reviewing the literature showed that smoking asthmatics are susceptible to several negative outcomes, especially the poor response to corticosteroids.

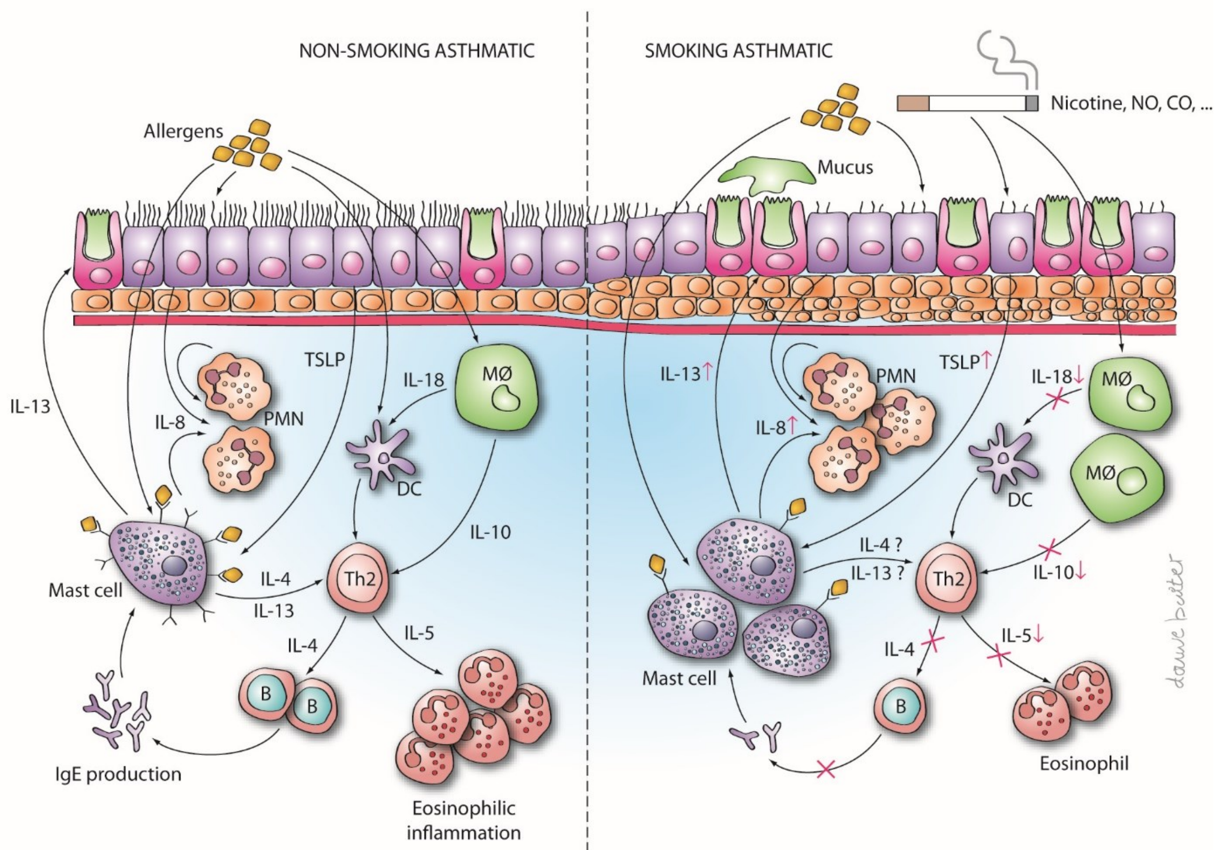

Figure 1. ***Smoking alters inflammatory pathways in asthma.*** *An update of the possible underlying mechanisms of smoke-induced changes in asthmatic airway inflammation. As shown in* ***the left panel****, DCs play a pivotal role in the induction of the Th2- type lymphocytes in nonsmoking asthma. This may be due to the lack of IL-12 production and upregulation of costimulatory molecules. Th2 cells produce IL-4, a cytokine necessary for IgE class switching in plasma cells, and IL-5, known to be involved in eosinophilic migration and activation. After the first contact with allergen, IgE will bind to its receptor on mast cells, which will crosslink after the second contact with the same allergen and induces degranulation of mast cells. A variety of mediators will be released, one of which is IL-13, which may contribute to development of airway remodeling. In allergic asthma, repeated exposure to allergens leads to TSLP production by epithelial cells, which can directly stimulate mast cell activation and release of its mediators. Repeated allergen exposure also activates MØ to produce IL-18 and IL-10, also important in driving a Th2-type response.* ***Right panel:*** *Smoking leads to increased exposure to a variety of toxic chemicals, present in cigarette smoke, such as nicotine, NO and CO. Exposure to smoke in asthma increases the number of MØ, which are involved in phagocytosis of smoke particles and apoptotic cells. These MØ produce less IL-18 and IL-10, which leads to less Th2-cell development, lower numbers of B cells and lower amounts of IL-4 and IL-5. This will lead to less activity and presence of eosinophils in smoking asthmatics and lower production of IgE, the latter being demonstrated in animal studies. Alongside this, exposure of epithelial cells to cigarette smoke is known to enhance production of mediators necessary for repair. In smoking asthma, increased levels of TSLP may further stimulate mast cells to release remodeling mediators such as IL-13, independently of Th2 cells. Increased mast cells, together with smoking-induced epithelial cell activation, may increase IL-8 production, contributing to increased infiltration of neutrophils (PMNs).*
↑ and ↓ indicate higher and lower cytokine levels in smoking compared with nonsmoking asthmatics.
CO: Carbon monoxide; DC: Dendritic cell; MØ: Macrophage; NO: Nitric oxide; PMN: Polymorphonuclear neutrophil; TSLP: Thymic stromal lymphopoietin.

Therefore, clinicians should always consider to prescribe stop-smoking interventions in the management of these patients, because up to one third of the asthmatic smokers show evidence of insensitivity to ICS [15]. One of the recent therapeutic strategies to control asthma in smoking individuals is combining ICS with a long-acting beta2 adrenergic (LABA) [16, 17]. Not surprisingly studies show that the association of ICS plus LABA (e.g. Fluticasone/Salmeterol) by smoking asthmatics results in more pronounced improvements in the airway hyperresponsiveness and airway caliber compared with the use of corticosteroid alone. In fact, in the presence of relative steroid resistance, the smooth muscle stabilization conferred by salmeterol is of greater clinical importance in patients who smoke than in those who do not smoke [16, 17]. Given that smoking predominantly affects the small airways, ICS in extra-fine particles associated with LABA may constitute a new perspective of treatment. Additionally, the use of leukotriene antagonists may become another therapeutic alternative [18-20].

Reviewing the literature showed how smoking cessation results in an improvement of symptoms and pulmonary function, but there is still a group of smokers that is unable to quit smoking. In fact, despite years of smoking prevention programs at a societal level, different studies show that sustained cessation (at 6 months to 1 year) in the overall population is limited (<50%), and the success rate of quitting on the initial try is low as 12% [16, 21]. A constant partnership between patient and provider is needed to succeed in smoking cessation. Unfortunately, frustration (practitioner and/or patient) sometimes results in an undesired outcome. If we accept that smoking is an addiction and not just a bad habit, we have to consider new therapeutic approaches for this population. The part of this claim is that nicotine induce an escalation of physical and psychological dependence through central and peripheral nervous system stimulation and tachyphylaxis [21].

Unfortunately, the clinical trials studying new drugs or therapeutic regimens for asthma generally exclude smokers. This exclusion was recently extended to former smokers [16, 17]. The rationale for such exclusion is that tobacco use by asthma patients is associated with numerous adverse outcomes, making it difficult to analyze the real efficacy of the drug being tested [4]. Consequently, there is a lack of specific information about the treatment of asthma in smokers. It is important to realize that the asthmatic smoker reflects a special phenotype with important therapeutic and prognostic clinical implications.

As a summary and conclusion, although quitting smoking can improve symptoms and lung function in asthmatic patients, the low rates of smoking cessation highlight the need for

improved strategies for managing these patients. Clinical trials assessing new therapies for asthma need to enroll smokers to identify treatments that are effective in the smoking asthma phenotype. In the meantime, combined behavioral interventions (health professional counseling, group therapy, and help lines) and first-line pharmacologic medications for smoking cessation (nicotine replacement therapy, bupropion and varenicline) [21, 22] are recommended to increase quitting rates, particularly as the extent of tobacco use increases. As a medical community, we must continue to expand our knowledge to better meet this challenge.

Old dilemma: is it Asthma with irreversible airway obstruction or COPD?

In the third chapter, we investigated whether it is possible to discriminate between asthma with irreversible airway obstruction and COPD. Although they have a completely different etiology and pathophysiology, they may become look-a-likes when ageing and smoking contribute to chronic airway obstruction and respiratory symptoms [10]. In the past, Bourdin et al tried to address this question by studying criteria in the bronchial biopsies from the asthma and COPD patients but they did not match these 2 groups regarding age, sex and lung function [23]. In our study, we investigated whether pathologists are able to differentiate between asthma or COPD, using bronchial biopsies. Biopsies from 24 asthma and 24 COPD patients were investigated to find out which criterium/criteria is/are helpful to discriminate between the two diseases. In this study asthma and COPD patients were carefully pair-wise matched based on ICS use, age, FEV1, and smoking habits. We also compared a group of 8 classic (non-obstructive) asthmatics with 8 severely obstructed COPD cases; we did so since discrimination between these 2 groups is not difficult. Ten (5 lung- and 5 general) pathologists, not aware of the patients' clinical background, examined bronchial biopsies using an interactive website. They were asked to diagnose asthma or COPD on biopsy findings in both a pair-wise and randomly mixed order of cases during 4 different phases. Correct diagnoses were scored between 63% and 73%, without important differences between pair-wise and randomly mixed examination or between general and lung pathologists. Surprisingly, these low scores contradicted with the high associations observed between pathological criteria in epithelium, basement membrane (BM) and submucosa and the correct diagnosis of asthma or COPD. Highest percentage of a correct diagnosis was found in young asthmatics who did not use ICS, and in COPD patients who did use ICS. In non ICS-users, a COPD diagnosis was favored if abnormal presence of glands, squamous metaplasia and

submucosal infiltrate was observed, and an asthma diagnosis was favored with goblet cells. In ICS-users the observation of abnormal presence of submucosal infiltrates, basement membrane thickening, eosinophils and glands associated with asthma. We concluded, that in spite of the poor ability of the pathologists to differentiate between asthma and COPD, they may improve their success rates if they use the pathological criteria described above, on condition they take the clinical setting into account, particularly the use of ICS.

Perspective

In general, careful history taking, physical examination, and lung function testing often lead to a clear diagnosis for asthma or COPD [24]. But, it is frequently difficult, if not impossible, to achieve an accurate diagnosis of either asthma or COPD in older patients particularly if they smoke (leading to having irreversible airway obstruction). The data present in our study provide important information about different pathological criteria which help pathologist to diagnose these problematic cases and to discriminate between those asthmatics with irreversible airway obstruction from COPD. However, because bronchoscopy is still an invasive procedure we anticipate that most clinicians will not embrace this idea. Less invasive biomarkers that predict treatment response is what the clinicians really need, and this need fits with the general concept of heterogeneity of airway obstructive diseases and the aim to define treatable traits in the frame of precision medicine. Does this mean that the Old Dilemma story ends at this point, and that bronchial biopsies should be forgotten? In our opinion there are at this moment no reliable non- invasive biomarkers that may help clinicians with the 'Old Dilemma' story: is this a patient with asthma or COPD? For the time being we have to rely on the arbitrary definitions of the GINA guidelines (which are a kind of biomarker). And for sure bronchial biopsy studies will be started to validate the GINA concepts in the next future. And probably these biopsy studies will be combined most likely with gene expression experiments and non-invasive tools, to explore using different treatment strategies. In that perspective the Old Dilemma "is it asthma with irreversible airway obstruction or COPD" will live on, yet in another form.

Based on our data and some other studies [23], there might become a possibility in the future to consider taking bronchial biopsy and use the pathological criteria (as described in our study) when in doubt for a COPD or asthma diagnosis. Based on our multicenter study data, the clinical information about the patient (e.g. use of ICS, smoking history, age) should be taken into account to make a definitive diagnosis.

Atopy, corticosteroid use, and respiratory symptoms in COPD

In chapter 4, we analysed the data from EUROSCOP, a large multi-center study performed in 39 centers in 9 European countries, which has been designed to assess the effect of 3-year treatment with inhaled budesonide on lung function decline in smoking COPD patients. The results of the original study showed a little improvement in lung function after 6 months in the treated group but no differences in long-term lung function decline [25]. However, the effect of atopy on lung function and respiratory symptoms was not evaluated in this large longitudinal study. Therefore, we assessed which factors associate with the presence of atopy in this COPD population, and whether there is a difference between atopic and non-atopic COPD patients regarding prevalence, incidence and remission of respiratory symptoms as well as lung function decline over the 3-year follow-up of the study. First of all, we found that the prevalence of atopy (as assessed by ImmunoCAP Specific IgE: Phadiatop test) was significantly higher in COPD patients who were males, had overweight/obesity and lower age. Regarding the effect of atopy on COPD symptoms we showed that atopy was significantly associated with a higher prevalence of cough and phlegm, but not with lung function levels or FEV1 decline. Regarding the effect of atopy on incidence and remission of respiratory symptoms, we found that atopic COPD patients who were not treated with budesonide more likely developed cough over time compared to non-atopic COPD patients, while those treated with budesonide more likely lost their cough compared to non-atopic COPD patients.

We were the first to publish about the effect of atopy on the long-term course of COPD [26]. Only an ATS abstract [27] reported data from 1424 COPD patients obtained from "The National Health and Nutrition Examination Survey (NHANES)" III (1988–1994) in Johns Hopkins University. This study found that individuals with indications of allergic disease more likely reported having episodes of sinusitis, and an additional trend towards more frequent reporting of cough and wheeze compared to non-allergic individuals. A problem of the NHANES derived study was the lack of a severity classification of atopy. They defined atopic COPD subjects (n = 346) on basis of the presence of any one of the following criteria: at least one positive skin prick test, self-reported doctor diagnosed hay fever, or symptoms induced by house dust, animals or pollen. Our study defined atopy objectively by specific IgE positivity and excluded subjects with a history of asthma, allergic rhinitis, or allergic eczema. As we excluded subjects with allergic diseases, we believe our data more closely reflect the effect of atopy on COPD-related cough. After we published our results [26], the complete

study of the NHANES study was published [28]. One year later, the same group showed the individuals with atopic COPD appear to be at higher risk of adverse respiratory health effects of indoor particulate matter exposure compared to non-atopic COPD, again showing the negative association of atopy with clinical effects in COPD. Increase in particulate matter (each 10 µg/m3) among the atopic individuals was significantly associated with higher risk of nocturnal symptoms, frequent wheezing, increased rescue medication use, higher St. George's Respiratory Quality of Life score, and higher breathlessness, cough, and sputum score [29].

Perspective

Data from our study and study by Hansel's group in the NHANES study [28] which both included a large population in Europe and the USA respectively showed that the presence of an atopic phenotype is associated with increased risk of respiratory symptoms among individuals with COPD. We therefore urge clinicians to remember not to ignore the presence of atopy in COPD patients, it may have detrimental effects!. Furthermore, because our observations suggest that atopic populations can get more remission of respiratory symptoms while taking ICS, studies are needed to determine whether or not pharmacologic treatment of atopy may lead to improvement in respiratory health in COPD. Based on the data from our study and others [28], it seems that atopic COPD patients benefit from corticosteroid treatment but there is very little clinical guidance for a differential management of atopics versus non-atopics with COPD. If we accept that atopic COPD patients from now on should be treated with ICS, this would widen the present indications for ICS as defined by GOLD (Global Initiative for Chronic Obstructive Lung Disease) [30].

Atopy and ICS use affect IL-17

In chapter 5, we studied the effect of atopy and ICS on IL-17 expression in the airways of asthmatic patients. One of the cytokines that was recently suggested to contribute to the pathogenesis of non-atopic (non-eosinophil/neutrophil-dominant) asthma is IL-17 [31]. There is no other study like ours' comparing IL-17 expression in well characterized atopic and nonatopic asthma patients so far. We investigated the expression of IL-17 in 114 asthmatic patients by counting the number of IL-17 positively stained cells in the submucosa of bronchial biopsies and exploring which cells types predominantly stain positive for IL-17. Our asthma cohort included 81 atopic (determined by positive Phadiatop) vs. 33 nonatopic

asthmatic patients. Among them, 51 patients were using ICS and 63 were not. We first divided our population based on the ICS or non-ICS use, then compared the IL-17 expression between atopic and non-atopic in each group. In the non-ICS user group, nonatopic asthmatics had more IL-17+ cells in the airway wall than atopic asthmatics. In line with this finding and expanding our observation, we found lower numbers of IL-17 positive cells with higher levels of the Phadiatop score. ICS use was associated with lower numbers of IL-17+ cells in both atopic and nonatopic asthmatics compared to asthmatics without ICS use.

Regarding the lung function data, in the nonICS users (both atopic and nonatopic subjects) with higher IL17 positive cell numbers had lower levels of reversibility. There was also a negative correlation between FEV1% predicted and IL-17 levels in the atopic individuals who did not use ICS. There was no association between current smoking and IL-17 levels.

Our regression analyses confirmed our data about the eliminating effect of atopy and ICS use on IL-17 levels. We demonstrated that the absence of atopy and non-ICS use most strongly contributed to a higher number of IL-17 expressing cells.

We showed that IL17-positive cells were predominantly neutrophils (all relevant details are in the chapter).

This is of interest since nonatopic asthmatics who did not use ICS had higher IL-17 expression in bronchial biopsies than atopic asthmatics, suggesting a potential role of IL-17 in the pathogenesis of nonatopic asthma. ICS use was associated with lower numbers of IL-17+ cells in both atopic and nonatopic asthmatics, suggesting a beneficial effect of ICS in general.

Importantly, IL-17+ cells were mostly neutrophils, which conflicts with the paradigm that lymphocytes (Th17) are the main source of IL-17. This supports the reports showing the early sources of IL-17 are the innate immune cells and they have a central role in the initiation of IL17-dependent immune responses, even before the first CD4+ T cell sees its cognate antigen and initiate the Th17 development program [32].

Perspective

IL-17 has recently gained more attention as a new target for treatment of several inflammatory diseases including asthma, psoriasis, rheumatoid arthritis, Crohn's disease and psoriatic arthritis This cytokine is upregulated at sites of inflammation and can synergize with other cytokines including TNF-α, to amplify the inflammatory response. Activation of these signaling pathways has been hypothesized to contribute to the underlying pathogenesis of these inflammatory diseases [33].

In our study we showed a significant negative correlation between IL-17+ cells numbers and

reversibility levels in the asthmatics who did not use ICS (both atopic and nonatopic subjects). Moreover, in atopic asthmatics not using ICS there was a negative correlation between FEV1% predicted and IL-17+ cells numbers. These correlations between levels IL-17+ cells and clinical data (respiratory function) are important as it shows the role of this cytokine in the respiratory function of asthmatic population. A clinical trial using anti–IL-17A (brodalumab) [34] did not find a beneficial treatment effect in asthma. Only the high-reversibility subgroup of their patients (post-bronchodilator FEV1 improvement ≥ 20%) showed improvement in Asthma Control Questionnaire (ACQ) score. The authors did not measure IL-17+ cells in airway biopsies, or IL17 levels in blood in their study. We found that the numbers of IL-17+ cells remarkably depend on the asthmatic phenotype (atopy vs. nonatopy) and use of ICS treatment (with vs without). Further clinical studies including more asthmatic patients and taking into account at least atopy and ICS use and measuring IL-17 levels in biopsies in the patients is required. Perhaps this mAb treatment (brodalumab) is only beneficial for nonatopic asthmatics who are not taking ICS and have levels of IL-17.

Corticosteroid insensitivity and CCL20

Th17-mediated neutrophilic airway inflammation has been implicated in a lower response to glucocorticoids in asthma [35]. One of the possible mechanisms behind glucocorticoid-insensitive asthma is CCL20 upregulation in epithelial cells in the airways of asthmatics using ICS which is Th17 cell and neutrophil chemoattractant. In chapter 6, we showed a novel mechanism in Th17-mediated glucocorticoid-insensitive inflammation in asthma. For this aim, we investigated effect of ICS on release of the CCL20 from airway epithelial cells. We assessed whether the epithelial release of CCL20 is sensitive to glucocorticoids and compared CCL20 levels in sputum from asthmatics using ICS and non-ICS users. We also investigated release of CCL20 by primary bronchial epithelial cells from asthma patients upon treatment with glucocorticoids in vitro. Interestingly, we found that CCL20 levels were higher in sputum of asthmatics who used ICS, and that glucocorticoids increased the release of CCL20 by primary bronchial epithelial cells in vitro. Data from asthmatics showed that CCL20 levels positively correlated with ICS dose and sputum neutrophils as expected. Regarding the mechanism responsible for CCL20 upregulation by glucocorticoids, we applied several studies using inhibitors for the glucocorticoid receptor, intracellular pathways and metalloproteases in bronchial epithelial cell line 16HBE. Inhibition of glucocorticoid receptor or ADAM17 abolished the budesonide-induced increase in CCL20 levels. Our data suggest

that increased production of CCL20 in human bronchial epithelium is mediated by the glucocorticoid receptor and dependent on ADAM17 activity. These findings may constitute a novel mechanism in Th17-mediated glucocorticoid-insensitive inflammation in asthma.

Perspective

In the previous section we explained the role of IL-17+ cells and its correlation with neutrophil numbers. In this section we described the role of a chemokine induced by this cytokine. IL-17 can induce expression of CC-chemokine ligand 20 (CCL20), which promotes trafficking of mucosal-associated cells expressing CC-chemokine receptor 6 (CCR6); notably, CCR6 is a characteristic receptor on IL-17-expressing cells such as Th17 cells and ILC3s [36]. CCL20 which is regulated by IL-17 serves to recruit CCR6 positive cells, including Th17 cells [37]. Our study showed the role of Th17 cells in glucocorticoid-insensitive inflammation in asthma. Th17 cells migrate toward CCL20, the ligand for CCR6, but also secrete CCL20. Disruption of the CCR6/CCL20 axis may also be a novel therapeutic approach in treating asthma patients who are so-called steroid resistant.

Smoking, ICS use, and HDAC-2 expression

In chapter 7, we investigated the effect of smoking and ICS use on HDAC-2 expression in bronchial biopsies from 123 asthma patients. As epithelial cells constitute the first barrier of the lungs against inhaled smoke, we studied this compartment for HDAC-2 expression using immunohistochemistry. The percentage of HDAC-2 nucleus positive cells was counted in intact epithelium. We compared percentage of HDAC-2 expression between smoking and non-smoking asthmatics, and evaluated effects of ICS use.

Our study for the first time demonstrates that smoking is associated with higher epithelial HDAC-2 expression in asthmatics which was compatible with earlier findings in epithelial cells from healthy smokers and COPD patients [38]. The interesting point of our study was that exsmoking asthmatics showed the same pattern as never smoking asthmatics (Figure 2), suggesting that the effects of smoking are reversible with respect to HDAC-2 expression.

Our study showed that smoking and ICS use independently associate with higher HDAC-2 expression in bronchial epithelial cells in asthma. Surprisingly, the lack of this effect by their combination again suggests that smoking reduces corticosteroid effectiveness in asthma. Of course, we have to be careful in our interpretations because this was an observational study

without random smoking or corticosteroid use.

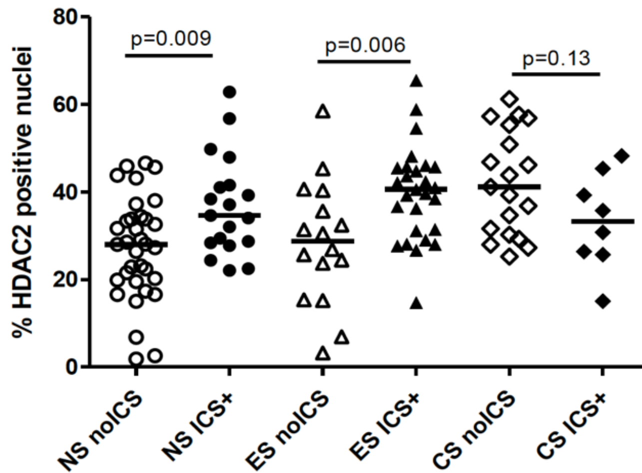

Figure 2. HDAC-2 expression in bronchial epithelial cells from asthmatic airways.
NS: Never smoker, ES: Ex-smoker, CS: Current smoker, ICS: Inhaled corticosteroid

Perspective

In general, decreased of HDAC-2 is suggested to be involved in corticosteroid resistance mechanisms in severe asthma and COPD [39]. Our data is opposite to what we expected, but we should realize that our data was from an observational study on mild to moderate asthma. We also did not study the expression of HDAC-2 in the submucosal airway tissues of asthmatic patients, so we don't know what the response of this compartment to ICS and smoking is. A randomized controlled trial comparing ICS, smoking, ICS plus smoking, and control in mild to severe non-smoking asthmatics, taking bronchial biopsies at baseline and after treatment, and measuring steroid sensitivity at baseline and after treatment, could give answers. However, such a trial will not start, not in the least for ethical reasons.

Uncontrolled asthma and activation of eosinophils

In chapter 8, we investigated the correlation between uncontrolled asthma (according to the GOAL study) and activation of eosinophils. This research was an answer on a letter by Persson [40], who responded on our previous publication entitled "Clinical control of asthma associates with measures of airway inflammation" [41]. We studied the effect of primary lysis/necrosis of eosinophils on clinical control of asthma. According to our previous paper we speculated that activation of eosinophils (EPX+ levels) in bronchial biopsies reflects the level of disease control better than the number of eosinophils itself [41]. Indeed, increased

EPX immunopositivity in our biopsies associated significantly with lower intact epithelium , particularly in uncontrolled asthma. This finding suggests that lysis of activated eosinophils and degranulation of toxic eosinophil proteins may damage the surrounding tissue in asthmatic airways as hypothesized by Persson [40], in this way contributing to uncontrolled asthma.

Perspective

The theory of eosinophils lysis by Persson is very interesting [40] as the occurrence of free eosinophil granules in airway tissues and sputum samples were either ignored or considered an artefact caused by sample handling in the past years. But tissue staining of eosinophil peroxidase (EPX) clearly demonstrated that free eosinophil granules were a real in vivo phenomenon which was not preceded by apoptosis. Interestingly, several studies show the positive correlation between the number of free eosinophil granules in the airway wall with disease activity in asthma and rhinitis [41-43]. These data support Persson's perception that eosinophil lysis releasing granules (plus many other biologically active nuclear/cytosolic molecules) may have a causative role in pathology and clinics of eosinophilic airways diseases.

We therefore recommend to investigate activation of eosinophils (EPX+ levels) in biopsy studies of asthma.

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Appendices

Nederlandse Samenvatting
Acknowledgments
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List of publications

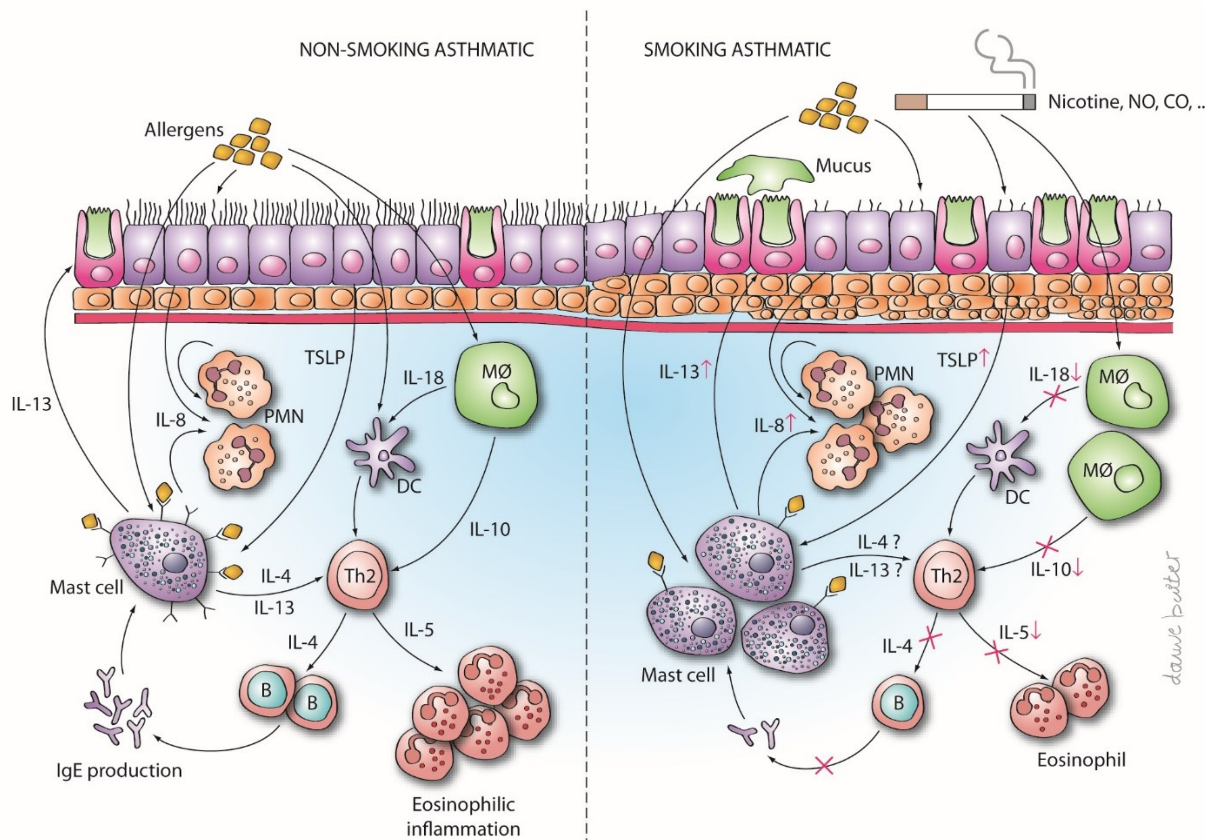
Samenvatting, discussie, conclusies en perspectieven

Doel van dit proefschrift was om de effecten van roken en atopie bij de nauw verwante ziektebeelden astma en COPD te onderzoeken. Hoe beïnvloeden roken en atopie de klinische expressie van astma en COPD, en de respons op corticosteroïden, in relatie met onderliggende pathologische en immunologische veranderingen? Een deel van het onderzoek richtte zich op astmapatiënten die door roken en veroudering een COPD-achtige verschijningsvorm kregen, resulterend in de vraag hoe deze vorm astma onderscheiden kan worden van "puur" COPD. In hoofdstuk 2 beschrijven wij de literatuur over de pathologische en klinische gevolgen van roken bij astma. In hoofdstuk 3 onderzochten wij of luchtwegbiopten van rokende astma patiënten met irreversibele luchtwegobstructie te onderscheiden zijn van luchtwegbiopten van COPD patiënten met dezelfde leeftijd en mate van luchtwegobstructie, en daarmee kunnen bijdragen aan de juiste diagnose. In hoofdstuk 4 en 5 werd de invloed van atopie onderzocht op het klinische fenotype en de corticosteroïdgevoeligheid van astma en COPD. Resultaten van een groot COPD-cohort werden gebruikt om te achterhalen of atopie geassocieerd was met de incidentie en remissie van respiratoire klachten bij COPD patiënten die resp. 3 jaar wel of niet behandeld werden met een inhalatiecorticosteroïd (ICS). In hoofdstuk 5 werd het effect van atopie en de bijdrage van ICS-gevoeligheid bestudeerd in luchtwegbiopten van astmapatiënten. Hierbij werd de ontsteking en expressie van IL-17 vergeleken tussen ICS gebruikers en niet-ICS gebruikers, rekening houdend met de aanwezigheid van atopie. In hoofdstuk 6 werd het effect bestudeerd van ICS op de uitscheiding van CCL20 (een chemoattractant van Th17+ en neutrofiele cellen) door luchtweg epitheliale cellen, alsmede de gevolgen m.b.t corticosteroïdgevoeligheid bij astma. In hoofdstuk 7 werd het effect van ICS en roken op de expressie van HDAC-2 in het epitheel van luchtwegbiopten van astmapatiënten onderzocht. Tenslotte, werd in hoofdstuk 8 (tekenen van) lysis van geactiveerde eosinofiele granulocyten onderzocht in relatie met het wel/niet hebben van goede controle van astma.

Astma, roken en corticosteroïdgevoeligheid

Hoofdstuk 2 is een review over het effect van roken op klinische en pathologische aspecten van astma. Er zijn overtuigende aanwijzingen dat actief roken het klinische fenotype van astma verandert evenals behandelresultaten. Een belangrijke waarneming daarbij is dat roken een "COPD-achtig" ziektebeeld veroorzaakt, met een persisterend matige controle van astma

[1-3]. In zijn algemeenheid is roken geassocieerd met aanwezigheid van ernstiger astma symptomen, een hogere frequentie van astma aanvallen, meer opnames in het ziekenhuis, en lagere kwaliteit van leven [4-5]. Deze verslechtering is mogelijk het gevolg van de inductie van relatieve corticosteroïdongevoeligheid, met daarmee een minder goede remming van ontstekingsprocessen in de luchtwegen [6-7]. In het review bediscussiëren we verschillen tussen rokende en niet-rokende astmatici m.b.t. de kliniek, longfunctie, corticosteroïdgevoeligheid, luchtwegontsteking en luchtwegremodellering. Mogelijke pathogenetische mechanismen die de link vormen tussen roken en de veranderde klinische expressie van astma worden besproken, evenals de positieve effecten van stoppen met roken. Figuur 1 beschrijft de voornaamste onderliggende immunopathologische mechanismen die een rol kunnen spelen bij de negatieve effecten van roken. In pathologisch opzicht is sigaretten roken geassocieerd met geringere aanwezigheid van eosinofiele granulocyten en toegenomen aanwezigheid van mestcellen in de submucosa van de luchtwegwand. Bij rokende astmatici wordt luchtwegwandremodellering gezien, in de vorm van een verdikte epitheliale laag en gobletcelhyperplasie [8-9]. De literatuur geeft aan dat het inflammatoire profiel en klinische fenotype van astma beïnvloed wordt door corticosteroïdgebruik, aantal pakjaren roken, de aanwezigheid van allergisch of niet-allergisch astma, en de ernst van astma [3,7,10]. Tevens geeft de literatuur aan dat stoppen met roken de kliniek van astma verbetert en de pathologische luchtwegveranderingen doet verminderen [8,11-13]. Helaas is het een vaststaand feit dat niet iedere rokende astmaticus kan stoppen met roken. Toekomstig onderzoek kan wellicht de complexe interactie tussen roken en astma ophelderen, en leiden tot de ontwikkeling van medicijnen die de roken-geïnduceerde corticosteroïdongevoeligheid opheffen.



Figuur 1. Roken verandert ontstekingsroutes in astma. Een update van de onderliggende mechanismen die mogelijk bijdragen aan roken-geïnduceerde veranderingen in de astmatische luchtwegontsteking. *In het* ***linker paneel*** *is te zien dat DC cellen een sleutelrol spelen bij de inductie van Th2 lymfocyten in niet-rokende astmatici. Dit is mogelijk het gevolg van een gebrek aan IL-12 productie en opregulatie van co-stimulerende moleculen. Th2-lymfocyten produceren IL-4, een cytokine nodig voor de IgE switch van plasmacellen, en IL-5, dat betrokken is bij de activatie en migratie van eosinofiele granulocyten. Na de eerste blootstelling aan een allergeen, bindt IgE aan receptoren op mestcellen, welke crosslinken na het tweede contact met hetzelfde allergeen, en vervolgens een degranulatie van mestcellen veroorzakend. Een breed scala aan mediatoren komt vrij, waaronder IL-13, wat bijdraagt aan remodellering van astmatische luchtwegen. Herhaalde blootstelling aan allergenen leidt in allergisch astma tot verhoogde TSL productie door epitheliale cellen, wat een rechtstreekse stimulatie van mestcellen en ontlading van mestcellen geeft. Herhaalde blootstelling aan allergenen activeert ook MØ cellen om IL-8 en IL10 te gaan produceren, wat mede bijdraagt aan de Th2 respons. In het* ***rechter paneel*** *is te zien dat roken associeert met (extra) blootstelling aan een breed palet van toxische chemicaliën aanwezig in sigarettenrook, zoals nicotine, NO en CO. Rookblootstelling verhoogt het aantal MØ cellen, welke betrokken zijn bij de fagocytose van rookdeeltjes en apoptotische cellen. Deze MØ cellen produceren minder IL-18 en IL10, wat leidt tot minder Th2 ontwikkeling, lager aantal B cellen, en lagere productie van IL-4 en IL-5. Dit leidt tot een lager aantal en verminderde activiteit van eosinofiele granulocyten in rokende astmatici, en lagere productie van IgE (zoals in dierstudies is aangetoond).*
Daarnaast is bekend dat blootstelling van epitheliale cellen aan rook leidt tot het extra vrijmaken van mediatoren betrokken bij reparatieprocessen. Verder kunnen verhoogde concentraties TSLP de mestcellen stimuleren om mediatoren uit te scheiden die betrokken zijn bij remodellering, zoals het cytokine IL-13, en dit onafhankelijk van Th2 cellen. Een verhoogd aantal mestcellen, samen met roken- geïnduceerde activatie van epitheelcellen, kan leiden tot een hogere IL-8 productie, welke bijdraagt aan rekrutering en infiltratie van neutrofiele granulocyten (PMSs).
↑ en ↓ wijzen op hogere en lagere cytokines uitscheiding bij rokende in vergelijking met niet-rokende astmatici. CO: koolstof monoxide, DC: dendritische cel, MØ: macrofaag, NO: stikstof monoxide, PMN: polymorfe neutrofiele granulocyt, TSLP: thymic stromal lymphopoetine.

Perspectief

Astma is nog steeds een belangrijk wereldwijd gezondheidsprobleem in veel landen; nieuwe

strategieën om deze ziekte beter onder controle te brengen zijn hard nodig, vooral in de 20% rokende astmatici (20% is ook ongeveer de rookprevalentie in de algemene populatie) [14]. Het review beschrijft de negatieve effecten van roken, met speciale aandacht voor de verminderde corticosteroïdgevoeligheid. Gezien de beschreven negatieve effecten van roken heeft iedere behandelaar de plicht om het roken te ontmoedigen, en de patiënt effectieve stop-roken interventies aan te bieden [15]. Een van de strategieën om een betere controle van astma te bewerkstelligen bij rokende astmatici is het toevoegen van een LABA aan ICS [16,17]. Het is geen verrassing dat de combinatie ICS-LABA bij rokende astmatici minder luchtwegobstructie en bronchiale hyperreactiviteit geeft dan ICS alleen. Opmerkelijk echter is wel dat de luchtwegstabilisatie door LABA meer uitgesproken is bij rokende dan niet-rokende astmatici [16,17]. Gezien het feit dat roken vooral de kleine luchtwegen treft, en inhalatie van kleine deeltjes ICS-LABA beter de perifere luchtwegen bereikt dan inhalatie van grote deeltjes, kan een lans gebroken worden om rokende astmatici preferentieel met kleine inhalatiedeeltjes te behandelen. Een andere manier om de perifere luchtwegen te bereiken is door systemische therapie, bijvoorbeeld door orale inname van een leukotriene antagonist [18-20].

Het review toont hoe stoppen met roken een verbetering van de kliniek van astma geeft, maar de werkelijkheid is dat helaas niet iedereen kan stoppen. In de algemene populatie zijn de stoppercentages laag (12%), terwijl minder dan 50% van de stoppers het lukt om blijvend te stoppen [16,21]. Een goede band tussen patiënt en behandelaar helpt, maar kan niet altijd de frustraties voorkomen die leiden tot persisterend roken. Wanneer we accepteren dat persisterend roken een uiting is van verslaafd zijn, en niet slechts een slechte gewoonte, dan ontstaat ruimte voor het toepassen van nieuwe therapieën bij deze groep van mensen. Het is de nicotine die een cascade van fysieke en psychologische verslaving veroorzaakt door stimulatie en tachyfylaxie van het perifere en centrale zenuwstelsel [21].
Helaas excluderen klinische studies die het effect van medicijnen op astma onderzoeken bijna altijd de rokende astmatici. Zelfs is er een trend om ex-rokers te excluderen [16,17]. De rationale hiervoor is dat roken door astmatici is geassocieerd met tal van negatieve uitkomsten, wat ten koste gaat van de evalueerbaarheid van het te testen medicijn [4].
Hierdoor is er relatief weinig bekend over het effect van medicatie bij rokende astmatici. Het geeft ook aan dat real-life studies, met minder restrictieve inclusie van astmatici, vaker overwogen zouden moeten worden.
Samenvattend verbetert stoppen met roken de kliniek van astma, maar helaas is een belangrijk

deel van de astmatici niet in staat om te stoppen. Klinische trials zijn nodig die ook rokende astmatici onderzoeken, opdat duidelijk wordt welke therapie het beste aanslaat bij dit fenotype. Rokende astmatici kunnen net als rokers zonder astma behandeld worden met gedragsinterventies (counseling, groepstherapie, telefonische helplijnen), medicamenteuze interventies (nicotine substitutie, bupropion, varenicline) [21,22].

Het oude dilemma: astma met niet-reversibele luchtwegobstructie of COPD?

In hoofdstuk 3 onderzochten wij of het mogelijk is om astma met irreversibele luchtwegobstructie te onderscheiden van COPD. Hoewel deze luchtwegziektes een compleet verschillende etiologie en pathofysiologie hebben, kunnen ze in de loop van de jaren op elkaar gaan lijken doordat het verouderingsproces en het persisterend roken gaan bijdragen aan chronische luchtwegobstructie en respiratoire klachten [10]. In het verleden trachtte Bourdin een antwoord op deze vraag te geven door pathologische criteria te formuleren voor de beoordeling van luchtwegbiopten van astma en COPD patiënten [23], echter de studie matchte astma en COPD patiënten niet op basis van leeftijd, geslacht, of longfunctie. In onze studie onderzochten wij ook of pathologen in staat zijn om onderscheid te maken tussen astma en COPD, zich baserend op microscopische bestudering van centrale luchtwegbiopten. Biopten van 24 astma en 24 COPD patiënten werden bekeken om uit te vinden welk(e) pathologisch(e) criterium (criteria) het best bijdragen aan een juist onderscheid. In tegenstelling tot de studie van Bourdin waren de patiënten nauwkeurig paarsgewijs gematched wat betreft ICS gebruik, leeftijd, FEV1, en rookgewoontes. Wij vergeleken ook 8 klassieke (niet-obstructieve) astmatici met 8 klassieke (ernstig obstructieve) COPD patiënten. Vijf pathologen gespecialiseerd in longziekten en vijf algemene pathologen, die niet geïnformeerd waren over de kliniek van de patiënt, kregen de mogelijkheid om op een interactieve website luchtwegbiopten te bekijken. Hun werd gevraagd om astma of COPD te diagnosticeren op basis van luchtwegbiopten die astma en COPD paarsgewijs of ‘at random’ aanboden. In het kader van reproduceerbaarheid vond het onderzoek in 4 fases plaats. De correcte diagnose werd in 63-73% van de gevallen gesteld, waarbij het weinig uitmaakte of de biopten paarsgewijs of at random werden aangeboden, en er was ook geen verschil tussen longpathologen en algemene pathologen. Tot onze verbazing contrasteerden de matige scores van de juiste diagnose (astma of COPD) met een aantal pathologische criteria die significant verschillend werden gescoord tussen astma en COPD. Het hoogste aantal correcte diagnoses werd gesteld in jonge astmatici en COPD patiënten die geen ICS gebruikten. Bij niet-ICS

gebruik wees de abnormale aanwezigheid van kliertjes, squameuze metaplasie en submucosale infiltratie in de richting van COPD, terwijl dit bij astma de aanwezigheid van eosinofiele granulocyten en gobletcellen was.

Perspectief

In het algemeen leidt een zorgvuldig afgenomen anamnese, goed uitgevoerd lichamelijk onderzoek, en adequaat reversibiliteitsonderzoek tot het juist stellen van de diagnose astma of COPD [24]. Bij oudere personen, vooral wanneer zij roken, en bij een niet-reversibele luchtwegobstructie, is dit een stuk lastiger, zo niet onmogelijk. Onze onderzoeksresultaten kunnen in een dergelijke situatie helpen de juiste diagnose te stellen. Echter, omdat veel clinici bronchoscopisch onderzoek belastend voor de patiënt vinden, zal dit niet vaak overwogen worden. Wat clinici werkelijk nodig hebben zijn minder invasieve biomarkers die de therapie respons voorspellen. Dit sluit aan bij het concept dat astma en COPD heterogene ziektes zijn, en dat er behoefte is aan een geïndividualiseerde behandeling; tegenwoordig spreekt men wel van ‘treatable traits’ en precisie-geneeskunde. Betekent dit het einde van het “oude dilemma” verhaal, en dat luchtwegbiopten niet aan de orde zijn? Volgens ons zijn er op dit moment geen betrouwbare biomarkers die clinici met zekerheid helpen om de juiste diagnose te stellen. We zullen ons voorlopig moeten behelpen met de arbitraire definities van GINA en GOLD, die in feite ook gebruik maken van biomarkers. Waarschijnlijk blijft onderzoek van luchtwegbiopten nodig om toekomstige concepten van GINA en GOLD te onderbouwen. Waarschijnlijk zal dit onderzoek gecombineerd gaan worden met gen-expressie experimenten en niet-invasieve meettechnieken, opdat verschillende behandelstrategieën gelinkt kunnen worden aan een onderliggend endotype. In dat opzicht zal het oude dilemma: “is het astma met irreversibele luchtwegobstructie of COPD” voortleven, alleen in een andere vorm.

Samenvattend suggereert onze studie en die van Bourdin [23] dat het nemen van luchtwegbiopten en het gebruik maken van boven beschreven pathologische criteria behulpzaam kan zijn wanneer men twijfelt aan de diagnose astma of COPD. Dit is echter alleen zinvol wanneer de patholoog rekening houdt met klinische informatie zoals ICS-gebruik, rookgewoontes, en leeftijd.

Atopie, corticosteroïdgebruik, en respiratoire klachten bij COPD

In hoofdstuk 4 maakten we gebruik van data van de EUROSCOP-studie, een groot placebo gecontroleerd onderzoek uitgevoerd in 39 centra in 9 Europese landen, met het doel om het effect van 3 jaren budesonide behandeling te bestuderen in relatie tot achteruitgang in longfunctie bij rokende COPD patiënten. De resultaten van de originele analyse lieten zien dat de longfunctie 6 maanden na start van budesonide significant beter was dan bij placebo gebruik, maar dat de (jaarlijkse) achteruitgang in longfunctie na 6 maanden hetzelfde was [25]. In EUROSCOP werd de invloed van atopie op longfunctie en respiratoire symptomen niet geanalyseerd. Wij analyseerden welke factoren invloed hebben op de aanwezigheid van atopie en of er een verschil is tussen atopische en niet-atopische COPD patiënten wat betreft de prevalentie, incidentie en remissie van respiratoire klachten, alsmede de achteruitgang van de longfunctie gedurende 3-jaar follow-up. Wij stelden op de eerste plaats vast dat de prevalentie van atopie (gemeten met de Phadiatop test) significant hoger was bij COPD patiënten van het mannelijk geslacht, aanwezigheid van overgewicht/obesitas, en lagere leeftijd. Daarnaast zagen we dat aanwezigheid van atopie significant associeerde met de ontwikkeling van hoestklachten in COPD patiënten die niet behandeld werden met budesonide, terwijl COPD patiënten die wel behandeld werden meer kans hadden op verdwijnen van hoestklachten. Onze studie was de eerste die het effect van atopie op het lange termijn beloop van COPD bestudeerde [26]. Slechts 1 abstract (op de ATS) rapporteerde hier eerder over [27], en wel over 1424 COPD patiënten afkomstig van The National Health and Nutrition Examination Survey (NHANES)" III (1988–1994) studie van Johns Hopkins University. Bij allergische personen werden vaker sinusitisperiodes en het optreden van hoesten en piepen gerapporteerd dan bij niet-allergische personen. Een nadeel van de NHANES studie was het ontbreken van een ernst-classificatie van atopie. De NHANES studie definieerde atopie op basis van de aanwezigheid van minstens 1 van de volgende criteria: minstens 1 positieve uitslag bij een huidallergietest, rapportage van een dokters diagnose hooikoorts, of klachten bij blootstelling aan huisstof, dieren of pollen. Onze studie definieerde atopie op basis van objectief gemeten aanwezigheid in bloed van specifiek IgE, en excludeerde personen met een geschiedenis met astma, allergische rhinitis en allergisch eczeem. Omdat we personen uitsloten met andere allergische ziekten geloven wij dat we met onze studie beter het effect van atopie op COPD-gerelateerd hoesten konden bestuderen.

Nadat we onze studie publiceerden werd alsnog de analyse op het NHANES cohort gepubliceerd [28]. Atopische COPD patiënten bleken in vergelijking met niet-atopische patiënten een hoger risico te hebben op respiratoire klachten bij blootstelling aan 'indoor

particulate matter', wat opnieuw de negatieve invloed van atopie op de kliniek van COPD toont. Verhoogde blootstelling aan 'particulate matter (iedere 10 µg/m3) ging bij deze atopische personen gepaard met een hoger risico op extra nachtelijke klachten, piepen, medicatiegebruik, kortademigheid, hoesten, slijm opgeven en lagere ziekte-gerelateerde kwaliteit van leven (gemeten met de st. George's Respiratory Quality of Life Questionnaire) [29].

Perspectief

Data van onze Europese EUROSCOP studie en Hansels Amerikaanse NHANES studie tonen beide aan dat de aanwezigheid van een atopisch fenotype geassocieerd is met een verhoogd risico op respiratoire klachten bij COPD. Daarom bevelen wij clinici aan om het vaststellen van atopie niet te vergeten bij de behandeling van COPD; aanwezigheid van atopie heeft immers een duidelijk negatief effect. Verder suggereren onze resultaten dat atopische personen eerder hun respiratoire klachten verliezen bij gebruik van ICS; hierdoor dringt zich ook de vraag op of andere medicamenteuze behandelingen van atopie verbetering geven van de pulmonale gezondheid van COPD patiënten. Als we accepteren dat atopische COPD patiënten vanaf nu behandeld zouden moeten worden met ICS, dan zou dat een verbreding zijn van de indicatiestelling zoals die door GOLD in 2007 geformuleerd werd [30].

Atopie en ICS gebruik bepalen de IL-17 expressie

In hoofdstuk 5 onderzochten we het effect van atopie en ICS gebruik op de expressie van IL-17 in de luchtwegen van astmapatiënten. Een van de cytokines die gesuggereerd werd een rol te spelen bij het ontstaan van niet-atopisch (niet-eosinofiel/neutrofiel dominant) astma is IL-17 [31]. Er is niet eerder een studie zoals de onze gepubliceerd die IL-17 expressie vergelijkt tussen atopische en niet-atopische astmapatiënten. Wij onderzochten IL-17 expressie in 114 goed gekarakteriseerde astmapatiënten door in luchtwegbiopten het aantal IL-17+ cellen in de submucosa te kwantificeren, en uit te zoeken welke cellen die IL17 expressie laten zien. Ons astma cohort bevatte 81 atopische en 33 niet-atopische astmatici, waarbij atopie bepaald werd door een positieve Phadiatop. In dit cohort gebruikten 51 patiënten ICS en 63 geen ICS. We verdeelden onze patiënten eerst op basis van dit ICS gebruik en vergeleken toen de IL-17 expressie tussen atopische en niet-atopische patiënten. In de niet-ICS gebruikers hadden niet-atopische astmatici een hoger aantal IL-17+ cellen dan de niet-atopische astmatici. In lijn,

associeerde een lager aantal IL-17+ cellen met een hogere Phadiatop-score. In de ICS gebruikers werden in vergelijking met de niet-ICS gebruikers lagere aantallen IL-17+ cellen gevonden bij zowel de atopische als niet-atopische astmatici. Wat betreft longfunctie, werd bij de niet-ICS gebruikers (zowel bij de atopische en niet-atopische personen) vastgesteld dat hogere aantallen IL-17+ cellen geassocieerd waren met minder reversibiliteit voor luchtwegverwijding. Ook werd een negatieve correlatie tussen de FEV1%predicted en IL-17 vastgesteld in atopici die geen ICS gebruikten. Er was geen associatie tussen IL-17 expressie en roken. Onze regressie analyse bevestigde dat atopie en ICS gebruik onafhankelijk bijdragen aan een lagere IL-17 expressie. Afwezigheid van atopie en ICS gebruik gaf de hoogste aantallen IL-17+ cellen.

Tevens lieten we zien dat het vooral de neutrofiele cellen waren die IL-17 positiviteit toonden. Dit is interessant omdat niet-atopische astmatici die geen ICS gebruikten een hogere expressie van IL-17+ cellen hadden dan atopische astmatici, wat suggereert dat IL-17 misschien een rol speelt in de pathogenese van niet-atopisch astma. ICS gebruik was geassocieerd met lagere IL-17 expressie in zowel de atopische en niet-atopische astmatici, wellicht wijzend op een gunstig effect van ICS. Van belang is dat de IL-17+ cellen hoofdzakelijk neutrofiele granulocyten bleken te zijn, wat conflicteert met het paradigma dat lymfocyten (Th17) de voornaamste bron vormen van IL-17. Deze bevinding past bij literatuur die meldt dat in eerste instantie IL-17 afkomstig is van cellen die de aangeboren afweer verzorgen, nog voordat CD4+ T cellen een antigen herkennen en TH17 reacties op gang brengen [32].

Perspectief

IL-17 heeft de laatste jaren meer aandacht gekregen als een ‘target’ voor nieuwe therapieën gericht op ziektes als astma, psoriasis, reumatoïde artritis, psoriatische artritis, en M Crohn. IL-17 is een cytokine dat opgereguleerd is ter plaatse van ontsteking, waar het samen met andere cytokines (zoals TNF-α), de inflammatoire cascade versterkt. Activatie van deze ontstekingsroutes wordt verondersteld bij te dragen aan de onderliggende pathogenese van boven beschreven chronische ontstekingsziektes [33]. Onze studie toonde een significante negatieve correlatie tussen het aantal IL-17+ cellen en reversibiliteit op luchtwegverwijding in astmatici die geen ICS gebruikten (zowel atopische als niet-atopische personen). Ook was er een negatieve correlatie tussen IL-17+ cellen en FEV1%pred in astmatici die geen ICS gebruikten, maar dan alleen in de atopische groep. Zulke correlaties zijn belangrijk omdat ze een duidelijke aanwijzing geven dat IL-17 belangrijk is voor de long gezondheid van astmatici. Een klinische trial die een monoclonaal antilichaam tegen IL-17A (brodalumab)

testte liet echter geen positief effect zien [34]. Alleen de subgroep met grote reversibiliteit (post-bronchodilatoire FEV1 verbetering >20%) toonde een verbetering in de Astma Controle Questionnaire (ACQ) score. De onderzoekers hebben geen IL17 in bloed of luchtwegbiopten gemeten. Onze studie toonde dat het aantal IL-17+ cellen in de luchtwegwand sterk afhangt van de aanwezigheid van atopie en het gebruik van ICS. Verder onderzoek is nodig dat rekening houdt met deze factoren, want wellicht is mAb behandeling tegen IL-17 alleen effectief bij niet-atopische astmatici, met laag ICS gebruik, en hoge expressie van IL-17.

Corticosteroïd ongevoeligheid en CCL20

Th17-gemedieerde neutrofiele luchtwegontsteking wordt in verband gebracht met een slechtere respons op corticosteroïden in astma [35]. Een van de potentiele mechanismen hierachter is opregulatie van CCL20 in luchtwegepitheel, wat een chemoattractant is voor Th17+ cellen en neutrofielen. In hoofdstuk 6 beschrijven we een nieuw mechanisme van TH17-gemedieerde corticosteroïd ongevoeligheid in astma. Hiertoe onderzochten we het effect van ICS op de uitscheiding van CCL20 door luchtweg epitheelcellen, en vergeleken CCL20 concentraties in sputum afkomstig van astmatici die wel of geen ICS gebruikten. Tevens onderzochten we in vitro de uitscheiding van CCL20 door primaire bronchiale epitheelcellen van astmatici die met een glucocorticoïd behandeld werden. We toonden aan dat CCL20 concentraties in sputum van ICS gebruikers hoger waren dan van niet-ICS gebruikers, en dat glucocorticoïd toediening in vitro de uitscheiding van CCL20 door primaire epitheelcellen verhoogde. Zoals verwacht correleerde CCL20 in sputum positief met de ICS dosis en sputum neutrofilie. Op zoek naar het mechanisme verantwoordelijk voor CCL20 opregulatie door corticosteroïden zijn verschillende experimenten verricht waarbij remmers van de corticosteroïd receptor, intracellulaire signaalroutes en metalloproteinases in een bronchiale epitheliale cellijn 16HBE onderzocht werden. Remming van de corticosteroïd receptor ADAM17 deed de budesonide-geïnduceerde uitscheiding van CCl20 verdwijnen.
Hiermee suggereren onze resultaten dat de verhoogde CCL20 productie gemedieerd wordt door de corticosteroïdreceptor en afhankelijk is van ADAM17 activiteit. Deze bevindingen vormen een nieuw mechanisme voor de TH17-gemedieerde corticosteroïd-ongevoelige ontsteking bij astma.

Perspectief

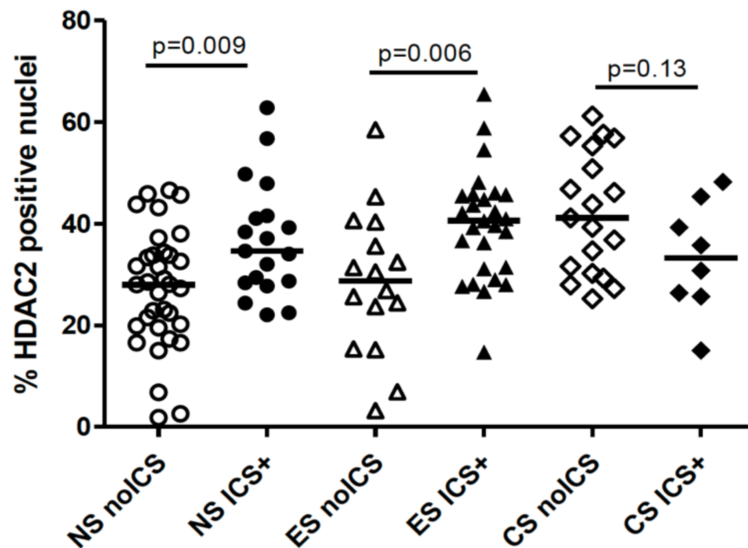
In de vorige sectie beschreven we de rol van IL-17+ cellen en de correlatie met het aantal neutrofiele granulocyten. In deze sectie beschreven we de rol van een chemokine dat verhoogd aanwezig is door dit cytokine. IL-17 kan de expressie van het CC-chemokine ligand 20 (CCL20) induceren, wat migratie van mucosaal-geassocieerde cellen met expressie van de CC-chemokine receptor 6 (CCR6) stimuleert. Daarbij moet gelijk de aantekening worden gemaakt dat CCR6 een karakteristieke receptor is op cellen die IL-17 tot expressie brengen, zoals Th17 cellen en ILC3s [36]. CCL20 gereguleerd door IL-17 zorgt er voor dat CCR6 positieve cellen gerekruteerd worden, zoals Th17+ cellen [37]. Onze studie toonde de rol van Th17+ cellen in de corticosteroïd-ongevoelige astmatische ontsteking. Th17+ cellen migreren naar CCL20, de ligand voor CCR6, maar scheiden ook CCL20 uit. Onderbreking van deze CCR6/CCL20 as zou een nieuwe therapeutische ‘target’ kunnen zijn bij astmatici die minder goed of niet reageren op corticosteroïden.

Roken, ICS gebruik, en HDAC-2 expressie

In hoofdstuk 7 onderzochten we het effect van roken en ICS gebruik op de expressie van HDAC-2 in luchtwegbiopten van 123 astmapatiënten. Omdat bronchiale epitheliale cellen de eerste barrière van de long vormen bij rookinhalatie, onderzochten we deze cellen in centrale luchtwegbiopten. Immunohistochemische HDAC-2 expressie werd gescoord middels bepaling van HDAC-2 positieve celkernen in intact luchtwegepitheel. We vergeleken HDAC2 expressie tussen rokende en niet-rokende astmatici, en evalueerden het effect van ICS-gebruik.

Onze studie toonde voor het eerst aan dat roken is geassocieerd met een hogere HDAC-2 expressie in luchtwegbiopten van astmapatiënten, een bevinding die in overeenstemming is met een eerdere studie bij gezonde rokers en COPD patiënten [38]. Een interessante bevinding van onze studie was dat ex-rokende astmatici hetzelfde patroon lieten zien als nooit-rokende astmatici (Figuur 2), wat suggereert dat de hogere HDAC-2 expressie door roken reversibel is. Onze studie toonde ook dat roken en ICS gebruik onafhankelijk van elkaar een hogere HDAC-2 expressie geven in het bronchiale epitheel van astmatici. Het is verrassend dat de combinatie van ICS en roken geen hogere expressie geeft, wat opnieuw suggereert dat roken de corticosteroïdgevoeligheid reduceert bij astma. Natuurlijk moeten we wel voorzichtig zijn met onze conclusies omdat het een observationele studie betreft zonder

randomisatie op roken en ICS gebruik.



Figuur 2. HDAC-2 expressie in bronchus epitheel van astmatische luchtwegen. *NS: Never smoker (nooit roker), ES: Ex-smoker (exroker), CS: Current smoker (actuele roker), ICS: Inhaled corticosteroid (inhalatie corticosteroïd).*

Perspectief

In het algemeen wordt gesuggereerd dat HDAC-2 een rol speelt bij corticosteroïdongevoeligheid bij ernstig astma en COPD [39]. Onze resultaten komen niet overeen met onze verwachtingen, waarbij moet worden aangetekend dat het om een observationele studie gaat bij mild tot matig ernstig astma. We onderzochten ook niet de expressie van HDAC-2 in submucosaal gelegen luchtwegcellen, dus we weten niet wat roken en ICS gebruik op HDAC-2 expressie veroorzaakt in dit gedeelte van de luchtwegwand.

Hypothetisch zou een gerandomiseerde gecontroleerde studie die het effect van resp. ICS, roken, ICS en roken, en geen ICS of roken bestudeert in milde tot ernstige niet-rokende astmatici, met luchtwegbiopten op baseline en na behandeling, en meting van corticosteroïdgevoeligheid op baseline en na behandeling, meer duidelijkheid kunnen geven. Het is duidelijk dat een dergelijke studie mede vanwege ethische redenen niet uitvoerbaar is.

Ongecontroleerd astma en de activatie van eosinofiele granulocyten

In hoofdstuk 8 onderzochten wij de relatie tussen ongecontroleerd astma (gedefinieerd volgens de Gaining Optimal Asthma Control (Goal)studie) en de activatie status van eosinofiele granulocyten. Dit was naar aanleiding van een vraag van Persson [40], die

reageerde op onze eerdere publicatie met de titel: “Clinical control of asthma associates with measures of airway inflammation” [41]. In deze publicatie speculeerden we dat de activatie van de eos (gemeten met EPX positiviteit) in luchtwegbiopten de controle van astma beter reflecteert dan het aantal eos zelf [41]. Inderdaad zijn hiervoor aanwijzingen omdat hogere EPX immunopositiviteit in luchtwegbiopten significant associeerde met verminderde epitheliale intactheid, vooral in ongecontroleerd astma. Dit suggereert dat lysis van geactiveerde eosinofiele granulocyten, en degranulatie van toxische eosinofiele eiwitten, het omgevende weefsel kan beschadigen (zoals gehypothetiseerd door Persson [40]), wat op deze manier bijdraagt aan ongecontroleerd astma.

Perspectief

Het theoretisch concept van eosinofiele cellysis van Persson [40] is erg interessant omdat de aanwezigheid van vrije eosinofiele granulae in luchtwegweefsel of sputum altijd genegeerd of beschouwd werd als een artefact ten gevolge van het bewerken van de monsters.

Immuunhistochemisch onderzoek van luchtwegweefsel met eosinofiel peroxidase maakt echter duidelijk dat de vrije eosinofiele granula een reëel in vivo fenomeen zijn dat niet vooraf wordt gegaan door apoptosis. Meer studies hebben de positieve correlatie tussen het aantal vrije eosinofiele granula en ziekteactiviteit gerapporteerd bij zowel astma als rhinitis [41-43]. Onze data bevestigt de hypothese van Persson dat granula (en andere biologisch actieve producten) die bij lysis van eosinofiele granulocyten vrijkomen een oorzakelijke rol spelen bij de pathologie en kliniek van eosinofiele luchtwegziektes. Daarom bevelen aan om voortaan ook de activatie status van eosinofiele granulocyten (EPX+ immunopositiviteit) te bestuderen bij bioptstudies van astma.

Tenslotte

Concluderend voegt dit promotieonderzoek enkele nieuwe stappen toe aan de moeilijke weg naar het begrijpen van de pathogenese en progressie van obstructieve longziektes, inclusief de effecten van roken, atopie en therapeutische modulatie (met ICS).

Duidelijk is dat actief roken de klachten en symptomen van astma verergert, en de gevoeligheid voor corticosteroïden vermindert. Het verandert het klassieke klinische en pathologische fenotype waarbij er een COPD-achtige luchtwegobstructie en luchtwegontsteking ontstaat na tientallen jaren roken. Dat veroorzaakt bij oudere personen het dilemma of het om astma of COPD gaat, en of dat gevolgen heeft voor de keuze van de juiste

therapie.

Een andere factor die invloed heeft op het klinisch fenotype en het ontstekingspatroon in obstructieve luchtwegziekten is de aanwezigheid van atopie. Wij lieten zien dat atopie in COPD patiënten gepaard gaat met extra respiratoire klachten zoals hoesten en slijm opgeven. Behandeling met inhalatiecorticosteroïden liet een gunstig effect zien in de atopische COPD patiënten: zij hadden in de loop van de jaren minder vaak last van hoesten dan de niet-atopische patiënten. Vice versa ontwikkelden atopische patiënten die niet met ICS behandeld werden vaker hoestklachten. Blijkbaar verandert atopie de onderliggende ontsteking van COPD patiënten, en daarmee hun gevoeligheid voor corticosteroïden. Atopische astmapatiënten en astmatici die inhalatiecorticosteroïden gebruiken hadden minder IL-17+ cellen in luchtwegbiopten dan niet-atopische astmatici en niet-ICS gebruikers. Naast IL-17 kan ook de expressie van HDAC-2 in de luchtwegen beïnvloed worden door corticosteroïd behandeling.

Hoewel corticosteroïden de hoeksteen van de behandeling van astma vormen, reageren sommige astmatici slecht op corticosteroïd behandeling, vooral de patiënten met ernstig astma, of zij die roken. We bespraken de rol van Th17 als één van de oorzakelijke mechanismen evenals CCL20, die een rol speelt bij de chemoattractie van neutrofiele granulocyten en een correlatie met de dosis ICS en neutro's in sputum toonde. Onze bevindingen wijzen hiermee op een nieuw mechanisme van TH17-gemedieerde corticosteroïd-ongevoelige luchtwegontsteking in astma.

Het brede spectrum van obstructieve luchtwegziektes wordt bepaald door zeer heterogene populaties van astma en COPD patiënten. Roken en atopie kan daarbij het ontstekingspatroon en het klinisch fenotype van een patiënt veranderen, met gevolgen voor corticosteroïdgevoeligheid. Met deze heterogeniteit zou beter rekening gehouden moeten worden bij het ontwerpen van toekomstige klinische studies.

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Acknowledgments

I am finally making my mind free by defending my PhD thesis after 7 years of its pushback and delay. I am definitely putting a heavy load of “a decade of PhD” thesis defense from my shoulders down and reporting a record for sure. I have a strange but good feeling now that I am defending the results of 42 months working on my PhD project in the department of pathology and medical biology of UMCG after such a long time!! I am happy that almost all of my results have been published a while ago, otherwise, I am not sure if I could fairly defend my data by knowing all of their details. Although there were a lot of struggling moments and obstacles during this period of time causing such delay for me, who was supposed to do her PhD defense 7 years ago (on October 9, 2013), but I learned a lot on the road of “chasing-defense” journey. I learned to stay strong, stay positive, and never give up especially as a hands-full mom of twins. Of course, it couldn’t be possible without the support from my wonderful husband and the great support and encouragement by my great supervisors; Nick and Wim. Thank you so much guys! I will never forget your amazing support and your kindness. Thank you so much for being so patient with me during this journey and always trying to help me to solve any issue on the road. Thanks for giving me the opportunity to perform my PhD under your supervision.

I believe my thesis book does not only contain the results of my PhD research, but also carries a big story of a main chapter of my life, reminding me the events causing such big delay. They weren’t all bad though during this “chasing-defense” journey. Several sweet and amazing moments happened on the road, foremost and important was getting my adorable twins; Roxanne and Ryan, being successful in my postdoctoral fellowship in the USA and much more. Nick maybe you’re right! We may make a movie from my “A Decade of PhD” thesis defense! in which I will be its superstar :) Thank you so much for your positive thoughts and trying to diminish my stressful time by your nice feedback during this long journey.

Sometimes I wish I could have multiple entry visas enabling me to go to the Netherlands for doing my PhD defense on the expected date, or sometimes I wish there was no travel ban for re-entering to the USA due to my nationality, and so and so about some other silly reasons.

There were a lot of funny moments that I called them a serial of the “First One” which didn’t happen:

I was supposed to be the first PhD student, among the Iranian people accepted as a group in UMCG in 2013, to do her PhD defense (due to having enough papers so allowing to move to the USA), which I wasn’t. I was supposed to be the first PhD student who does her PhD defense online in UMCG (due to travel ban in my case), which I wasn’t. I am not even now the first one who is doing her PhD defense online due to the COVID-19 crisis, as many of the thesis defenses nowadays are done online. I need to finish up before the next unexpected event coming down from the sky :)

Regardless whatever reasons caused such silly delay, I am happy that I finally could make it in which Wim and Nick play the major roles for helping me to make it. Being in touch with my supervisors Nick and Wim was one of the nice gifts that I got during this journey. My dear Nick, thank you so much. You are very very kind and patient. Your support was not only on my research but also you helped us to get ‘een mooi klein flatje’ in Haren. I learned a lot from you and also from my dear Wim, working in the pathology lab under your supervision was definetly a great honor for me and helped me to learn a lot from you about immunohistochemistry (IHC). So, I would like to express my special gratitude and appreciation to both of you who were not only my great supervisors but also always my amazing friends. I would also like to express my special appreciation and thanks to my other supervisors, Dirkje and Machteld, who this thesis would not have been completed without

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I would like to thank Hanneke for her kindness to me and my husband. Also, thanks to my other friend in Haren: Ali Baker, heel veel bedankt voor je hulp. Your amazing help is not countable, thanks for being such a great Dutch teacher for us, for inviting us to participate in several memorial and fun events in Haren and helping us for storing Mohammad's art paintings. My dearest Esther, you are a wonderful and patient Dutch teacher. Thanks for coming to our place for teaching us Dutch language. I would also like to thank Annemieke for inviting us to their regular events in Haren. You were so nice with us. Hartelijk bedankt! Thanks also to Cara for helping us to interview with 'Haren de Krant' and publishing 'Iraanse droom komt in Haren uit'.
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Biography

Fatemeh Fattahi was born in Arak, Iran. She started her research works, as a research scientist (under supervision of Prof. Pourpak and Prof. Moin), just after her graduation from medical school in Immunology, Asthma and Allergy Research Institute (IAARI) at Tehran University of Medical Sciences in October 2004. During more than 4 years of her research work in IAARI, she was involved in various projects. One of her projects was studying adverse drug reactions/allergies in hospitalized patients in several main university hospitals in Tehran. The results of this project were published in eight papers in the prestigious international journals. She received an award for this project when it was selected as the best project in the 7th Avicenna Festival, January 2006 in Tehran, Iran. Fatemeh was also involved in a national project studying the chronic granulomatous disease (a primary immunodeficiency disorder) in Iran and could publish the 3rd largest study, regarding the number of the cases, on this topic in the world. In addition of performing several projects and publishing numerous papers, she became executive manager of a large conference of "The 2nd Iranian Asthma Meeting" (with more than 1000 national and international participants) in Tehran in 2005. She also received several national and international awards and grants for taking part and/or for presenting her data (as oral or poster) in the conferences. Supervising several master-level students for their MSc degree thesis projects and teaching "Methodological Research and Statistical Analysis (mainly by SPSS software)" (2 university credits) to MSc students are among the list of her other scientific activities in Iran. Fatemeh was also involved in several research projects in Research Center for HIV/AIDS at Tehran University of Medical Sciences (under supervision of Prof. Mohraz) and Royan Institute.
Fatemeh's research and executive scientific activates in IAARI resulted in receiving the "Best Junior Investigator" award in Tehran University of Medical Sciences during her fellowship in Iran. In October 2009, she moved to the Netherlands and started her PhD project at the Department of Pathology and Medical Biology at the UMCG under supervision of Prof. Timens, Dr. ten Hacken, Dr. Hylkema and Prof. Postma. During 3.5 years of her PhD project, she performed her research studies about effect of smoking and atopy on the inflammatory pattern of lung as well as on the corticosteroid responsiveness in the affected patients with asthma or COPD. The results of her PhD project are presented in this thesis book. On April 2013, Fatemeh moved to US and shortly after she started doing her research work as a postdoctoral fellow/research scientist at the University of Michigan Medical School in Ann Arbor, Michigan successfully.

List of publications:

Scientific publications from Postdoctoral fellowship (2013-now)

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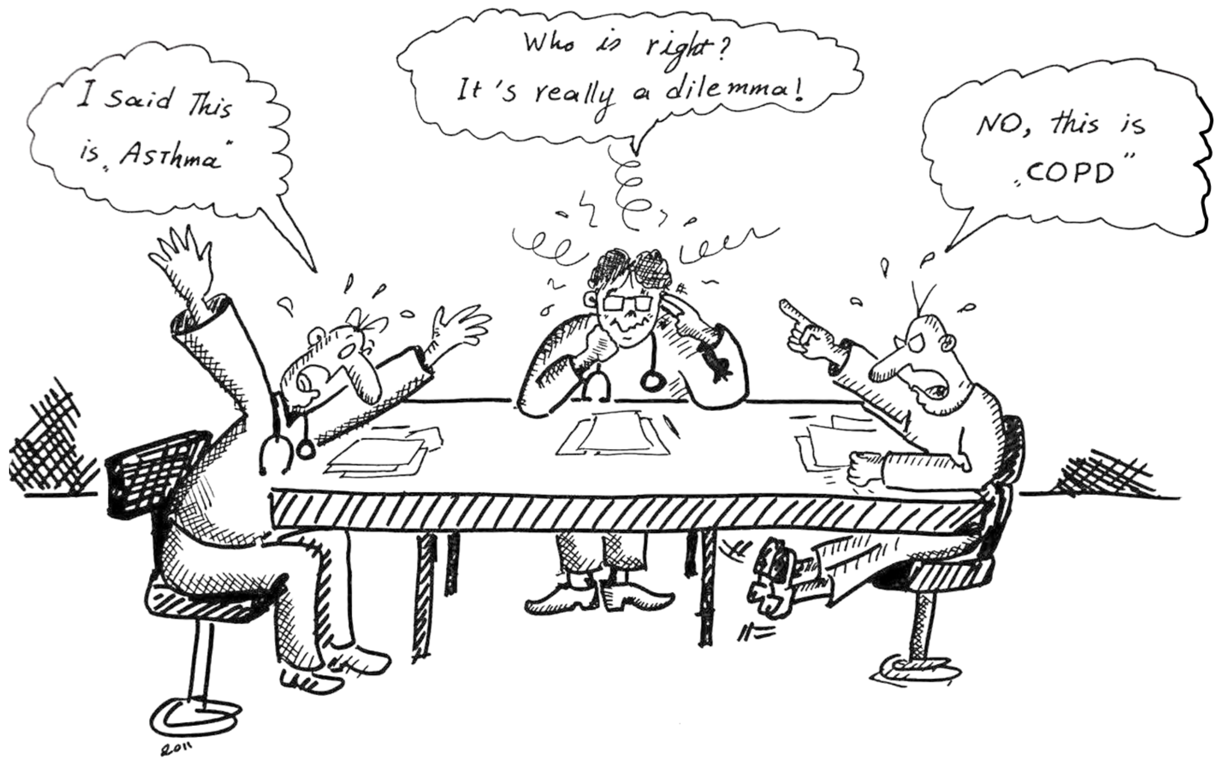
I said This is „Asthma"
Who is right? It's really a dilemma!
NO, this is „COPD"
2011