On phenotyping in asthma and COPD

Telenga, Evert Dirk

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STELLINGEN

1 Rokende en voormalig rokende astmapatiënten reageren minder goed op een kortdurende behandeling met inhalatiecorticosteroïden dan astmapatiënten die nooit gerookt hebben (dit proefschrift).

2 Astmapatiënten met ernstig overgewicht hebben een fenotype dat gekarakteriseerd is door meer neutrofiele inflammatie, zowel systemisch als in de luchtwegen, in vergelijking met astmapatiënten zonder overgewicht (dit proefschrift).

3 Astmapatiënten met luchtwegobstructie laten een minder sterke verbleking van de huid zien na blootstelling aan corticosteroïden dan astmapatiënten zonder luchtwegobstructie, hetgeen wijst op verminderde gevoeligheid voor corticosteroïden (dit proefschrift).

4 Mensen met asymptomatische bronchiale hyperreactiviteit hebben geen dysfunctie van de kleine luchtwegen onder normale omstandigheden, maar wel tijdens een bronchiale provocatietest (dit proefschrift).

5 Zelfs bij gezonde mensen zorgt roken voor verdikking van de luchtwegwand (dit proefschrift).

6 Lipiden van de 'sphingolipiden pathway', een pathway betrokken bij apoptose en inflammatie, zijn opgereguleerd in sputum bij COPD patiënten in vergelijking met gezonde rokers (dit proefschrift).

7 Work expands so as to fill the time available for its completion (Parkinson, 1955).

8 For every action towards graduation there is an equal and opposite distraction (J. Cham, 2001).

9 Een gezonde roker is als een witte raaf.

10 In het huidige tijdperk past 4 jaar promotiewerk uiteindelijk in zes verhuisdozen en op één USB-stick.

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General Introduction

CHAPTER 1

OBSTRUCTIVE AIRWAY DISEASES

Asthma and Chronic Obstructive Pulmonary Disease (COPD) are obstructive airway diseases. In both diseases, narrowing of the airways leads to expiratory airflow limitation, causing a sensation of breathing discomfort called dyspnea.[1] Airflow limitation can be assessed by spirometry and the forced expiratory volume in 1 second (FEV_1) and FEV_1 divided by forced vital capacity (FVC) are the main markers for airflow limitation.[2]

Asthma

Asthma is an inflammatory disease, characterized by episodic shortness of breath often accompanied by cough.[3] The episodic symptoms can be triggered by exposure to allergens, exposure to aspecific stimuli (e.g. cigarette smoke, perfume, cold air) or by exercise. There is a link between asthma and allergy, with many asthma patients having a history of atopic signs and symptoms, such as eczema and allergic rhinitis. A typical presentation of allergic asthma is called the 'atopic march' referring to the progression of symptoms from eczema in infants and toddlers to allergic rhinitis and finally to asthma in children.[4] However, other presentations of asthma, like non-allergic asthma and adult-onset asthma are also common.[5,6] The prevalence of asthma has increased markedly over the last decades, especially in the Western world and it is estimated that as many as 300 million people suffer from asthma worldwide.[7]

In the last years there has been increasing interest in the involvement of the small airways in asthma. The small airways are airways with an internal diameter <2 mm. In the past, the small airways were called the 'quiet zone', as they contribute only 10% to the total airway resistance under normal circumstances.[8] However, recent studies have shown that the small airways are the predominant site of airflow obstruction in asthma patients.[9–11]

Inflammation of the airways is a cardinal feature of asthma; however the type and severity of inflammation differs between patients. Furthermore, the type and severity of inflammation does not always correlate with the severity of symptoms. Studies investigating induced sputum of asthma patients have shown 4 categories of airway inflammation: 1) eosinophilic, 2) neutrophilic, 3) mixed eosinophilic and neutrophilic and 4) paucigranulocytic, i.e. normal levels of both eosinophils and neutrophils.[12,13] It appears that different phenotypes of asthma are characterized by distinct patterns of inflammation. For instance, patients with early-onset allergic asthma usually have eosinophilic inflammation.

Chronic Pulmonary Disease

COPD is defined by chronic airflow limitation, which is not fully reversible with bronchodilators.[14] Cigarette smoking is the major risk factor for developing COPD in the Western world. Cigarette smoke and other noxious particles or gases cause an abnormal inflammatory response in smokers who are susceptible to their effects. This abnormal inflammatory response in susceptible smokers induces a faster decline in lung function than

in non-susceptible smokers and non-smokers.[15] Smoking cessation can reduce the decline in lung function, but the lost lung function will not be recovered. COPD is a major health problem with high morbidity and mortality. It is estimated that 65 million people have moderate to severe COPD worldwide and it is currently the fourth leading cause of death in the world.[14] Moreover, the burden of COPD is increasing and the World Health Organization predicts that COPD will be the third leading cause of death in 2030.[16]

The inflammatory response occurs in different compartments of the lung: the lung parenchyma, the large airways (i.e. those with an internal diameter >2 mm) and the small airways (those with an internal diameter <2 mm). As COPD is a heterogeneous disease, the level of inflammation per compartment differs widely between patients. In the parenchyma, the inflammation causes destruction of alveolar walls leading to emphysema. In emphysema, the elastic recoil of the lung is reduced. In addition, the loss of alveolar walls limits the total area available for gas exchange, resulting in an impaired diffusion capacity.[17] Inflammation of the airways leads to remodeling with thickening of the bronchial wall, peribronchial fibrosis, and mucous plugging caused by hyperplasia of goblet cells, subepithelial seromucinous glands and Clara cells in peripheral airways.[18–21] A study by Hogg *et al.* showed that there is mostly narrowing and obliteration of the small airways in COPD, which is associated with the severity of the airflow limitation.[22] In this study, Hogg et al. provide convincing evidence that this narrowing and obliteration of small airways precedes the occurrence of parenchymal changes.

PHENOTYPES OF OBSTRUCTIVE AIRWAY DISEASES

Asthma and COPD are both complex, heterogeneous diseases. This leads to different presentations of the diseases, called phenotypes. These phenotypes are determined by genetic, epigenetic, environmental and lifestyle variations. The study of these phenotypes is relevant as prognosis and treatment responses may differ widely between the distinct phenotypes.[23–26] In this thesis, we investigate phenotypes of obstructive airway diseases. It consists of two parts. The first part describes which factors determine corticosteroid sensitivity as a subphenotype of asthma and COPD. The second part describes the different methods that can be used to investigate obstructive airway diseases and may help to identify new phenotypes in the future.

Corticosteroid sensitivity

Corticosteroids are anti-inflammatory drugs used in the treatment of both asthma and COPD. They exhibit their anti-inflammatory effect by binding to the glucocorticoid receptor, which then translocates to the nucleus where it represses the expression of pro-inflammatory genes and induces the expression of anti-inflammatory genes.[27] Inhaled corticosteroids (ICS) exert their anti-inflammatory effect on the airways and have limited uptake in the

systemic circulation. Therefore they give fewer side effects than orally administered corticosteroids.[28,29]

In asthma, ICS are the cornerstone of treatment.[3] They reduce asthma symptoms, improve asthma control, quality of life, lung function and bronchial hyperresponsiveness (BHR), control airway inflammation, reduce the frequency and severity of exacerbations, and reduce asthma mortality.[3,30–34] The effects of ICS in COPD are less pronounced. Although ICS improve symptoms and quality of life and reduce exacerbation frequencies,[14,35–41] most studies so far have not shown an effect of ICS on lung function decline or mortality on the long-term.[36,42–45] However, two studies do show less long-term decline in lung function with ICS treatment, suggesting that they may have an effect on the long-term, at least in a in a subgroup of COPD patients.[46,47] It has been suggested that ICS are less effective in COPD than in asthma because the type of inflammation in COPD is different than in asthma. However, a reduced responsiveness to corticosteroids is also seen in different phenotypes of asthma.[48] Several mechanisms for reduced corticosteroid responsiveness have been identified, such as more neutrophilic inflammation, epigenetic changes, e.g. reduced expression of histone deacetylases (HDAC) and DNA methylation, more expression of the less active β isoform of the glucocorticoid receptor and increased expression of pro-inflammatory transcription factors, such as nuclear factor-kappa B and activator protein 1.[49–59]

The first part of this thesis will be about the response to corticosteroids in different phenotypes of asthma and COPD. **Chapter 2** is a review on the use of corticosteroids in the treatment of COPD. COPD patients appear to respond less to corticosteroid treatment than asthmatics and cigarette smoking is the main risk factor for COPD. It could be envisaged that smoking asthmatics also respond less well to ICS. Therefore, the inflammatory profile and the response to corticosteroid treatment in asthma patients who smoke are being discussed in **Chapter 3**. To assess if other phenotypes of asthma are also less sensitive to corticosteroid treatment, the inflammatory profile and the response to corticosteroid treatment in obese asthma patients are being discussed in **Chapter 4**. Finally, **Chapter 5** discusses a test to assess corticosteroid sensitivity, the corticosteroid skin-blanching test, and shows that lower corticosteroid sensitivity is associated with a lower level of lung function, female gender and absence of allergy.

METHODS TO INVESTIGATE OBSTRUCTIVE AIRWAY DISEASES

The second part of this thesis will discuss methods to investigate obstructive airway diseases. These methods can provide new insights in the processes that occur in the lungs of patients with obstructive airway diseases and may help to identify new phenotypes.

Impulse oscillometry (IOS) to detect small airways dysfunction in bronchial hyperresponsiveness

An important feature of asthma is bronchial hyperresponsiveness (BHR), the narrowing of the airways in response to a stimulus that is innocuous in a healthy subject, such as cigarette smoke, perfume, cold air and dry or moist air.[3] BHR can be assessed by provocation tests with stimulating agents, such as methacholine, histamine or adenosine 5'-monophosphate. The severity of BHR is expressed as the concentration of a stimulus that induces a 20% fall in FEV_1. It is generally acknowledged that obstruction of the large airways contributes to more severe BHR. However, recently there have been studies suggesting that asthmatics with BHR have more small airways obstruction.[60,61] **Chapter 6** discusses the contribution of the small airways to BHR.

Besides baseline measurements of small airways obstruction, there is also interest in determining the extent of small airways obstruction during BHR, e.g. during bronchial provocation tests. Although spirometric measurements such as the FVC, the forced expiratory flow between 25-75% of FVC (FEF_{25-75}) and forced expiratory flow at 50% of FVC (FEF_{50}) are indicators of small airways obstruction, these values are also dependent on the large airways and therefore not suitable to measure small airways obstruction during provocation tests. With the increasing interest in the small airways, there has been a renewed interest in the use of IOS to measure small airways obstruction.[62–64] During IOS measurements, an impulse generator produces pressure oscillations of different frequencies which are superimposed on normal breathing and conducted throughout the airways. The resulting changes on flow and pressure for each frequency can be measured at the mouth and resistance and reactance of the airways can be calculated for each frequency.[65] Based on the principal that lower frequencies are conducted throughout the entire lung and that higher frequencies only reach the large airways, the resistance in the small airways can be calculated. IOS measurements are easy to perform for patients, since they are effort independent and take only a few seconds to perform. As the technique measures both the resistance in the small and large airways, it can be used to assess changes in small airways resistance after each step during a provocation test.[66,67] **Chapter 7** discusses small airways function during a methacholine provocation test in healthy controls, subjects with asymptomatic BHR and asthma patients.

Airway wall thickness on CT scans

An important part of obstructive airway diseases is remodeling of the airways. The chronic inflammatory processes in airways of asthma and COPD patients induce changes in the epithelium, the reticular basement membrane, extra-cellular matrix and smooth muscle.[68–70] Both the inflammatory processes and the remodeling lead to thickening of the airway walls. Airway remodeling is difficult to study *in vivo*, since it normally requires biopsies taken from the airway, an invasive and time consuming procedure. A new tool to study airway remodeling is measuring airway wall thickness non-invasively by computed tomography (CT). This may enable us to study remodeling in an asymptomatic stage and at different time points during disease progression. This may give new insights into the pathological processes leading to remodeling in asthma and COPD. So far, studies have shown that airway wall thickness is higher in COPD patients and asthma patients than in healthy controls.[71–73] However, there are some limitations to studies performed to date. Earlier studies measured the wall thickness manually, which is less accurate than automated assessments.[74] Furthermore, only airways that ran perpendicular to the transverse CT slices were evaluated, limiting the number of airways measured.[75–79] Advances in multi-detector row CT scanners, development of multiplanar, three-dimensional segmentation of airways and automated measurements of airway walls, have made it possible to measure the thickness of airway walls in many airways throughout the airway tree.

Before remodeling of the airways in obstructive airway disease can be investigated with CT-scans it is important to know the normal situation first. What is a normal airway wall thickness and how is the airway wall thickness affected by age, gender and lifestyle? At the moment there is very limited data on this. **Chapter 8** describes the effect of age and gender on airway wall thickness in a group of well characterized, healthy controls. Additionally, it describes the effect of cigarette smoking on airway wall thickness, in this group that was specifically selected for their normal lung function.

Lipidomics in obstructive airway diseases

To better understand the pathobiological basis of obstructive airway diseases research has turned to the study of biological networks, also called '-omics'. The first of these biological networks to be studied was the genome. The study of the whole genome has identified genes involved in asthma and COPD.[80,81] After genomics followed studies into gene expression (transcriptomics), metabolites (metabolomics) and proteins (proteomics).[82–85] A new '-omic' is lipidomics, which studies lipids. Lipids play multiple roles in cellular function. They are the main components of membranes, regulate cellular signaling and are used as fuel for many cell types. It is estimated that there may be tens of thousands of structurally distinct lipids.[86] Lipidomics has been developed to characterize all lipids in a particular cell type or body fluid and to understand the influence of lipids on a biological system with respect to cell signaling, membrane architecture, transcriptional and translational modification, cell-cell and cell-protein interactions and responses to environmental changes over time.[87] Recently, there

is increased interest in the immunomodulatory properties of lipids. A group of lipids, the sphingolipids, has been shown to be involved in apoptosis on one hand and cell survival on the other hand.[88–92] Therefore, it is possible that differential lipid expression may be involved in obstructive airway diseases. **Chapter 9** describes the effects of cigarette smoke on the expression of lipids *in vitro* and in induced sputum of healthy controls and COPD patients, as measured by liquid chromatography–mass spectrometry.

OUTLINE OF THIS THESIS

Chapter 2 Corticosteroids in Chronic Obstructive Pulmonary Disease: A review
This chapter reviews the use of corticosteroids in COPD patients, with regard to lung function, symptoms, quality of life, exacerbations and long-term lung function decline and mortality.

Chapter 3 Inflammation and corticosteroid responsiveness in ex-, current- and never-smoking asthmatics
This chapters discusses the question whether ex-, current- and never-smoking asthmatics have a different inflammatory profile and if the short- and long-term corticosteroid treatment response is different between these groups.

Chapter 4 Obesity in asthma: more neutrophilic inflammation as a possible explanation for a reduced treatment response
In this chapter the baseline differences in asthma severity and inflammation in obese and lean asthma patients are investigated. Moreover, this chapter addresses the question whether obese asthma patients have a reduced corticosteroid treatment response?

Chapter 5 Skin-blanching is associated with FEV_1, allergy, age and gender in asthma families
A large group of asthma patient and non-asthmatic individuals were evaluated with question whether the skin-blanching response to corticosteroids is lower in asthma patients in than non-asthmatic subjects. Additional questions were if the skin-blanching response to corticosteroids is lower in asthma patients with airway obstruction than in patients without, and which clinical and inflammatory parameters influence the variability in skin-blanching response.

Chapter 6 Small airways in asthma: their independent contribution to severity of hyperresponsiveness
This chapter investigates whether asthmatics with small airways obstruction express more severe BHR than those without small airways obstruction. Moreover, given the considerable effect of the large airway parameters, we questioned whether more severe small airways obstruction is associated with more severe BHR independently of FEV_1.

Chapter 7 Less small airway dysfunction in asymptomatic bronchial hyperresponsiveness than in asthma

This chapter investigates asymptomatic BHR. It investigates whether subjects with asymptomatic BHR have less small airways obstruction than asthma patients, but more than healthy controls at baseline and during methacholine provocation.

Chapter 8 Airway wall thickness on HRCT scans decreases with age and increases with smoking

This chapter evaluates the association between age, gender and cigarette smoking and airway walls thickness measured by high resolution CT-scans in a group of well characterized healthy subjects. Additionally, it evaluates whether a higher airway wall thickness is associated with lower levels of lung function in these healthy subjects.

Chapter 9 Untargeted Lipidomic Analysis in COPD: Uncovering Sphingolipids and Lysophosphatidylethanolamines

This chapter investigates lipid expression in induced sputum comparing 1) smokers with and without COPD and 2) current-smokers without COPD and never-smokers without COPD with liquid chromatography – mass spectrometry (LC-MS) and tandem mass spectrometry (MS/MS). Changes in lipid expression after 2-month smoking cessation are investigated in smokers with and without COPD. Additionally, lipid expression is assessed in BEAS-2B cell lines after 6-month exposure to cigarette smoke extract.

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CHAPTER 2

Corticosteroids in Chronic Obstructive Pulmonary Disease: a Review

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ABSTRACT

Importance to the field: Chronic Obstructive Pulmonary Disease (COPD) is a disease characterized by chronic airflow obstruction and a progressive lung function decline. Although widely used, the efficacy of inhaled corticosteroids (ICS) in the treatment of COPD remains a matter of debate.

Areas covered in this review: This article reviews the evidence about the effects of inhaled corticosteroids in COPD.

What the reader will gain: Short-term treatment with ICS improves lung function and quality of life; in addition, several studies with longer follow-up have shown less decline over time in quality of life, and a lower number of exacerbations. By contrast, long-term studies were unable to show substantial improvement in the decline of lung function in COPD. Based on these findings, it was concluded that the use of ICS did not influence the natural course of COPD. However, this conclusion has been challenged by two subsequent studies, TORCH and GLUCOLD, which both showed a reduction in lung function decline over time with the use of ICS. These two studies indicate that ICS might indeed influence the natural course of the disease, at least in a subgroup of COPD patients.

Take home message: Further studies are needed to identify which individuals have a favorable short- and long-term response to ICS treatment.

INTRODUCTION

Chronic Obstructive Pulmonary Disease (COPD) is a chronic disease with a major health impact throughout the world. It is one of the most common diseases and has a prevalence that increases with age, affecting 10% of adults > 65 years.[1] COPD is the fourth most common cause of death worldwide and its mortality rate continues to increase.[2,3] COPD has been defined as a disease characterized by chronic airflow limitation, which is not fully reversible with bronchodilators.[1] The airflow limitation is associated with an abnormal inflammatory response of the lungs to noxious particles or gases. Patients with COPD experience a more rapid decline in their lung function than is normal for their age. Disease severity is classified in stages according to the guidelines from the Global Initiative for Obstructive Lung Disease (GOLD), ranging from stage I, mild COPD, to stage IV, very severe COPD.[1] The major risk factor for developing COPD in the Western world is exposure to tobacco smoke. Smoking accelerates lung function loss and increases bronchial hyperresponsiveness, airway inflammation, and even mortality in COPD.[1–4] To date, smoking cessation is the only intervention that reduces mortality and rate of decline of lung function.[1–5] Next to smoking cessation, many studies have investigated the efficacy of anti-inflammatory treatment with inhaled corticosteroids (ICS).

Currently available ICS are beclomethasone, budesonide, ciclesonide, fluticasone, mometasone and triamcinolone. They can be delivered to the airways either by dry-powder inhalers or pressurized metered-dose inhalers. The type of inflammation in COPD appears to be less responsive to the anti-inflammatory actions of ICS than the inflammation in asthma, and there is no consensus on whether ICS can attenuate the accelerated lung function loss and influence the natural course of the disease. Because of the high prevalence of COPD and the large impact on daily living, as well as the high socioeconomic impact associated with the management of COPD, it is important to clearly identify the role of ICS in the treatment of COPD. The current GOLD guidelines recommend treatment with ICS only for patients with severe COPD (GOLD stage III or IV) and more than 3 exacerbations per 3 years.[1] Several review articles have already been published on this subject.[5–10] Recent data from new studies indicate that ICS may also have long-term beneficial effects in patients with less severe COPD and infrequent exacerbations.

This article discusses the effects of ICS/ with or without long-acting β2-agonist (LABA) treatment in COPD with regard to lung function, quality of life, exacerbations, comorbidity and mortality, airway inflammation, bronchial hyperresponsiveness, prediction of corticosteroid response, and adverse effects. For this manuscript, we performed a non-systematic Pubmed search of published data with the following search terms: 'chronic obstructive pulmonary disease', 'COPD', 'inhaled corticosteroids', 'FEV_1', 'bronchial hyperresponsiveness', and 'inflammation'. We did not limit our search based by date of publication and used only articles published in English.

EFFECTS OF ICS ON LUNG FUNCTION

Despite debatable efficacy, ICS are widely used in the treatment of patients with COPD. The effects of ICS on symptoms and lung function have been investigated in a number of studies. On the short term, it is clear that ICS improve the level of the forced expiratory volume in 1 second (FEV_1).[11–18] However, whether treatment with ICS has positive long-term effects by reducing the accelerated decline in FEV_1 remains uncertain. Several long-term therapy studies (follow-up ≥1 year) have been performed and their results are summarized below and in **table 1**. Vestbo and colleagues have investigated the effects of budesonide (400 µg b.i.d.) in 290 patients with mild to moderate COPD who were followed-up for 3 years.[19] They did not find any improvement in the annual rate of FEV_1 decline with budesonide (-46 ml/year) versus placebo (-49 ml/year) (p=0.70).

In the European Respiratory Society Study on Chronic Obstructive Pulmonary Disease (EUROSCOP), the effect of budesonide 400 µg b.i.d. was evaluated in 1277 subjects with moderate COPD who were all current smokers. During the first 6 months of the study, FEV_1 improved by 17 ml per year in the budesonide treatment group compared with a decrease of 41 ml per year in the placebo group. However, the annual rate of decline in FEV_1 between 9 months until the end of the follow-up at 3 years did not differ between budesonide and placebo (-57 vs -67 ml/year, p=0.39).[16]

In the Inhaled Steroids in Obstructive Lung Disease in Europe (ISOLDE) study, 751 subjects were treated with either fluticasone 500 µg b.i.d. or placebo for a period of 3 years. No significant difference in annual decline in FEV_1 was observed (-59 ml in the placebo group vs -50 ml in the fluticasone group, p=0.16).[20] In the Lung Health Study (LHS), 1116 subjects with an FEV_1 between 30 and 90% were enrolled in a randomized trial of inhaled triamcinolone at a dose of 600 µg b.i.d. The rate of decline was similar in the triamcinolone and placebo group (-44 vs -47 ml/year, p=0.50).[21] Based on these findings, it was concluded that the use of ICS does not influence the natural course of COPD. However, this conclusion has been challenged by two subsequent studies.

First, the Toward a Revolution in COPD Health (TORCH) study was performed. This large study evaluated the effect of fluticasone, salmeterol and their combination on mortality over 3 years in 5343 patients with COPD. In a post-hoc analysis, all active treatments reduced the rate of lung function decline compared to placebo (placebo decline of 55 ml/year, salmeterol decline of 42 ml/year, fluticasone decline of 42 ml/year and salmeterol/fluticasone decline of 39 ml/year; p≤0.003) **(Figure 1)**.[22]

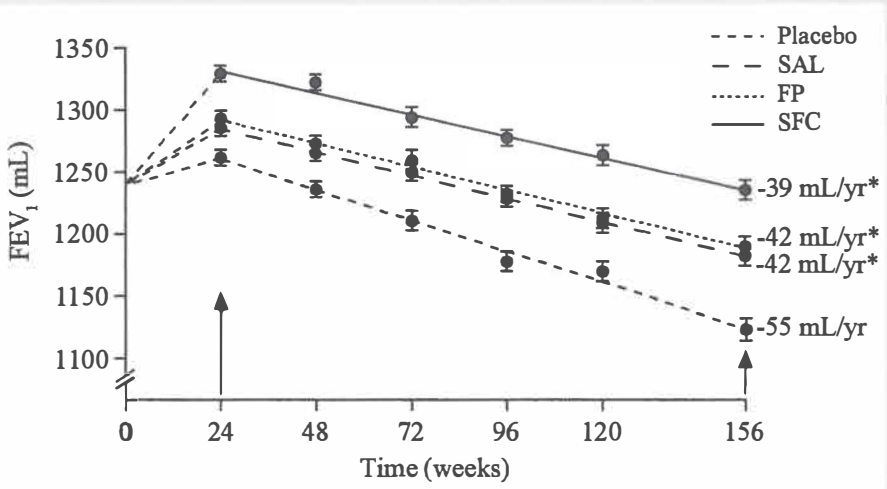
Second, the Groningen Leiden Universities Corticosteroids in Obstructive Lung Disease (GLUCOLD) study investigated the long-term effects of fluticasone with or without salmeterol in patients with COPD who had not used ICS for at least 6 months prior to inclusion. In the GLUCOLD study, patients were assigned to one of the following four treatment arms: 6 months (n=31) or 30 months (n=26) fluticasone 500 µg b.i.d., 30 months (n=30) fluticasone/salmeterol 500/50 b.i.d., or 30 months (n=29) placebo. Patients who

Table 1: Study summary and results for long term clinical studies

Study	n	Age (years)	BDR	PC_{20} Mch	FEV_1 (%pred)	Packyears	Current smoking (%)	Medication	Treatment period	Effect					
										Short term improvement of FEV_1?	Decline of FEV_1	Mortality	Exacerbations	Quality of life	BHR
Vestbo [19]	203	59	7.6 (init)	?	%87%	?	77%	BUD/ PLAC	3 y		-			-	?
EUROSCOP [16]	912	52	2.8 (pred)	?	77%	39	100%	BUD/ PLAC	3 y	+	-	-	?	-	?
ISOLDE [20]	751	64	4.4 (pred)	?	50%0%	44	38%	FP/ PLAC	3 y	+	-	-	+	+	?
LHS [21]	1116	56	6.6 (init)	*	68%	?	90%	TRI / PLAC	40 mo	-	-	-	?	-	+
TORCH [11,22]	6112	65	3.7 (pred)	?	44%	49	43%	SFC/ FP/ SAL/ PLAC	3 y	+	+	-	+	+	?
GLUCOLD [23]	116	62	6.9 (pred)	0.6	63%	43	63%	SFC/ FP/ PLAC	30 mo	+	+	-	?	+	+
TRISTAN [15]	1465	63	3.8 (pred)	?	45%	43	51%	SFC/ FP / SAL / PLAC	1 y	+	+	?	+	+	?
Calverley [14]	1022	64	6 (pred)	?	36%	39	35%	BF / BUD / FT / PLAC	1 y	?	+	?	+	+	?
Szafranski [26]	812	64	6 (pred)	?	36%	45	34%	BF / BUD / FT / PLAC	1 y	?	+	-	+	+	?

+ = positive effect treatment; - = no effect of treatment; ? = effect not reported; FP = fluticasone propionate; SFC = salmeterol/fluticasone; SAL = salmeterol; BF = budesonide/formoterol; BUD = budesonide; FT = formoterol; BDP = beclomethasone; TRI = triamcinolone ; PLAC = placebo; BDR = bronchodilator responsiveness in % of predicted (pred) or initial (init); * = no PC_{20} available, however 82% responded to concentrations ≤25 mg/ml, 48% responded to concentrations ≤5 mg/ml and 13% responded to concentrations ≤1 mg/ml.

Figure 1: Long-term therapy with inhaled corticosteroids (ICS) + long-acting β2-agonist (LABA) reduces decline of lung function in the TORCH study



From Celli et al.22 Reproduced with permission

* = p≤0.003; FP = fluticasone propionate; SAL = salmeterol; SFC = salmeterol/fluticasone combination therapy.

were treated with placebo, or with fluticasone for the first 6 months followed by 24 months placebo, experienced a considerable decline in FEV_1 of -87 and -65 ml/year over 24-months follow-up respectively. Remarkably, treatment with fluticasone or fluticasone/salmeterol significantly improved the rate of decline in FEV_1, being close to zero for fluticasone and only -16 ml/year for fluticasone/salmeterol ($p<0.001$).[23]

From the above, it can be seen that the effect of treatment with ICS on the long-term decline in FEV_1 was larger in the TORCH and the GLUCOLD study than in earlier studies. These findings can be explained in the following manner. The TORCH study was carried out in a large number of patients, which increases the power to detect differences. Furthermore, the GLUCOLD study was carried out in patients who had not used ICS for at least 6 months, and 95% of the patients had never used ICS at all. This prevented selective drop-out (the withdrawal from the study of patients due to withdrawal effects of ICS in the placebo arm, as reported in two double-blind studies).[24,25] This selective drop-out causes a diminution of the FEV_1 decline in the placebo arm, and hence less contrast with ICS. Third, the majority of patients in the GLUCOLD study demonstrated airway hyperresponsiveness as well as a modest reversibility of FEV_1 (6.9% of the predicted value). It could well be that these characteristics, which are classically attributed to asthma, might identify a subgroup of COPD patients with a favorable response to corticosteroids. In this context, it is important to note that patients with a previous or concurrent diagnosis of asthma were carefully excluded in the GLUCOLD

study by excluding patients with a history of asthma. Moreover, only patients older than 45 years of age, with a smoking history ≥10 packyears, were included by reviewing family practice charts (for earlier diagnosis of asthma), and by clinical judgment of chest physicians.

QUALITY OF LIFE

Patients with COPD experience a reduced health-related quality of life (HRQL), which is related to the severity of the disease and decreases further over time.[27–29] The effects of ICS on HRQL have been investigated in a number of studies. First, in the ISOLDE study HRQL was measured with the St. George's Respiratory Questionnaire (SGRQ).[20] Treatment with ICS significantly reduced the decline of HRQL compared to placebo (2.0 units/year and 3.2 units/year respectively; p=0.004). Second, TORCH showed an improvement with fluticasone/salmeterol of 3.0 units of the SGRQ compared to baseline after three years of treatment (p<0.001 vs placebo), with an improvement of 1.8 units in the fluticasone group (p<0.001 versus placebo). In comparison, the placebo group showed a deterioration of 0.2 units in the SGRQ.[11] The effect of treatment was largest in patients with more severe COPD (GOLD stage III and IV). The SGRQ improved by 5.9 units with combination therapy in patients with GOLD stage IV and 3.3 units with GOLD stage III. However, in patients with less severe COPD (GOLD stage 2), the SGRQ still improved significantly by 2.3 units.[30] Finally, the GLUCOLD study found an improvement in dyspnea, the SGRQ activity score, and clinical COPD questionnaire (CCQ) after 30 months of treatment with fluticasone with or without salmeterol compared to placebo (all p-values ≤0.036).[23] For the SGRQ, a difference of 4 points is considered clinically important. **Table 2** shows the annual change in SGRQ for different treatments. In conclusion, long-term treatment with ICS – with or without a LABA – improves the quality of life of patients with COPD. It has been speculated that this improvement may, at least partially, be attributed to a reduction of exacerbations.[31]

Table 2: Change in quality of life measured with the St. George's respiratory questionnaire

Study	n	Age (years)	Medication	Treatment period	Baseline score	Change / year			
						Placebo	LABA	ICS	ICS + LABA
ISOLDE [20]	751	64	FP/ PLAC	3y	48.8	3.0	–	2.0#	–
TORCH [11]	6112	65	SFC/ FP / SAL / PLAC	3y	49.0	0.2	-0.8	-1.8*	-3.0#
TRISTAN [15]	1465	63	SFC/ FP / SAL / PLAC	1 y	48.2	-2.3	-3.6	-3.1	-4.5#
Szafranski [26]	812	64	BF / BUD / FT / PLAC	1 y	51-54	-0.03	-3.6*	-1.9*	-3.9#

A negative change signifies an improvement in quality of life; # = p<0.05 compared to placebo; * = p compared to placebo not known; – = group not present in study; BDP = beclomethasone; BF = budesonide/formoterol; BUD = budesonide; FP = fluticasone propionate; FT = formoterol; ICS = inhaled corticosteroids; LABA = long-acting β2-agonist; PLAC = placebo; SFC = salmeterol/fluticasone; SAL = salmeterol.

EXACERBATIONS

Prevention of exacerbations with ICS

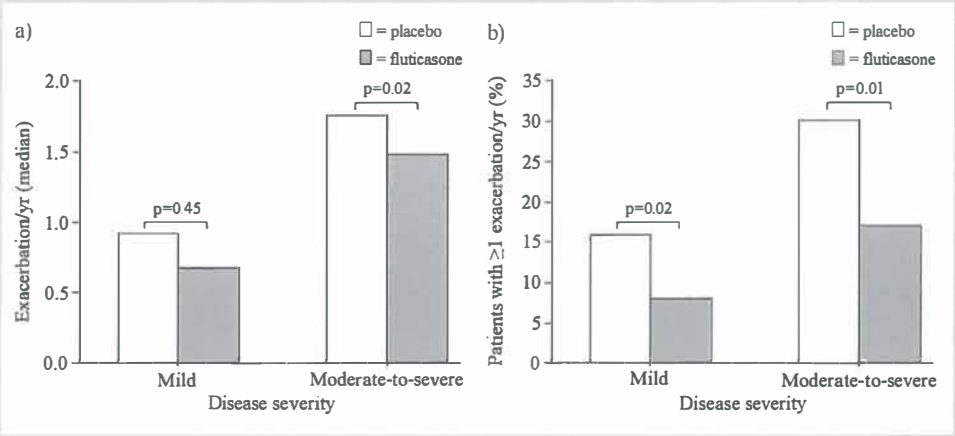
Many definitions of exacerbations can affect the outcome of intervention studies.[32] In the GOLD guidelines, an exacerbation is defined as: "an event in the natural course of the disease characterized by a change in the patients baseline dyspnea, cough, and/ or sputum that is beyond normal day-to-day variations, is acute in onset, and may warrant a change in regular medication in a patient with underlying COPD".[1]

Exacerbations have a major impact on the quality of life of COPD patients.[33] Seemungal and colleagues have reported that it can take a long time to recover from a COPD exacerbation.[34] In their study, 25% of patients still had increased symptoms and a lower FEV_1 at 5 weeks after the onset of their exacerbation. Furthermore, COPD patients with frequent exacerbations may suffer from a larger annual decline in FEV_1.[33] In a prospective 4-year study, 109 COPD patients were divided into two groups: those experiencing more than the median number of 2.9 exacerbations per year (frequent exacerbators) and those with less than 2.9 exacerbations per year (infrequent exacerbators). Although no differences in age, sex, lung function, smoking habits, and ICS use were observed at baseline, frequent exacerbators experienced a significantly larger annual decline in their FEV_1 than infrequent exacerbators (40 vs 32 ml/year; $p<0.05$).[35] These findings suggest that exacerbations may contribute to lung function loss in COPD. Finally, exacerbations have a large societal and economic impact, mainly due to hospitalizations and absence from work.[36–38] In summary, it is clear that reducing the number of exacerbations is an important treatment goal in COPD.

A number of studies have now demonstrated that ICS reduce the number of exacerbations [11,14,15,20,26,39] and that withdrawal of ICS causes an increase in exacerbation rates.[24,25] In a recent meta-analysis, Yang and colleagues have shown that the use of ICS reduces the number of exacerbations with 26% compared to placebo.[13] However, this may not be relevant to all patients with COPD, since some will not experience an exacerbation for several years. In this context, the findings of de Oca *et al.* are of interest. They showed that patients with more severe COPD (FEV_1 < 50% predicted) have a higher risk to experience a COPD exacerbation.[40] For this reason, the current GOLD guidelines recommend to prescribe ICS in patients with an FEV_1 below 50% predicted (GOLD stage III and IV).[1] However, a post-hoc analysis of the TORCH study found that patients with COPD GOLD stage II also experience exacerbations quite frequently, with a mean number of 0.82 exacerbations per year. Interestingly, treatment with fluticasone or fluticasone/salmeterol resulted in a similar proportionate reduction in the number of exacerbations. These findings suggest that treatment with ICS may also be worthwhile in patients with mild to moderate COPD, especially when they suffer from frequent exacerbations.[30] The latter is also in agreement with the findings of the ISOLDE study. Although ICS did not significantly reduce the number of exacerbations in all patients with mild COPD (now labeled moderate, GOLD stage II) **(Figure 2a)**, the number of patients with mild COPD who experienced more than one exacerbation per year was reduced from

16 to 8% **(Figure 2b)**.[41] **Table 3** shows the exacerbation rates of several long-term studies for different treatment groups. Altogether, ICS reduce the number of exacerbations in patients with COPD GOLD stages II-IV and reduce exacerbation frequency in patients with frequent exacerbations. As it can take several weeks to recover from an exacerbation, this reduction may be clinically relevant for a patient's welfare and possibly contribute to the observed long-term improvement in quality of life.

Figure 2: Exacerbation rates of the ISOLDE trial



a) In patients with mild chronic obstructive pulmonary disease (COPD), inhaled corticosteroids (ICS) do not reduce the number of exacerbations per year. b) However ICS do reduce the percentage of patients that have more ≥1 exacerbation per year. Indicating that ICS are effective in patients with mild COPD and frequent exacerbations. From Jones et al.41 Reproduced with permission.

Table 3: Influence of ICS on annual exacerbation rates

Study	n	Age (years)	Medication	Treatment period	Exacerbation rate (no./patients/year)			
					Placebo	LABA	ICS	ICS + LABA
ISOLDE [20]	751	64	FP/ PLAC	3y	1.32	-	0.99#	-
TORCH [11]	6112	65	SFC/ FP / SAL / PLAC	3y	1.13	0.97#	0.93#	0.85#
TRISTAN [15]	1465	63	SFC/ FP / SAL / PLAC	1 y	1.30	1.04#	1.05#	0.97#*
Calverley [14]	1022	64	BF / BUD / FT / PLAC	1 y	1.80	1.85	1.60	1.38#
Szafranski [26]	812	64	BF / BUD / FT / PLAC	1 y	1.87	1.84	1.59	1.42#
Kardos [39]	994	64	SFC / PLAC	44 wk	-	1.40	-	0.92*

= p<0.05 compared to placebo; * = p<0.05 compared to LABA; – = group not present in study; BDP = beclomethasone; BF = budesonide/formoterol; BUD = budesonide; FP = fluticasone propionate; FT = formoterol; LABA = long-acting β2-agonist; PLAC = placebo; SFC = salmeterol/fluticasone; SAL = salmeterol.

Treatment of Acute Exacerbations

It is well known that systemic corticosteroids are effective in the treatment of COPD exacerbations. They improve FEV_1 and oxygenation and reduce the length of hospital stay.[42] Few studies have been carried out to determine the optimum dose of systemic steroids with regard to efficacy and safety. Niewoehner *et al.* investigated the effect of treatment duration and found that a 2-week treatment period was as effective as 8 weeks treatment.[43] It has been shown by de Jong and colleagues in a study of 210 COPD patients that prednisolone (60 mg once daily) is equally effective when administered orally compared to intravenously.[44] They did not find any differences in treatment failure, length of hospitalization or FEV_1 after one week treatment. Several studies have investigated the efficacy of combination therapy with ICS/LABA in the treatment of mild to moderate COPD exacerbations. Bathoorn and colleagues have shown that outpatient treatment with inhaled budesonide/formoterol (320/9 µg q.i.d) for 2 weeks significantly improves symptom scores, but not (significantly) the level of FEV_1 when compared to placebo.[45] In addition, a reduction in sputum eosinophils but not neutrophils or macrophages was found with budesonide/formoterol. The improvements in inflammatory and clinical parameters were similar between budesonide/formoterol and oral prednisolone (30 mg once daily). These findings are in agreement with those of Ställberg and colleagues, who compared the efficacy of inhaled budesonide/formoterol (320/9 µg q.i.d.) to oral prednisolone (30 mg once daily) in patients with an acute COPD exacerbation. In a non-inferiority analysis, no differences were found between budesonide/formoterol and oral corticosteroids. Both were equally effective with regard to change in FEV_1, symptoms, quality of life, treatment failure and need for rescue medication.[46] Similar results were found in studies with nebulized corticosteroids versus systemic corticosteroids.[47,48] Altogether, inhaled corticosteroids in high doses are effective also in the treatment ofCOPD exacerbations.

COMORBIDITY AND MORTALITY

COPD increases mortality. It accounted for 4% of all deaths in the USA in 1995, making it the fourth highest cause of death.[49] In COPD patients, the most important predictors for mortality are a lower lung function and higher age.[50,51] Several studies have shown that the risk to develop cardiovascular diseases and lung cancer is increased in patients with COPD. For example, Mannino and colleagues have shown in a large population-based study of 20,296 subjects that more severe COPD is associated with the presence of higher comorbidity. In a logistic regression model adjusting for age, sex, race, smoking, body mass index and education, patients with COPD GOLD stage III or IV had an increased risk for hypertension [odds ratio (OR) 1.6, 95% confidence interval (CI) 1.3 – 1.9] and cardiovascular disease (OR 2.4, 95% CI 1.9 – 3.0].[52] In addition, a 10 year, follow-up study found that patients with COPD have a markedly increased risk of developing lung cancer compared with controls matched for age, sex, occupation and smoking history (8.8% in 113 COPD patients and 2.0% in 113 matched

controls).[53] These risks are reflected in the leading causes of death of COPD patients, which are respiratory failure, followed by acute myocardial infarction, other ischemic heart disease and lung cancer.[11,54] The exact mechanism linking COPD with these comorbid diseases is not yet entirely clear, although it is speculated that continuous low-grade systemic inflammation, oxidative stress or hypoxemia, and a shared genetic predisposition could play a role.[55–57] There are several indications that ICS might have a positive effect on these comorbidities. A Canadian case control study found an association was found between the use of low dose (50 – 200 µg/day) ICS and a decreased risk of acute myocardial infarction.[58] In addition, the risk of developing lung cancer was found to decrease by 61% in 10,474 patients with COPD when they used more than 1200 µg/day ICS.[59] Based on the above studies, among others, it could be speculated that ICS with or without LABA might reduce overall mortality in COPD. However, this has recently been the subject of intense debate. No effects on mortality were found in the LHS, EUROSCOP and ISOLDE trials separately,[16,20,21] but a meta-analysis of these studies did suggest overall survival benefit.[60] Subsequently, the TORCH study was carried out, which was specifically designed to investigate the effect of ICS with or without LABA on mortality.[11] This study found no reduction in mortality with fluticasone mono-therapy when compared to placebo (16% versus 15.2%; p=0.53). However, with fluticasone/salmeterol combination therapy, an absolute risk reduction of 2.6% was found. Although this difference did not quite reach statistical significance (p=0.052), these findings suggest that ICS/LABA combination therapy reduce overall mortality in COPD. A subsequent meta-analysis (now also including the TORCH data) found that all-cause mortality can be reduced with ICS/LABA combination therapy compared to placebo (OR 0.79; 95% CI 0.65 – 0.98);[61] however there was no significant difference between the combination and LABA alone (OR 0.89; 95% CI 0.73 – 1.08), suggesting an effect of LABA more than of ICS.[62]

THE EFFECTS OF ICS ON AIRWAY INFLAMMATION IN COPD

Increased numbers of neutrophils, macrophages, lymphocytes (especially $CD8^+$ lymphocytes), eosinophils, and B-cells (mainly in lymphoid follicles) in the airway wall and parenchyma have been described in patients with COPD when compared to healthy smokers or ex-smokers.[63–67] In the airway lumen, predominantly neutrophils and eosinophils are increased.[65,68–70] Several studies have shown an association between the extent of airway inflammation and the severity of COPD. For example, in a recent cross-sectional study of surgically resected lung tissue from 150 COPD patients, Hogg and colleagues have shown that the percentage of airways containing neutrophils, macrophages, $CD4^+$ lymphocytes, $CD8^+$ lymphocytes, B-cell lymphoid follicles increases with disease progression as reflected by the GOLD stage.[64] In addition, they showed that these inflammatory differences were coupled to an increased thickness of the airway wall due to an increase in epithelium, airway smooth muscle, lamina propria and adventitial compartments. Many studies have

investigated the anti-inflammatory effects of inhaled corticosteroids in COPD **(table 4)**.[71–92]

In a study of 55 COPD patients who received either salmeterol/fluticasone 50/500 µg b.i.d., fluticasone 500 µg b.i.d. or placebo, Bourbeau *et al.* showed a significant decrease in $CD8^+$ cells and $CD68^+$ macrophages in bronchial biopsies.[73] Similarly, Barnes *et al.* demonstrated a decrease in $CD8^+$, $CD45^+$ and $CD4^+$ cells after 13 weeks treatment with fluticasone/salmeterol 500/50 µg b.i.d., although no difference in $CD68^+$ cells was found.[71] They also found a decrease in the percentage of neutrophils and total number of eosinophils in sputum. In the GLUCOLD study, 6 months of treatment with fluticasone decreased the number of bronchial $CD3^+$, $CD4^+$, $CD8^+$ cells, and mast cells compared to placebo (difference: -55%, p=0.004; -78%, p<0.001; -57%, p=0.010; -38%, p=0.039, respectively).[23] After 30 months of treatment the decrease of $CD3^+$, $CD4^+$ and $CD8^+$ cells was maintained and mast cells decreased even further (-56%, p=0.001 compared to 6 months). Furthermore, treatment with fluticasone decreased the percentage of sputum neutrophils, macrophages and lymphocytes after 30 months treatment, but not after 6 months treatment. However, it should be taken into account that a number of studies did not show any effects of ICS on inflammation, this could be due to the small size of most studies and their relative short time of follow-up.[74,82,84,88] It has been suggested that the relative glucocorticoid insensitivity in COPD may be due to a reduction of histone deacetylase (HDAC). Ito *et al.* have shown that HDAC levels in blood macrophages and lung biopsies are reduced in patients with COPD compared to healthy smokers. In addition, they observed that lower HDAC levels were associated with more severe COPD.[93,9494] Interestingly, it appears that low-dose theophylline increases HDAC activity, which may improve glucocorticoid responsiveness.[95] However, whether change in HDAC activity accompanies steroid sensitivity on an individual level has not yet been determined. In conclusion, the type of inflammation in COPD is complex and appears to be less responsive to treatment with ICS than the inflammation seen in asthma. The diversity of the airway inflammatory process in COPD hampers the interpretation of the data; it is presently unknown which component of COPD contributes to which part of the clinical presentation.

BRONCHIAL HYPERRESPONSIVENESS

Although bronchial hyperresponsiveness (BHR) is often thought to be a hallmark characteristic of asthma, it also occurs in up to two-thirds of patients with COPD.[98,99] It has been shown that the presence of BHR in COPD is associated with a more rapid decline in lung function over time.[100–103] For this reason, it may be important to assess whether treatment influences the severity of BHR.[104] Thus far, few studies have investigated the effects of ICS on BHR in COPD.[21,90,105–108][21,93]In five studies, no improvement in PC_{20} methacholine and/or PC_{20} histamine was found after treatment with ICS.[90,105–108] However, those studies were small with a maximum number of 58 patients included. Interestingly, two larger studies did find

Table 4: Study summary and results for studies investigating the effect of inhaled corticosteroids on local inflammation

Study	n	Age (years)	FEV (%pred or L)	Packyears	Current smoking (%)	Medication	Treatment period	Specimen	Effect of treatment
Hattotuwa [81]	30	65	46%	63	77%	FP/ PLAC	3 mo	Biopsy	Epithelium: CD4/CD8 ↓, mast cells ↓
Bourbeau [73]	51	64	58%	58	55%	SFC/ FP/ SAL/ PLAC	12 wk	Biopsy	SFC: CD8+ ↓, CD68+ macrophage ↓; FP: no significant effect
Gizycki [80]	24	65	50%	60	?	FP/ PLAC	3 mo	Biopsy	Mucosal mast cells ↓, neutrophils ↑
Barnes [71]	140	64	58%	42	87%	SFC/ PLAC	13 wk	Biopsy / sputum	Biopsy: CD8+ ↓, CD45+ ↓, CD4+ ↓; Sputum: neutrophils ↓, eosinophils ↓
Verhoeven [90,96]	23	55	63%	25	100%	FP/ PLAC	6 mo	Biopsy / BAL	Biopsy: trends for CD8+, MBP+, CD68+ and tryptase+ cells
Lapperre [23]	114	62	63%	43	63%	SFC/ FP/ PLAC	30 mo	Biopsy / sputum	Biopsy: MucosalCD3+ ↓, CD4+ ↓, CD8+ ↓ and mast cells ↓; Sputum: neutrophils ↓, macrophages ↓ and lymphocytes ↓
Ozol [85]	22	65	60%	45	0%	BUD/ PLAC	6 mo	BAL	Neutrophils ↓, IL-8 ↓
Thompson [89]	30	50	73%	60	100%	BDP / PLAC	6 wk	BAL	Total cells ↓, albumin ↓, lactoferrin ↑ in placebo; lysozyme ↑ in placebo
Confalonieri [77]	34	58	60%	49	100%	BDP/ PLAC	2 mo	Sputum	Total cells ↓, Neutrophils ↓
Brightling [74]	49	±62	±1.30 L	40	48%	MOM/ PLAC	6 wk	Sputum	No significant effect on eosinophils
Culpitt [78]	40	67	41-56%	±52	?	BUD/ PRED/ PLAC	4 wk	Sputum	Eosinophils ↓, if baseline eosinophils >3%
Sugiura [88]	25	43 - 73	50%	>20	44%	FP/ PLAC	4 wk	Sputum	No effects on total cells, subcells, IL-8, MMP-1, MMP-9, TIMP-1, SLPI or IL-8
Keatings [82]	21	45 - 78	40%	48	46%	BUD/ PRED	2 wk	Sputum	No effect on total cells, subcells, TNF, MPO, HNL, ECP, EPO or IL-8
Boorsma [72]	19	63	65%	37	68%	BUD/ PRED/ FLAC	6 mo	Sputum	Neutrophils ↓, no effect on neutrophils
Yildiz [97]	18	64	42%	54	67%	FP/ PLAC	2 mo	Sputum	Total cells ↓, eosinophils ↓
Loppow [84]	19	55	83%	48	?	FP	4 wk	Sputum	No effects on total cells, subcells, LDH, ECP, IL-8 or elastase
Burnett [76]	18	67	1.20 L	?	0%	BDP/ PLAC	4 wk	Sputum	Eosinophils ↓

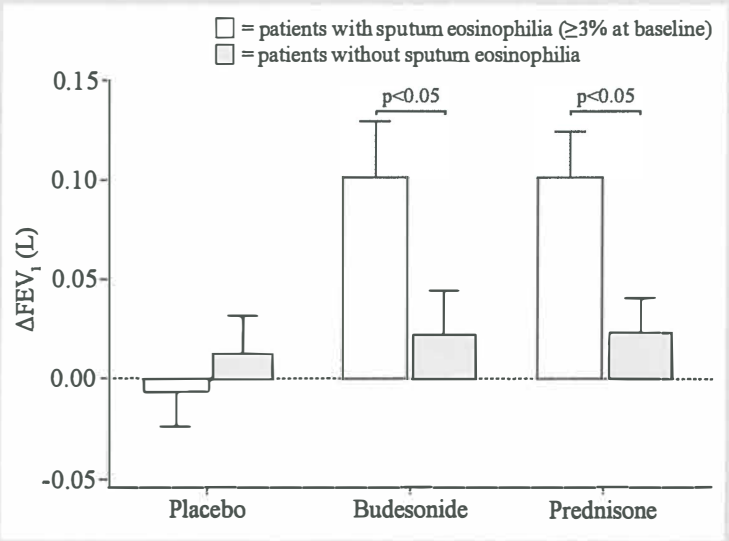
BAL = bronchoalveolar lavage; BDP = beclomethasone; BUD = budesonide; ECP = eosinophil cationic protein; EPO = eosinophil peroxidase; FP = fluticasone propionate; HNL = human neutrophil lipocalin; IL = interleukin; :DH = lactate dehydrogenase; MBP+ = myelin basic protein; MMP = matrix metalloproteinase; MOM = mometasone; MPO = myeloperoxidase; PLAC = placebo; PRED = prednisolone; SAL = salmeterol; SFC = salmeterol/ fluticasone; SPLI = secretory leukoprotease inhibitor; TIMP = tissue inhibitor of MMP; TNF = tumor necrosis factor.

an effect of ICS on BHR. In the 116 patiens in the LHS, the responsiveness to methacholine improved significantly with triamcinolone 600 µg b.i.d. (p=0.02).[21] In agreement with this, the GLUCOLD study (n=116) showed an improvement of 1.5 doubling dose concentrations after 6 and 30 months treatment with fluticasone or fluticasone/salmeterol compared to placebo (p=0.036).[23] Altogether, ICS appear to improve BHR both on the short- and long-term, in COPD.

PREDICTION OF CORTICOSTEROID RESPONSE IN COPD

So far, a limited number of studies have investigated which clinical features might predict corticosteroid response in COPD and their results are summarized below. First, Soriano and colleagues have performed a meta-analysis in 3911 patients who were randomized to ICS or placebo and followed-up for a period between 12 and 36 months.[109] They showed a slightly better short-term improvement in FEV_1 with ICS in females. Another finding was that continued smoking attenuated the increase in FEV_1 related to ICS treatment during the first 6 months of treatment. However, no differences on the rate of FEV_1 decline on the longer term were observed between males and females and smokers or ex-smokers. Second, several studies have investigated the association between bronchodilator responsiveness (BDR) and treatment response to ICS. Bleecker and colleagues were able to demonstrate in a large study of 358 COPD patients that the improvement in FEV_1 after 8 weeks treatment was significantly larger in reversible patients than in non-reversible patients.[110] Similarly, Kitaguchi and colleagues found a larger improvement in FEV_1 after 2 months of treatment with ICS, when patients with COPD demonstrated BDR (increase in FEV_1 >12% and 200 ml after 20 µg of procaterol) compared with those who did not demonstrate BDR.[111] By contrast, two further studies were unable to find a difference in corticosteroid responsiveness between COPD patients with and without BDR, but these studies were small and therefore hampered by a lack of power.[112,113] Third, several studies have reported that a higher baseline percentage of sputum eosinophils in COPD is associated with a larger increase in FEV_1 and health status after short-term treatment (between 2 and 6 weeks) with inhaled or oral corticosteroids **(Figure 3)**.[75,79,83] In this context, the findings of Siva and colleagues may also be of interest. They showed that a treatment regimen aimed to reduce the percentage of sputum eosinophils reduces the number of severe exacerbations in COPD.[114] Finally, it has been shown in a group of 30 COPD patients who were all responsive to methacholine that those patients who were also responsive to inhaled mannitol (n=7), an indirect stimulus, had a significantly higher increase in FEV_1 after three months treatment with ICS when compared to mannitol negative patients (p=0.001).[115] However, this contrasts with findings of Rutgers and colleagues, who did not find any improvement in FEV_1 with 6 weeks of ICS treatment in 22 COPD patients, all of whom were hyperresponsive to adenosine 5'-monophosphate (AMP), which is also an indirect stimulus.[116] Therefore, it remains unclear whether measurement of

Figure 3: Patients with a high percentage of sputum eosinophils (≥3%) have a larger improvement of their FEV1 after treatment with budesonide or prednisone



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hyperresponsiveness with an indirect stimulus is able to predict a favorable corticosteroid response in COPD. Finally, it has been suggested that patients with frequent exacerbations may benefit more from ICS treatment than patients with fewer exacerbations. However, to our knowledge this has not yet been formally demonstrated in a clinical study. Altogether, the presence of BDR and eosinophilic airway inflammation may predict a better short-term response to ICS. It remains unclear whether these features can also predict a better ICS response on the long term. This has to be sorted out in future studies, especially since the data from the TORCH study and the GLUCOLD study have now shown that inhaled corticosteroids may slow the progression of COPD, at least in a subset of patients.

COMBINATION THERAPY WITH ICS AND LONG-ACTING B_2-AGONISTS

Corticosteroids can interact with β_2-agonists in a beneficial way; they increase the expression of β_2-receptors on inflammatory and airway smooth muscle cells and inhibit β_2-receptor desensitization and downregulation, at least in vitro.[117–120] In turn, β_2-agonists are capable of potentiating the anti-inflammatory actions of corticosteroids by increasing the expression of glucocorticoid receptors.[121–123] In asthma, it has been shown that adding a long-acting β_2-agonist to maintenance treatment with ICS improves symptoms and lung function and reduces the number of asthma exacerbations. In the TRial of Inhaled STeroids ANd long-

acting β_2-agonists agonists (TRISTAN) study, 1465 patients with COPD were treated with fluticasone/salmeterol, fluticasone alone, salmeterol alone or placebo for 1 year. Patients who were treated with fluticasone/salmeterol had a significantly larger increase in FEV_1 than those with either treatment alone.[15] These findings are in agreement with those of Szafranski *et al.*,[26] who treated 812 COPD patients with budesonide/formoterol, budesonide alone, formoterol alone, or placebo. Treatment with budesonide/formoterol improved both FEV_1 and quality of life to a greater extent than either treatment alone. Finally, Calverley *et al.* performed a study in 1022 COPD patients with moderate to severe COPD (mean FEV_1 36% predicted). [14]During a run-in period of two weeks, all patients were treated with oral prednisolone and formoterol. After this run-in period, patients were randomized to budenosonide/formoterol, budesonide alone, formoterol alone, or placebo. Combination therapy prolonged the time to first exacerbations compared to mono-therapy or placebo (budesonide/formoterol 254 days, budesonide 178 days, formoterol 154 days and placebo 96 days). In addition, patients who were treated with combination therapy had a slower decline in FEV_1 and a greater improvement in quality of life. Furthermore, a recent meta-analysis showed that combination therapy resulted in a reduced number of COPD exacerbations has been demonstrated with combination therapy, compared to ICS alone.[61] Finally, combination therapy with fluticasone/salmeterol 500/50 µg b.i.d. has been shown to reduce the number of CD8+ lymphocytes and macrophages in the airway wall to a greater extent than fluticasone 500 µg b.i.d. monotherapy.[73] However, many of these results are from studies with a short follow-up of a maximum of 1 year. In this context, it is important to mention two other studies with a longer follow-up (30 months to 3 years). The TORCH study showed no supplementary effect of adding salmeterol to fluticasone, that is a similar annual decline in FEV_1 occurred with fluticasone/salmeterol (39 ml/year) and with fluticasone (42 ml/year) ($p=0.445$).[22] In addition, the GLUCOLD study, which had a follow-up of 30 months, did not find any beneficial effect of adding salmeterol to fluticasone with regard to parameters of airway inflammation and even showed a significantly worse outcome for quality of life.[23] In conclusion, combination therapy with ICS/LABA may improve symptoms and lung function and reduce the number of exacerbations on the short term. However, there is currently no evidence that combination therapy with ICS/LABA is more effective in improving symptoms, FEV_1 and inflammation on the long term.

ADVERSE EFFECTS OF ICS

Local effects are the most common adverse effects seen in ICS treatment. Oropharyngeal candidiasis is described in many studies with an incidence ranging between 1 and 10%. Other symptoms that are significantly increased are pharyngeal irritation and hoarseness.[11,16,20,21] Although systemic effects of ICS appear to be small, there has been concern with their use on the long term. The possible systemic adverse effects of ICS are discussed below.

Serum cortisol

A small yet significant decrease in serum cortisol level after treatment with fluticasone 500 µg b.i.d. was seen in the ISOLDE study.[20] However, only 5% of the patients had a cortisol level below the normal range and no signs of hypoadrenalism or other clinical effects were observed in this study.

Bone density and fractures

There has been concern that ICS may induce osteoporosis on the long term. In the LHS, a reduced bone density in the lumbar spine and femoral neck was seen after 3 years treatment with triamcinolone. However, no reduction in bone mineral density or increased risk of fracture was seen in EUROSCOP or the TORCH study.[11] These findings were confirmed in a recent meta-analysis, which found no increased risk of fracture or loss of bone density.[124]

Cataract

A British study using a general practitioners database of 15,479 cataract patients and 15,479 controls, found an increased risk to develop cataract with the use of ICS, with an odds ratio of 1.58 (95% CI 1.46 – 1.71). However, after correction for use of systemic corticosteroids and consultation rate the odds ratio was reduced to 1.10 (95% CI 1.00 – 1.20).[125] In addition, no increased risk was seen after three years of treatment with ICS in either EUROSCOP, the LHS, the ISOLDE or the TORCH studies.[11,21]

Pneumonia

Ernst and colleagues demonstrated, in a population-based case-control study that, the use of ICS in COPD is associated with an increased risk of pneumonia.[126] In this study, a cohort of 175,906 patients with COPD was investigated and followed-up over the period 1988 – 2003. The patients who were hospitalized for pneumonia (a total of 23,942) more often used ICS than patients who did not develop pneumonia. In addition, a dose-response relationship was found, with an increase in the risk to develop pneumonia at a higher ICS dose. This is in agreement with the findings of the TORCH study, which found a 60% increased risk to develop pneumonia in patients who were treated with fluticasone or fluticasone/salmeterol when compared to salmeterol or placebo (the chance to develop pneumonia was 19.6% in the combination group and 18.3% in the fluticasone group, compared with 13.3% in the salmeterol group and 12.3% in the placebo group).[11] This increase in pneumonia in the TORCH study with ICS and combination therapy was not accompanied by increased but decreased exacerbation rates (and a trend for decreased mortality) compared to placebo. Several meta-analyses support these findings.[5,61,127] However, there are several limitations on the abovementioned studies and meta-analyses. First, a chest radiograph was not required to confirm the diagnosis of pneumonia. This may be important, since the signs and symptoms of COPD exacerbations and pneumonia often overlap. Furthermore, the meta-analyses did not have access to individual patient data and could therefore not adjust for potential

confounding factors such as age, lung function, or other clinical features. Finally, the meta-analyses were heavily weighted by studies using fluticasone. In this context, the recent findings of Sin and colleagues may also be of interest.[128] They a performed a meta-analysis of seven large clinical trials with budesonide of which they did have access to the individual patient data. In this meta-analysis, they were unable to find an increased risk to develop pneumonia with budesonide. Altogether, further studies are needed which are specifically designed to investigate whether the use of ICS increases the risk of pneumonia.

Hyperglycemia

No increased risk for developing diabetes was found in the LHS after three years treatment with triamcinolone.[21] A cohort study in 1698 subjects using ICS did not find an association between serum glucose levels and ICS use in non-diabetic subject; however, serum glucose levels in subjects with self reported diabetes were associated with the dose of ICS used.[129]

Skin bruising

Several studies have shown that long-term ICS use increases skin bruising.[16,20,21] Furthermore, it was found that high-dose ICS was associated with a decrease of skin thickness of 15 to 19% compared with the skin thickness of controls.[130]

CONCLUSION

The role of ICS in the treatment of COPD has been the subject of much debate. Although ICS clearly improve symptoms and lung function on the short term and on exacerbation frequency on the long term, several studies were unable to demonstrate an effect on decline in lung function of ICS on the long term. For this reason, it was concluded that ICS do not influence the natural course of COPD. However, this conclusion has been challenged by the TORCH and the GLUCOLD studies, which found a reduction in the decline in FEV_1 with ICS. Altogether, there is now more evidence that COPD can be a treatable disease, at least in a subset of patients. This subset of patients comprises those with a favorable response to treatment with ICS (i.e. reducing progression of lung function loss, reducing exacerbation rate and improving quality of life). However, it is questionable whether all COPD patients should be treated, as the long-term use of ICS is accompanied by side effects, such as oral candidiasis and hoarseness. In addition, there is now considerable evidence that the use of ICS increases the number of cases of pneumonias. For this reason, it is becoming increasingly important to identify those subjects with COPD with or without a favorable response to ICS. This seems to be the case for patients with frequent exacerbations. Otherwise, few studies have been performed on this subject. It has been shown that the presence of BDR and eosinophilic airway inflammation are associated with a better response to ICS on the short term. However, it is currently unclear whether these features also predict a favorable response on the long term.

EXPERT OPINION

The question remains: which COPD patients to treat and which not to treat? The current GOLD guidelines recommend treatment with ICS only for patients with severe COPD (GOLD stage III or IV) and more than 3 exacerbations per 3 years. These guidelines might be too conservative for several reasons. First, the GLUCOLD and TORCH studies showed that ICS improve lung-function decline in patients with moderate COPD. Second, several studies have shown a smaller annual decline in quality of life in all COPD stages with the use of ICS. Finally, ICS reduce the number of exacerbations to a similar proportionate extent in moderate COPD (GOLD II) as in more severe COPD. For this reason, ICS should also be considered with moderate COPD (GOLD II) and in patients of all severity, who experience repeated COPD exacerbations.

As the use of ICS is associated with side effects, it is now important to identify those patients with COPD with a more favorable response to ICS. Additionally, an increased percentage of sputum eosinophils and a larger bronchodilator response seem promising to predict a favorable response to ICS on the short term. However, whether they predict a better response to ICS on the longer term is unknown. A very promising approach to predict a better response both in the short and long term might be pharmacogenetics. A recently published study by Kim *et al.* showed that the rs242941 polymorphism in the corticotrophin-releasing hormone receptor 1 (*CRHR1*) gene in COPD patients is associated with a better improvement in lung function after twelve weeks of treatment with fluticasone/salmeterol.[131] The recent findings of Woodruff and colleagues are also of interest.[132] By using gene expression microarrays, they identified three genes that were upregulated in patients with asthma, but not in healthy controls, namely calcium activated family member 1 (*CLCA1*), *periostin*, and serine peptidase inhibitor B2 (*SERPINB2*). Treatment with inhaled corticosteroids downregulated these three genes and markedly upregulated expression of FK506 binding protein 51 (*FKBP51*). Interestingly, the baseline expression of *CLCA1*, *periostin*, *SERPINB2* correlated directly with fluticasone-induced improvement in FEV_1, whereas the baseline level of *FKBP51* correlated inversely with improvement in FEV_1. These findings elegantly demonstrate usefulness of the microarray technique in identifying those asthma patients who benefit the most from ICS. It is tempting to speculate that gene expression microarrays may also be able to identify COPD patients who benefit the most from treatment with ICS.

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CHAPTER 3

Inflammation and Corticosteroid Responsiveness in Ex-, Current- and Never-smoking Asthmatics

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ABSTRACT

Background: It has been suggested that smoking asthmatics benefit less from corticosteroid treatment than never-smoking asthmatics. We investigated differences in blood and sputum inflammatory profiles between ex-, current-, and never-smokers and assessed their ICS treatment response after 2-week and 1-year treatment.

Methods: We analyzed FEV_1, PC_{20} methacholine and PC_{20} AMP, (differential) cell counts in sputum and blood in ex-, current- and never-smokers at baseline (n=114), after 2-week treatment with fluticasone 500 or 2000 µg/day (n=76) and after 1-year treatment with fluticasone 500 µg/day or a variable dose of fluticasone based on a self-management plan (n=64).

Results: A total of 114 patients were included (29 ex-, 30 current- and 55 never-smokers. At baseline, ex- and current-smokers had less eosinophils in sputum and blood than never-smokers. Blood neutrophil counts were higher in current- than in never-smokers. A higher number of cigarettes smoked daily was associated with lower blood and sputum eosinophils. After 2-week ICS treatment, FEV_1 %predicted improved less in current-smokers than never-smokers (2.4% versus 8.1%, p=0.010) and ex-smokers tended to improve less than never-smokers (4.1%, p=0.067). In contrast, no differences in ICS treatment response in lung function or inflammatory cells were found between the three groups after 1 year.

Conclusion: Ex- and current-smokers have less eosinophils and more neutrophils in their sputum and blood than never-smokers. Although ex- and current-smokers have a reduced short-term corticosteroid treatment response, we did not find a difference in their long-term treatment response.

INTRODUCTION

Asthma is a chronic inflammatory airway disease in which a variety of inflammatory cells and mediators play a role. Inhaled corticosteroids (ICS) are the cornerstone of treatment, since they exert broad anti-inflammatory effects. They have been shown to improve symptoms and lung function as well as bronchial hyperresponsiveness and markers of airway inflammation in blood, induced sputum and bronchial biopsies.[1] In addition, the use of ICS reduces the number of asthma exacerbations.[2]

About 20-30% of asthma patients smoke and another 20-40% are ex-smokers.[3–6] Current-smokers appear to have a different airway inflammatory profile than never-smokers, with less eosinophilic and more neutrophilic inflammation.[7–12] Thus far, very little is known about the inflammatory profile of ex-smokers.

The few studies investigating the effects of smoking on the short-term efficacy of oral or inhaled corticosteroid treatment in asthma, demonstrate that the forced expiratory volume in one second (FEV_1) improves significantly in never-smokers, but not in current-smokers.[7,13–15] However, none of these studies found statistically significant differences in improvement in FEV_1 when directly comparing never- and current-smokers. The only study that included ex-smokers, showed no improvement in FEV_1 or asthma control after 2-week oral corticosteroid treatment in ex- and current-smokers.[15]

We aimed to investigate whether ex-, current- and never-smokers with asthma have different inflammatory profiles and if current number of cigarettes or packyears smoked affect this. Furthermore, we assessed whether the short- and long-term responsiveness to corticosteroids after 2-week and 1-year treatment is different between ex-, current- and never-smoking asthmatics. We have analyzed this in a relatively large group of 114 well-characterized patients with allergic, mild to moderately severe asthma.[16]

PATIENTS AND METHODS

Patients

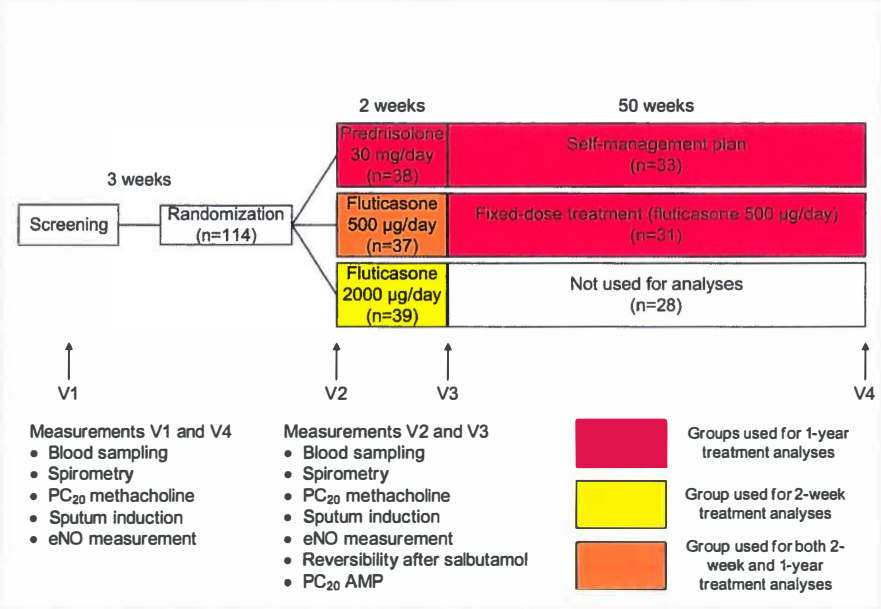
Patients with a diagnosis of asthma, 18-65 years old, were included if they met the following criteria: provocative concentration of methacholine inducing a 20% fall of FEV_1 (PC_{20} methacholine) ≤8 mg/ml, at least one positive skin-prick test out of 17 common aero-allergens, reversibility to salbutamol 200 µg ≥9% of the predicted FEV_1 and the ability to expectorate sputum after hypertonic saline inhalation. This study was conducted in accordance with the amended declaration of Helsinki and the study was approved by the medical ethics committee of the University Medical Center Groningen and all participants gave their written informed consent.

Study design

Figure 1 shows the outline of the study. ICS were tapered before enrollment in the study, as described in the original manuscript.[16] After discontinuation of ICS completely for three weeks, or earlier, if they experienced symptoms of an asthma exacerbation, patients were randomized to 3 treatment arms, with minimization according to smoking status, age, previous dose of ICS, FEV_1 %predicted, reversibility after 200 µg of salbutamol, PC_{20} methacholine, and serum IgE. Patients were first treated for 2 weeks with either prednisolone 30 mg/day, fluticasone 500 µg/day or fluticasone 2000 µg/day via Diskhaler, followed by another 50 weeks of treatment as follows.

The prednisolone 30 mg/day group was treated according to a self-management plan. They first received fluticasone 200 µg/day and were instructed to change the dose according to a self-management plan (Appendix). The fluticasone 500 µg/day group continued with the same dose for another 50 weeks. The fluticasone 2000 µg/day arm followed a program with step-down and eventually complete discontinuation of corticosteroids. The latter is not in agreement with the current guidelines and therefore this arm was removed from our long-term analyses. During the first 2 weeks, the study had a double-blind, double-dummy design, followed by 50 weeks open label treatment. Rescue medication consisted of salbutamol 400 µg via Diskhaler. No other concomitant pulmonary medication was allowed.

Figure 1: Flow-chart of the study



FEV_1 = forced expiratory volume in one second, PC_{20} = provocative concentration causing a 20% fall in FEV_1, AMP = adenosine-5'-monophosphate.

Patients with an exacerbation were treated with a standardized 7-day course of oral prednisolone. Patients were withdrawn if they required >1 hospitalization, >4 courses of oral prednisolone or >2 courses within 3 months. Requirement of >2000 µg fluticasone in the self-management group additionally led to withdrawal.

Lung function and bronchial hyperresponsiveness

FEV_1 was measured with a calibrated, water-sealed spirometer according to standardized guidelines before and 20 minutes after 200 µg of salbutamol.[17] Provocation tests were performed using a 2-minutetidal breathing method, adapted from Cockcroft and coworkers.[18] After an initial nebulized saline challenge, subjects inhaled doubling concentrations of the provocative agent (methacholine-bromide 0.038 to 19.6 mg/l or adenosine-5'-monophosphate (AMP) 0.04 to 320 mg/ml) at 5 minute intervals. All calculations of PC_{20} were performed with a base-2 logarithm, reflecting doubling concentrations and normalizing the distribution.

Sputum induction and processing

Sputum was induced by inhalation of hypertonic saline as previously described.[16] Fifteen minutes after salbutamol (200 µg) inhalation, hypertonic saline (3%, 4%, and 5%) was nebulized for each concentration during 7 minutes. Whole samples were processed according to the method of Fahy *et al.* with some modifications.[19]

Cell counts in blood were performed by flow cytometry. Eosinophilic cationic protein (ECP) in serum and sputum were measured with a fluoroenzyme assay (ImmunoCAP ECP, Pharmacia, Uppsala, Sweden). Exhaled nitric oxide (NO) was measured by tidal breathing method using a chemiluminescence analyzer (CLD 700 AL, ECO physics, Switzerland) as described previously.[16]

Statistical methods

In case of non-normal distribution, log-transformation was performed to obtain normally distributed variables. Baseline differences between ex-, current- and never-smokers were tested by analysis of variance (ANOVA), Kruskal-Wallis or Chi-square test. If a significant difference between the three groups was found, we performed post-hoc tests with Holm's Bonferroni correction for multiple testing. Short-term treatment effects were analyzed only in the two groups using ICS (i.e. fluticasone 2000 µg/day or 500 µg/day). To test for changes after treatment within a group (i.e. ex-, current- or never-smokers), we performed paired t-tests. To test for differences in corticosteroid treatment responsiveness between groups, we performed linear regression analyses with change from baseline of each variable as outcome variable and smoking status as the predictor variable and age, gender and type of treatment as covariates. In addition, we adjusted for the baseline value of each variable, since this has been shown to be one of the major predictors of treatment response.[20] To test the effect of current and cumulative smoke exposure on baseline differences and treatment

response, we performed linear regression analyses with either the number of cigarettes/day or packyears as predictor variables. We added age as a covariate in these analyses. The reported correlation coefficient (b) signifies the change in an outcome variable (e.g. FEV_1) for every unit increase of the predictor variable (e.g. cigarettes/day). In all regression analyses with absolute FEV_1 we corrected for age, gender and height.

RESULTS

Patient characteristics

114 patients were included, 29 ex-smokers, 30 current-smokers and 55 never-smokers. Their baseline characteristics, after tapering of ICS (visit 2), are presented in **table 1**. During the ICS tapering period, 16 patients returned to the hospital earlier due to symptoms compatible with an asthma exacerbation. From these 16 patients, 6 still used ICS at the start of the treatment period (2 ex-smokers, 2 current-smokers and 2 never-smokers) with a median beclomethasone equivalent dose of 450 µg/day (range 400 – 800 µg/day); the remaining 10 patients had discontinued ICS completely for a median period of 12 days (range 2 – 21 days). Ex-smokers had a median smoking cessation period of 7 years (interquartile range (IQR) 1.5 -15.5 years) and had smoked a median of 6.9 packyears (IQR 3.5 – 20.8). Current-smokers had smoked 7.4 packyears (IQR 2.5 – 14.1) and smoked a median of 8.0 cigarettes/day (IQR 4.6 – 15.0).

Baseline differences between ex-, current- and never-smokers

Ex-smoking asthmatics were significantly older than current- and never-smokers (median 38 versus 27 and 25 years, respectively; p=0.001). Sputum eosinophil percentages and blood eosinophil counts were significantly lower in ex- and current-smokers than in never-smokers. Serum ECP, a marker of eosinophil activation, was significantly lower in ex- than never-smokers. Blood neutrophil counts were higher in current- than in never-smokers. Blood neutrophil counts of ex-smokers were between those of never- and current-smokers, but not significantly different from either group. FEV_1, reversibility to salbutamol, bronchial hyperresponsiveness to methacholine or AMP and exhaled NO were comparable between ex-, current- and never-smokers.

Association between current and cumulative smoke exposure and baseline clinical and inflammatory parameters

In current-smokers, a higher number of cigarettes smoked daily was associated with lower sputum eosinophil percentages, blood eosinophil counts and serum ECP **(table 2)**. Furthermore, it was associated with less severe bronchial hyperresponsiveness to both methacholine and AMP (0.1 doubling dose per cigarette/day for methacholine and 0.2

Table 1: Differences in clinical and inflammatory variables between ex-, current- and never-smokers at baseline

	Ex-smokers (n=29)	Current-smokers (n=30)	Never-smokers (n=55)	p-value
Age (years)	38 (28 – 41)	27‡ (25 – 37)	25‡ (25 – 35)	0.001
Gender (male/female) #	13 / 16	10 / 20	16 / 39	0.349
Daily number of cigarettes	–	8.0 (4.6 – 15.0)	–	
Packyears (number)	6.9 (3.5 – 20.8)	7.4 (2.5 – 14.1)	–	
Duration of smoking cessation (years)	7.0 (1.5 – 15.5)	–	–	
Still using ICS after tapering (yes/no)#	2 / 27	2 / 28	2 / 53	0.645
Treatment # (prednisolone/FP500/ FP2000)	10 / 11 / 8	11 / 6 / 13	17 / 20 / 18	0.508
FEV_1 (L)	2.9 (2.3 – 3.4)	2.8 (2.4 – 3.4)	3.0 (2.3 – 3.4)	0.911
FEV_1 (%predicted)	79 (68 – 89)	78 (70 – 91)	82 (62 – 94)	0.957
Reversibility (%predicted)	11 (9 – 17)	11 (9 – 15)	13 (9 – 18)	0.150
PC_{20} methacholine (mg/ml) §	0.7 (0.06 – 7.9)	0.8 (0.03 – 7.3)	0.4 (0.02 – 7.8)	0.123
PC_{20} AMP (mg/ml) §	10.3 (0.2 – 640)	7.2 (0.2 – 640)	3.6 (0.02 – 640)	0.179
Sputum eosinophils (%)	2.8* (1.1 – 6.0)	4.7* (0.8 – 10.7)	7.7 (3.8 – 14.3)	0.015
Blood eosinophils (10^3/μL)	0.27* (0.14 – 0.43)	0.28* (0.15 – 0.41)	0.44 (0.34 – 0.61)	0.001
Sputum ECP (μg/L)	33 (19 – 124)	67 (16 – 126)	49 (17 – 163)	0.979
Serum ECP (μg/L)	10* (8 – 17)	14 (9 – 23)	22 (12 – 29)	0.001
Sputum neutrophils (%)	39 (22 – 53)	42 (26 – 65)	29 (20 – 50)	0.175
Blood neutrophils (10^3/μL)	3.9 (3.0 – 4.6)	4.1* (3.4 – 5.3)	3.1 (2.6 – 4.1)	0.003
Exhaled NO (ppb)	15 (11 – 21)	12 (6 – 17)	16 (12 – 21)	0.058

Values are presented as medians with interquartile ranges, unless stated otherwise, # = number, § geometric mean (range), prednisolone = prednisolone 30 mg once daily, FP500 = fluticasone propionate 500 μg/day, FP2000 = fluticasone propionate 2000 μg/day, FEV_1 = forced expiratory volume in one second, PC_{20} = provocative concentration causing a 20% fall in FEV_1, AMP = adenosine-5'-monophosphate, ECP = eosinophilic cationic protein, NO = nitric oxide, ppb = parts per billion, * = $p<0.05$ compared to never-smokers with Holm's Bonferroni correction, ‡ = $p<0.05$ compared to ex-smokers with Holm's Bonferroni correction.

Table 2: Association between the amount of smoke exposure, as reflected by the number of cigarettes smoked daily and number of packyears and clinical and inflammatory variables at baseline

	Cigarettes/day		Packyears	
	b	p-value	b	p-value
FEV_1 (L)	0.13	0.508	-0.01	0.450
FEV_1 (%predicted)	0.06	0.897	-0.31	0.034
Reversibility (%predicted)	-0.22	0.162	-0.00	0.968
PC_{20} methacholine (doubling concentrations)	0.11	0.050	0.04	0.123
PC_{20} AMP (doubling concentrations)	0.19	0.031	0.02	0.593
Sputum eosinophils (%) *	-0.06	0.024	-0.01	0.496
Blood eosinophils (10^3/μL) *	-0.02	0.021	0.00	0.645
Sputum ECP (μg/L) *	-0.04	0.313	-0.00	0.839
Serum ECP (μg/L) *	-0.04	0.025	0.00	0.829
Sputum neutrophils (%) *	-0.00	0.779	0.00	0.713
Blood neutrophils (10^3/μL) *	0.00	0.673	-0.00	0.338
Exhaled NO (ppb)	-0.09	0.708	0.10	0.254

b = unstandardized regression coefficient, FEV_1 = forced expiratory volume in one second, PC_{20} = provocative concentration causing a 20% fall in FEV_1, AMP = adenosine-5'-monophosphate, ECP = eosinophilic cationic protein, NO = nitric oxide, ppb = parts per billion, * variable log-transformed.

doubling concentrations per cigarette/day for AMP). In ex- and current-smokers, a higher number of packyears was associated with a lower FEV_1 %predicted (p=0.034).

Short-term efficacy of ICS treatment in ex-, current- and never-smokers

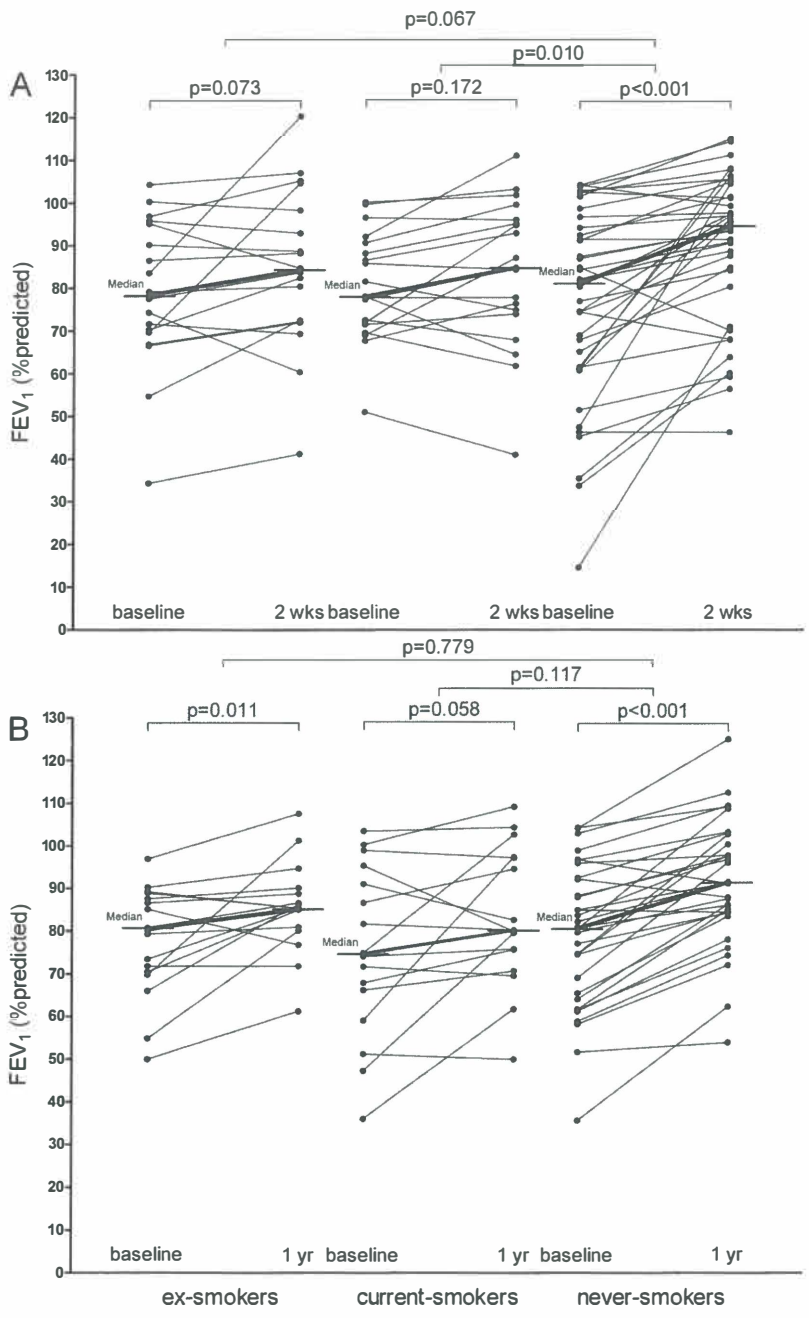
76 patients were treated with fluticasone 2000 µg/day or 500 µg/day. After 2-week treatment, FEV_1 %predicted levels improved significantly in never-smokers (8.1%, p<0.001, **table 3**), but not in ex- or current-smokers (4.1%, p=0.073 and 2.4%, p=0.172 respectively). The magnitude of improvement in FEV_1 %predicted was significantly lower in current- than in never-smokers (p=0.010, **figure 2A**) and tended to be lower in ex- than in never-smokers (p=0.067). Sputum eosinophil percentages and ECP concentrations improved less in current- than never-smokers and tended to improve less in ex- than never-smokers. No significant differences in short-term ICS-induced improvements in bronchial hyperresponsiveness and exhaled NO were observed between the three groups. A higher number of packyears smoked was associated with less improvement in FEV_1 %predicted (-0.55% per packyear, p=0.025, supplementary table 1). The number of cigarettes smoked daily was not associated with the short-term ICS response in current-smokers.

Table 3: Treatment differences between ex-, current- and never- smokers after 2-week ICS treatment

	Ex-smokers		Current-smokers		Never-smokers	
	(n=19)	p-value	(n=19)	p-value	(n=38)	p-value
Age (years)	38 (28 – 44)		27 (25 – 42)		25 (25 – 36)	
Gender (male/female)	9 / 10		4 / 15		12 / 26	
Treatment (FP500/FP2000)	11 / 8		6 / 13		20 / 18	
ΔFEV_1 (L)	0.14 (-0.07 – 0.31)	0.051	0.08 * (-0.05 – 0.32)	0.113	0.30 (0.18 – 0.77)	<0.001
ΔFEV_1 (%predicted)	4.1 # (-2.1 – 9.2)	0.073	2.4 * (-4.7 – 8.7)	0.172	8.1 (4.6 – 20.4)	<0.001
ΔReversibility (%predicted)	-4.8 (-8.1 – -0.3)	0.022	-4.3 (-7.9 – 2.3)	0.025	-6.8 (-9.7 – -2.9)	<0.001
ΔPC_{20} methacholine (doubling concentrations)	1.3 # (0.4 – 2.0)	0.001	1.4 (0.3 – 2.4)	0.001	2.3 (1.1 – 3.2)	<0.001
ΔPC_{20} AMP (doubling concentrations)	3.1 (0.4 – 5.7)	0.001	0.9 # (0.1 – 5.6)	0.007	5.1 (2.2 – 6.4)	<0.001
ΔSputum eosinophils (%)	-1.4 (-5.7 – -0.7)	<0.001	-1.0 ‡ (-4.5 – 0.0)	0.004	-6.3 (-14.5 – -2.2)	<0.001
ΔBlood eosinophils (10^3/µL)	-0.05 (-0.16 – 0.02)	0.018	-0.02 (-0.15 – 0.03)	0.060	-0.16 (-0.28 – -0.01)	0.002
ΔSputum ECP (µg/L)	-12 (-82 – -1)	0.022	-10 *§ (-48 – 9)	0.219	-31 (-135 – -1)	<0.001
ΔSerum ECP (µg/L)	-1.3 (-4.5 – 2.1)	0.564	-2.4 # (-15.0 – -4.2)	0.303	-6.9 (-14.6 – -1.0)	<0.001
ΔSputum neutrophils (%)	-3.3 (-17.4 – 7.8)	0.270	-2.7 § (-15.0 – 4.2)	0.496	0.5 (-7.8 – 6.3)	0.382
ΔBlood neutrophils (10^3/µL)	0.31 (-0.57 – 0.78)	0.625	0.27 § (-0.47 – 0.97)	0.902	0.22 (-0.26 – 0.79)	0.563
ΔExhaled NO (ppb)	-3.3 (-6.8 – 0.00)	0.039	-3.6 (-6.8 – 3.1)	0.248	-5.1 (-8.4 – -2.1)	<0.001

Values are presented as median change from baseline with interquartile range. ICS = inhaled corticosteroids, FP500 = fluticasone propionate 500 µg/day, FP2000 = fluticasone propionate 2000 µg/day, FEV_1 = forced expiratory volume in one second, PC_{20} = provocative concentration causing a 20% fall in FEV_1, AMP = adenosine-5'-monophosphate, ECP = eosinophilic cationic protein, NO = nitric oxide, ppb = parts per billion,* Significantly different from never-smokers (p<0.05), # Trend for difference from never-smokers (0.05<p≤0.1), ‡ Significantly different from ex-smokers (p<0.05), § Trend for difference from ex-smokers (0.05<p≤0.1).

Figure 2: Change in FEV1 % predicted after 2-week and 1-year treatment with ICS



A = 2-week treatment, B = 1-year treatment, FEV1 = forced expiratory volume in one second.

Long-term efficacy of ICS treatment in ex-, current- and never-smokers

Data from 64 patients treated for 1-year with fluticasone 500 µg/day or a variable dose of fluticasone according to the self-management plan were available **(table 4)**. In the self-management group, the median daily dose of fluticasone over the 50 week period was 275 µg/day (range 200-1375 µg/day), which was significantly lower than the 500 µg/day used by the fixed-dose group. The level of FEV_1 %predicted improved significantly in ex- and never-smokers, (5.1%, p=0.011 and 10.2%, p<0.001 respectively) and tended to improve in current-smokers (3.1%, p=0.058). There was no significant difference in the magnitude of improvement in FEV_1 between the three groups (figure 2B). The treatment-induced changes in PC_{20} methacholine and numbers and percentages of inflammatory cells in blood and sputum did also not differ significantly between ex-, current- and never-smokers. A higher number of packyears was associated with less improvement in FEV_1 %predicted (p=0.032, supplementary table 2). In addition, the severity of PC_{20} methacholine improved less with a higher number of packyears smoked (p=0.043). The number of cigarettes smoked daily was not associated with the magnitude of improvement in FEV_1 or PC_{20} methacholine.

Table 4: Treatment differences between ex-, current- and never-smokers after 1-year ICS treatment

	Ex-smokers		Current-smokers		Never-smokers	
	(n=16)	p-value	(n=16)	p-value	(n=32)	p-value
Age (years)	37 (27 – 40)		29 (25 – 36)		25 (25 – 34)	
Gender (male/ female)	9 / 12		7 / 10		10 / 27	
Treatment (FP500/ self management)	11 / 10		6 / 11		20 / 17	
ΔFEV_1 (L)	0.15 (0.00 – 0.60)	0.010	0.17 (-0.07 – 0.82)	0.052	0.35 (0.22 – 0.71)	<0.001
ΔFEV_1 (%predicted)	5.1 (0.4 – 13.9)	0.011	3.1 (-1.7 – 21.5)	0.058	10.2 (6.4 – 20.1)	<0.001
ΔPC_{20} methacholine (doubling concentrations)	2.7 (1.5 – 5.7)	0.002	2.3 (1.4 – 3.1)	<0.001	4.4 (2.1 – 5.5)	<0.001
ΔSputum eosinophils (%)	-2.7 (-4.5 – -0.3)	0.005	-2.0 (-14.3 – -0.1)	0.029	-7.0 (11.9 – -1.3)	<0.001
ΔBlood eosinophils (10^3/µL)	-0.04 (-0.05 – 0.02)	<0.001	-0.04 (-0.08 – 0.05)	<0.001	-0.16 (-0.26 – -0.04)	<0.001
ΔSputum ECP (µg/L)	11 (-5 – 33)	0.194	-11 (-165 – 6)	0.096	-19 (-73 – 3)	0.023
ΔSerum ECP (µg/L)	0.1 (-3.1 – 2.3)	0.576	-1.8 (-12.8 – 2.9)	0.268	-9.2 (-19.3 – -4.1)	<0.001
ΔSputum neutrophils (%)	8.5 (-18.2 – 23.3)	0.126	4.8 (-22.2 – 24.3)	0.641	6.9 (-5.1 – 24.6)	0.077
ΔBlood neutrophils (10^3/µL)	0.32 (-0.51 – 0.70)	<0.001	-0.15 (-0.87 – 0.21)	<0.001	-0.40 (-0.78 – 0.39)	<0.001
ΔExhaled NO (ppb)	-4.9 (-7.1 – 2.1)	0.182	-6.1 (-9.3 – 2.9)	0.033	-4.4 (-7.9 – -1.4)	0.002

Values are presented as median change from baseline with interquartile range. ICS = inhaled corticosteroids, FP500 = fluticasone propionate 500 µg/day, FEV_1 = forced expiratory volume in one second, PC_{20} = provocative concentration causing a 20% fall in FEV_1, AMP = adenosine-5'-monophosphate, ECP = eosinophilic cationic protein, NO = nitric oxide, ppb = parts per billion.

Effect of inflammation on improvement in lung function

To investigate if the baseline type and level of inflammation was associated with the corticosteroid treatment response, we analyzed the independent associations between the improvement in FEV_1 %predicted after 2-week and 1-year ICS treatment and eosinophils in sputum and blood and smoking status. Sputum: higher percentages of sputum eosinophils were significantly associated with a greater improvement in FEV_1 %predicted both after 2-week and 1-year treatment (b = 0.252, p= 0.005 and b=0.232, p=0.002 respectively, supplementary table 3), whereas sputum neutrophils were not independently associated with improvement in FEV_1 %predicted. Blood: higher levels of blood eosinophil and lower levels of blood neutrophils were independently associated with a higher improvement in FEV_1 %predicted after 2-week ICS treatment (b=0.529, p=0.022 and b=-0.343, p=0.049 respectively, supplementary table 4). After 1 year ICS treatment blood eosinophil levels were still significantly associated with improvement in FEV_1 %predicted. Smoking status was not significantly associated with improvement in FEV_1 % predicted, when inflammation was taken into account (supplementary tables 3 and 4).

DISCUSSION

Our study shows that current-smokers with asthma have a different type of inflammation, i.e. they have less eosinophils and more neutrophils in their sputum and blood than never-smokers, even though the severity of airflow obstruction and bronchial hyperresponsiveness is comparable. Moreover, a higher number of cigarettes smoked daily was associated with a lower percentage of eosinophils in sputum, suggesting that the type of airway inflammation may be influenced by the amount of smoke exposure. Interestingly, the inflammatory profile of a group of asthmatics with a median smoking cessation of 7 years was more similar to that of the current-smoking than that of the never-smokers, suggesting that effects of smoking may persist for a long time after smoking cessation in asthmatics. Additionally, we show that current-smokers have a blunted short-term corticosteroid treatment response. Again, ex-smokers are more similar to current-smokers than to never-smokers, with a trend for a blunted response. However, we found no evidence for a blunted response in both ex- and current-smokers on the long-term.

After short-term treatment with ICS, current-smokers had less improvement in FEV_1 than never-smokers, as reported earlier.[7,13,15] We extend these findings by showing that ex-smokers also tend to respond less to corticosteroid treatment than never-smokers on the short-term. Thus far, the efficacy of corticosteroid treatment in ex-smokers has only been investigated in one study with 15 asthmatic ex-smokers.[15] Comparable to our findings, they observed that the short-term improvement in FEV_1 after 2-week treatment with oral corticosteroids in ex-smokers was intermediate between current- and never-smokers.

Interestingly, we found that the long-term effects of 1-year ICS treatment were not significantly different in ex- and current-smokers compared to never-smokers. This observation is in line with a study in 492 current- and 2,432 never-smokers, showing that 400 µg/day budesonide or placebo for 3 years was equally effective in current- and never-smokers.[21] Furthermore, in a large, real-life study in 619 asthmatics, the level of improvement in FEV_1 and asthma control was similar in ex-, current- and never-smokers after 1-year treatment with small particle budesonide/formoterol formulation.[22] Taken together, these findings suggest that ex- and current-smokers with asthma have a lower corticosteroid treatment response on the short-term than never-smoker, whereas the long-term response is similar between the three groups. We extend these observations by showing that 1-year ICS treatment response is not driven by smoking per se. Rather the underlying inflammatory process present drives the ICS response over 1 year, i.e. a better response with higher sputum and blood eosinophils, independent of smoking. In this context, the findings of Tomlinson and colleagues are of interest.[14] They found a reduced short-term response to inhaled beclomethasone in current-smokers with asthma, which could be overcome by increasing the dose of beclomethasone from 400 µg/day to 2000 µg/day. It is tempting to speculate that the blunted corticosteroid treatment response in ex- and current-smokers can also be overcome by prolonged treatment, although this remains to be formally demonstrated in future prospective studies.

We did not find any differences in the level of lung function or severity of bronchial hyperresponsiveness between ex-, current- and never-smokers at baseline. However, we did observe a lower level of eosinophilic inflammation in blood and sputum and higher blood neutrophil counts in current-smokers than in never-smokers. These findings are consistent with earlier studies.[7–12] Additionally, we demonstrated that the level of eosinophilic inflammation was also lower in ex-smokers and very similar to that seen in current-smokers. To date, only one other study, also from our research group, reported on the inflammatory profile in ex-smoking asthmatics.[23] This study demonstrated that ex-smoking asthmatics have lower percentages of eosinophils in airway wall biopsies than never-smokers and that the percentage of sputum neutrophils is significantly higher in ex- than in never-smokers. The above findings suggest that smoking does not only have an acute effect on airway inflammation, but also a chronic effect that may persist for years after smoking cessation.

More severe neutrophilic inflammation in asthma has been associated with a reduced corticosteroid treatment response.[24,25] Therefore, the shift from eosinophilic to neutrophilic inflammation that we observed in ex- and current-smokers may be a possible explanation for the reduced short-term corticosteroid treatment response in ex- and current-smokers. Support for the hypothesis that the type of inflammation in ex- and current-smokers influences the corticosteroid treatment response is provided by our observation that smoking status was not independently associated with improvement in FEV_1 %predicted, whereas less eosinophilic inflammation in sputum and blood was independently associated with lower improvement in FEV_1 % predicted, both after 2-week and after 1-year ICS treatment. In addition, higher levels of blood neutrophils were also independently associated with lower improvement in FEV_1%

predicted after 2-week ICS treatment. Interestingly, after 1-year ICS treatment there were no longer any significant differences in inflammation between ex-, current- or never-smokers (supplementary table 5). This suggests that long-term ICS treatment is able to correct the inflammatory differences in ex- and current-smokers, thereby normalizing their ICS treatment response. Other possible explanations for a lower corticosteroid responsiveness in ex- and current-smokers are epigenetic changes, e.g. reduced expression of histone deacetylases (HDAC) [26] and DNA methylation [27], more expression of the less active β isoform of the glucocorticoid receptor [28-30] and increased expression of pro-inflammatory transcription factors, such as nuclear factor-kappa B and activator protein 1.[31,32] Finally, NO in cigarette smoke reduces the affinity of the glucocorticoid receptor for corticosteroids and reduces the binding of corticosteroids to the glucocorticoid receptor.[33]

There are several strengths to our study. Our patients were extensively characterized, including lung function, bronchial hyperresponsiveness and inflammation in sputum and blood, at baseline and after 2-weeks and 1-year treatment with ICS. Our study also has some limitations. First, we performed post-hoc analyses on data from a study that was not originally designed to investigate the effects of smoking on inflammation or corticosteroid treatment response. Our study was originally a three-arm study (figure 1). However, in the 2-week treatment analyses we included only patients treated with ICS, and in the 1-year treatment analyses we excluded one group of patients who were treated according to a program with step-down and eventually complete discontinuation of corticosteroids, which is not in agreement with the current guidelines. Due to this study design, the short- and long-term corticosteroid response was not investigated in the same groups. In this context, it is important to mention that the randomization of the study was performed with minimization for smoking status, age, previous dose of ICS, FEV_1 %predicted, reversibility after 200 µg of salbutamol, PC_{20} methacholine, and serum IgE. This minimization ensures comparable treatment arms with minimal baseline differences. Therefore, we believe that the different treatment arms are comparable. Second, current- and never-smokers were significantly younger than ex-smokers and therefore we had to adjust for age in all analyses.

In conclusion, ex- and current-smokers have a different type of inflammation with less eosinophils and more neutrophils in their blood and sputum. These differences in the type of inflammation were present even several years after smoking cessation. Although we agree with the literature that ex- and current-smokers have a blunted short-term response to ICS, we did not find a difference in their long-term treatment response. Therefore, they should not be withheld from ICS treatment.

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APPENDIX

Self-management plan

PB= personal best

1 If PEF ≥80% of PB, continue maintenance treatment: bronchodilators as needed and same dose of fluticasone twice daily
2 If PEF <80% and >60% of PB, double the dose of inhaled fluticasone for one month. If after one month PEF is ≥80% of PB and symptoms are similar to baseline then return to previous dose. If PEF is still below 80% of PB then again double the dose of inhaled fluticasone for one month (therefore 4 times the original dose). If PEF is ≥80% of PB but symptoms are not back to baseline then continue with this dose.
3 If PEF <60% of PB, double the dose of inhaled fluticasone for one month and start with a course of oral prednisolone 30 mg/day for 7 days. After one month, if PEF is ≥80% and symptoms are similar to baseline then return to previous dose.
4 If PEF is still below 80% of PB then again double the dose of inhaled fluticasone for one month (therefore 4 times the original dose). If PEF is ≥80% of PB but symptoms are not back to baseline then continue with this dose.
5 If symptoms increase with 1 on a scale of 4 during more than 1 day, even in case PEF ≥80% of PB, double the dose of inhaled fluticasone and start with a course of oral prednisolone 30 mg/day for 7 days. After one month if symptoms are similar to baseline then return to previous dose. If symptoms are not back to baseline, again double the dose of inhaled fluticasone and start with a course of oral prednisolone (30 mg/day) for 7 days.

Symptom score

How was your asthma during the day?
0 = no asthma, normal unrestricted activity
1 = Wheezing or short of breath on strenuous exercise/hurrying, otherwise asthma not unduly troublesome.
2 = Wheezing or short of breath most of the day- normal activities difficult
3 = Asthma bad- could not go to work or to do household or carrying out usual activities at all because of short of breath

How was your asthma at night?
0 = Good night, slept well, no asthma
1 = Good night, slept well, but woke once with wheeze and cough
2 = Woken 2-3 times by cough/ wheeze/breathlessness/asthma
3 = Bad night, awake most of the night with cough/wheeze/breathlessness/asthma

Supplementary table 1: Association between the amount of smoke exposure, a s reflected by the number of cigarettes smoked daily and number of packyears and improvement in clinical and inflammatory variables after 2-week ICS treatment

	Cigarettes/day		Packyears	
	b	p-value	b	p-value
ΔFEV_1 (L)	-0.01	0.528	-0.01	0.529
ΔFEV_1 (%predicted)	-0.48	0.242	-0.55	0.025
ΔReversibility (% of the predicted FEV_1)	-0.26	0.199	-0.13	0.394
ΔPC_{20} methacholine (doubling concentrations)	-0.06	0.365	0.00	0.987
ΔPC_{20} AMP (doubling concentrations)	-0.14	0.342	0.08	0.502
ΔSputum eosinophils (%) *	0.04	0.288	0.01	0.812
ΔBlood eosinophils (10^3/μL) *	0.00	0.917	0.00	0.804
ΔSputum ECP (μg/L) *	0.05	0.282	0.03	0.387
ΔSerum ECP (μg/L) *	0.02	0.328	0.01	0.763
ΔSputum neutrophils (%) *	0.00	0.924	0.00	0.850
ΔBlood neutrophils (10^3/μL) *	-0.00	0.915	0.00	0.950
ΔExhaled NO (ppb)	0.08	0.788	0.18	0.367

b = unstandardized regression coefficient, ICS = inhaled corticosteroids, FEV_1 = forced expiratory volume in one second, PC_{20} = provocative concentration causing a 20% fall in FEV_1, AMP = adenosine-5'-monophosphate, ECP = eosinophilic cationic protein, NO = nitric oxide, * variable log-transformed.

Supplementary table 2: Association between the amount of smoke exposure, as reflected by the number of cigarettes smoked daily and number of packyears and clinical and inflammatory variables after 1-year ICS treatment

	Cigarettes/day		Packyears	
	b	p-value	b	p-value
ΔFEV_1 (L)	-0.02	0.349	-0.03	0.034
ΔFEV_1 %pred	-0.42	0.437	-0.66	0.032
ΔPC_{20} methacholine (doubling concentrations)	-0.02	0.730	-0.14	0.043
ΔSputum eosinophils (%) *	0.07	0.600	0.04	0.752
ΔBlood eosinophils (10^9/L) *	-0.02	0.446	-0.02	0.281
ΔSputum ECP (μg/L) *	0.02	0.577	0.02	0.718
ΔSerum ECP (μg/L) *	0.03	0.305	0.05	0.015
ΔSputum neutrophils (%) *	0.03	0.179	-0.01	0.686
ΔBlood neutrophils (10^9/L) *	-0.02	0.599	-0.01	0.832
ΔExhaled NO (ppb)	0.03	0.917	-0.08	0.670

b = unstandardized regression coefficient, ICS= inhaled corticosteroids, FEV_1 = forced expiratory volume in one second, PC_{20} = provocative concentration causing a 20% fall in FEV_1, ECP = eosinophilic cationic protein, NO = nitric oxide, ppb = parts per billion, * variable log-transformed.

Supplementary table 3 Independent associations between improvement in FEV_1 %predicted after 2-week or 1-year ICS treatment and smoking status and sputum eosinophil and neutrophil percentages

	2 week		1 year	
	b	p-value	b	p-value
Sputumeosinophils (%) *	0.252	0.005	0.232	0.002
Sputum neutrophils (%) *	-0.133	0.173	-0.021	0.828
Ex- vs. never-smokers	-0.203	0.170	-0.084	0.542
Current- vs. never-smokers	-0.184	0.229	0.002	0.990
Baseline FEV_1 (%)	-0.212	0.003	-0.179	0.044

b = unstandardized regression coefficient, ICS= inhaled corticosteroids, FEV_1 = forced expiratory volume in one second, * variable log-transformed.

Supplementary table 4 Independent associations between improvement in FEV_1 %predicted after 2-week or 1-year ICS treatment and smoking status and blood eosinophil and neutrophil levels

	2 week		1 year	
	b	p-value	b	p-value
Blood eosinophils (10^9/L) *	0.529	0.022	0.469	0.025
Blood neutrophils (10^9/L) *	-0.343	0.049	-0.207	0.288
Ex- vs. never-smokers	-0.256	0.086	-0.062	0.680
Current- vs. never-smokers	-0.226	0.148	0.036	0.825
Baseline FEV_1 (%)	-0.280	0.000	-0.193	0.044

b = unstandardized regression coefficient, ICS= inhaled corticosteroids, FEV_1 = forced expiratory volume in one second, * variable log-transformed.

Supplementary table 5 Differences in clinical and inflammatory variables between ex-, current- and never-smokers at baseline

	Ex-smokers (n=21)	Current-smokers (n=17)	Never-smokers (n=37)	p-value
Treatment # (self management / FP500)	10 / 11	11 / 6	17 / 20	
FEV_1 (L)	3.4 (2.6 – 3.6)	3.0 (2.5 – 3.7)	3.3 (3.2 – 3.7)	0.15
FEV_1 (%predicted)	85 (80 – 90)	80 (72 – 97)	91 (84 – 102)	0.55
PC_{20} methacholine (mg/ml) §	8.8 (0.05 – 39)	3.6 (0.14 – 39)	6.5 (0.04 – 39)	0.46
Sputum eosinophils (%)	0.7 (0.0 – 1.8)	0.3 (0.0 – 5.4)	1.3 (0.0 – 3.5)	0.75
Blood eosinophils (10^3/µL)	0.25 (0.13 – 0.37)	0.24 (0.15 – 0.40)	0.31 (0.19 – 0.45)	0.26
Sputum ECP (µg/L)	49 (18 – 107)	34 (27 – 103)	26 (14 – 47)	0.50
Serum ECP (µg/L)	10 (8 – 17)	13 (7 – 21)	13 (9 – 17)	0.50
Sputum neutrophils (%)	38 (23 – 53)	46 (34 – 58)	32 (23 – 62)	0.94
Blood neutrophils (10^3/µL)	3.5 (3.1 – 4.7)	4.1 (3.5 – 5.0)	3.2 (5.6 – 3.7)	0.44
Exhaled NO (ppb)	10.2 (4.5 – 16.5)	8.0 (5.2 – 10.5)	8.9 (6.7 – 15.9)	0.21

Values are presented as medians with interquartile ranges, unless stated otherwise, # = number, § geometric mean (range), self management = treatment according to self management plan in the online supplemental appendix, FP500 = fluticasone propionate 500 µg/day, FEV_1 = forced expiratory volume in one second, PC_{20} = provocative concentration causing a 20% fall in FEV_1.

CHAPTER 4

Obesity in Asthma: More Neutrophilic Inflammation as a Possible Explanation for a Reduced Treatment Response

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ABSTRACT

Background: The incidence of asthma and obesity is increasing worldwide, and reports suggest that obese patients have more severe asthma. We investigated whether obese asthma patients have more severe airway obstruction and airway hyper-responsiveness and a different type of airway inflammation than lean asthmatics. Furthermore, we assessed the effect of obesity on corticosteroid treatment response.

Methods: Patient data from 4 well-documented asthma cohorts were pooled (n=423). We evaluated FEV_1, bronchial hyper-responsiveness (PC_{20}) to either methacholine/histamine or adenosine 5'-monophosphate (AMP) (differential) cell counts in induced sputum and blood and corticosteroid treatment response in 118 patients.

Results: At baseline, FEV_1, PC_{20} methacholine or histamine and PC_{20} AMP values were comparable in 63 obese (BMI ≥30 kg/m²) and 213 lean patients (BMI <25 kg/m²). Obese patients had significantly higher blood neutrophils. These higher blood neutrophils were only seen in obese women and not in obese men. After two-week treatment with corticosteroids, we observed less corticosteroid-induced improvement in FEV_1 %predicted in obese patients than in lean patients (median 1.7% vs 6.3% respectively, p=0.04). The percentage of sputum eosinophils improved significantly less with higher BMI (p=0.03) and the number of blood neutrophils increased less in obese than in lean patients (0.32 x10³/μL vs 0.57 x10³/μL, p=0.046).

Conclusions: We found no differences in asthma severity between obese and nonobese asthmatics. Interestingly, obese patients demonstrated more neutrophils in sputum and blood than nonobese patients. The smaller improvement in FEV_1 and sputum eosinophils suggests a worse corticosteroid treatment response in obese asthmatics.

INTRODUCTION

Obesity is a worldwide epidemic, and the prevalence of obesity has tripled in the last two decades.[1] More than 1.5 billion of adults are overweight [body mass index (BMI) >25 kg/m^2] and more than 500 million are obese (BMI >30 kg/m^2). More recently, the presence of overweight and obesity has also been associated with asthma.[2–4] Prevalence of asthma is higher in obese than in nonobese adults (9% vs 5%), and the presence of overweight and obesity increases the relative risk of having asthma by 2.0– and 2.7–fold, respectively.[2,3] Further, there exists a dose-response effect of BMI, in that overweight increases the odds to develop asthma by 38% and obesity by 92%.[4]

An increased body weight has also important consequences in patients with established asthma. It has been suggested that obese asthma patients respond less to treatment with inhaled corticosteroids, which may be due to a different type of inflammation.[5–9] In this context, studies on the systemic effects of obesity are of interest. Obesity is associated with a chronic low-grade inflammatory state that may contribute to inflammation at sites distant from the adipose tissue such as the lungs.[10–16] In agreement with this, Haldar et al. identified a phenotype of asthma consisting of predominantly female, obese asthma patients.[17] Patients with this phenotype had high percentages of sputum neutrophils and more asthma symptoms. Finally, the presence of obesity may be associated with more severe airway obstruction in asthma. However, there is controversy about this in the literature.[5,7,8,8,9,18–21]

The aim of this study was to investigate whether obese patients have more severe asthma than lean patients, that is, patients without obesity or overweight (BMI<25 kg/m^2), and whether they have a different type of airway inflammation. Furthermore, we investigated whether obesity affects corticosteroid responsiveness in asthma. To this end, we combined individual patient data from four studies previously performed in the Groningen Research Institute for Asthma and COPD (GRIAC).

MATERIALS AND METHODS

We pooled the data from four clinical studies that were conducted in the University Medical Center Groningen. Patient characteristics and measurements in the studies are summarized in **table 1**.[22–26] All studies were approved by the Medical Ethics Committee of the University Medical Center Groningen, and all patients provided written informed consent.

Table 1: Baseline characteristics and measurements in the studies included in the analyses

	Baseline measurements								
	Number	BMI	BMI ≥30	Age	FEV_1 % predicted	BHR		Sputum	Blood
						Mch/ Hist	AMP		
Study 1 [22]	118	24 (22 – 28)	19 (16.1)	27 (22 – 38)	81 (68 - 92)	+	+	+	+
Study 2 [23, 24]	144	27 (25 – 29)	30 (20.8)	49 (38 – 57)	91 (79 - 102)	-	+	+	+
Study 3 [25]	140	24 (22 – 26)	13 (9.3)	31 (25 – 46)	66 (53 - 76)	+	-	-	-
Study 4 [26]	21	25 (22 – 26)	1 (4.8)	31 (26 – 39)	107 (88 - 112)	+	+	+	+

Values are presented as numbers (with percentage) or medians (with interquartile range). AMP = adenosine 5'-monophosphate; BMI = body mass index; BHR = bronchial hyperresponsiveness; FEV_1 = forced expiratory volume in 1 second; Mch/ Hist = methacholine / histamine; + = measurement performed; - = measurement not performed.

Patients

All patients had a doctor's diagnosis of asthma and bronchial hyper-responsiveness, defined as a provocative concentration causing a 20% fall in FEV_1 (PC_{20}) methacholine <8 mg/ml or PC_{20} histamine <8 mg/ml. Further inclusion criteria were dependent on the different study protocols. From study 3, only the subpopulation of patients who were 18–60 years, with episodic respiratory symptoms, PC_{20} histamine <8 mg/ml and <10 packyears smoking, was used for analysis.[25] In studies 1, 3 and 4 inhaled corticosteroids were tapered before the baseline visit.[22,25,26] In studies 1 and 4 inhaled corticosteroids were tapered and when possible discontinued completely, at least 3 weeks before the measurements. When patients experienced a worsening of their asthma before complete discontinuation for 3 weeks, they were asked to visit the hospital earlier. Patients experiencing an exacerbation for which an oral prednisolone course was necessary were not included. In study 3, inhaled corticosteroids were discontinued for 4 weeks before the measurements. In study 2, patients were not required to stop inhaled corticosteroids. In **table 2**, the number of patients who still used inhaled corticosteroids at the time of the measurements is presented.

Table 2: Baseline characteristics of obese (BMI ≥30 kg/m2) and lean (BMI <25 kg/m2) patients*

	BMI <25 (n=213)	BMI ≥30 (n=63)	p-value
Age (years)	29 (24 – 42)	45 (36 – 52)	**<0.001** #
Males (n, %)	105 (49)	22 (35)	**0.03** ‡
Use of ICS at baseline (n, %)	19 (9)	12 (19)	**0.04** ‡
Current-smoking (n, %)	42 (20) n=207	10 (16)	0.3 ‡
FEV_1 pre bronchodilator (L)	2.9 (2.2 – 3.5) n=212	2.5 (2.0 – 3.0)	0.9
FEV_1 %predicted pre bronchodilator	77 (61 – 92) n=212	84 (67 – 98)	0.7
PC_{20} methacholine/ histamine (mg/ml) £	0.26 (0.02 – 7.31) n=166	0.35 (0.02 – 5.74) n=33	0.5
PC_{20} AMP (mg/ml) £	9.8 (0.02 – 640) n=124	14.5 (0.02 – 640) n=50	0.5
AMP dose response slope (%/mg/ml)	2.27 (0.27 – 8.33) n=124	1.02 (0.04 – 9.77) n=49	0.98
Blood eosinophils (10^3/μL)	0.27 (0.18 – 0.43) n=122	0.21 (0.13 – 0.33) n=49	0.6
Sputum eosinophils (%)	3.3 (1.0 – 8.5) n=115	0.5 (0.2 – 4.9) n=40	0.3
Blood neutrophils (10^3/μL)	3.4 (2.5 – 4.4) n=122	4.2 (3.3 – 5.0) n=49	0.001
Sputum neutrophils (%)	36 (22 – 60) n=115	52 (39 – 70) n=40	0.095

*All values are presented as median with interquartile range unless stated otherwise; £ geometric mean with range; # tested by Mann-Whitney U test; ‡ tested by Fisher's exact test; all other variables tested by linear regression analysis with the presence of obesity as an independent variable and age and gender as covariates (except for FEV_1 % predicted); n (number of patients) is only given for variables that were not available in all individuals in the specific BMI group; AMP = adenosine 5'-monophosphate; BMI = body mass index; FEV_1 = forced expiratory volume in 1 second; ICS = inhaled corticosteroids.

Data pooling

We collected the following available data: BMI, FEV_1, PC_{20} methacholine / histamine, PC_{20} adenosine 5'-monophosphate (AMP) and inflammatory cell differential counts in sputum and blood. We pooled individual patient data from all four studies, and duplicates were removed. Five patients were excluded because either their weight or height could not be retrieved. The corticosteroid treatment response in obese and nonobese patients was analyzed in study 1. In this study, all 118 patients were treated with either fluticasone 500 μg/day, fluticasone 2000 μg/day or prednisolone 30 mg/day for 2 weeks. More information about the design of this study can be found in the original publication or in the appendix and supplementary figure 1.[22]

Measurement of lung function and bronchial hyperresponsiveness

In all four studies, spirometry was performed according to international guidelines, and reference values were obtained from Quanjer et al.[27] Provocation tests were performed with a 2-min tidal-breathing method. Patients inhaled increasing concentrations of a direct

stimulus, either histamine (0.03 to 32 mg/ml) or methacholine (0.03 to 16 mg/ml), or the indirect stimulus AMP (0.04 to 320 mg/ml). The challenge was discontinued when FEV_1 had fallen by 20% or more from the pre-challenge level or when the highest concentration had been administered. PC_{20} was calculated by linear interpolation between the last two data points of the logarithmic concentration-response curve. If a patient responded to an initial saline challenge or the lowest concentration, half of the lowest concentration was used as PC_{20} value. If a patient did not respond at the highest concentration, twice the highest concentration was used as the PC_{20} value.[25] The results from the provocation tests with histamine and methacholine were analyzed together, since PC_{20} values for histamine and methacholine are equivalent on a mg-for-mg basis.[28] All calculations of PC_{20} were performed with a base-2 logarithm, as this reflects doubling concentrations. We also calculated the dose response slope. This was calculated as the percentage fall in FEV_1 per concentration of either methacholine or histamine or AMP.

Sputum induction and processing

Sputum induction was performed following a standard protocol. Fifteen minutes after inhalation of 200 µg salbutamol, hypertonic saline (3%, 4%, and 5%) was nebulized with an ultrasonic nebulizer (Ultraneb 2000, DeVillbiss, Somerset, PA, USA) and inhaled for seven minutes. The output of the nebulizer was calibrated at 1.5 ml/min. After each concentration, patients were encouraged to cough and expectorate sputum. All sputum inductions and sputum processings were performed in the same laboratory according to standard operating procedures that were the same in all studies. Whole samples were processed according to the method of Fahy et al. with some modifications.[29] An equal volume of dithiothreitol 0.1% (Sputalysin 10%, Behring Diagnostics Inc, Sommervillle, NY, USA) was added to the weight of the sputum and after 15 minutes filtered through a nylon (48 µm) gauze. A haematocytometer was used to count the total cell number, viability, and squamous epithelial contamination of the cell suspension. The sputum sample was centrifuged (10 min, 450g, 4° Celsius). The cell pellet was resuspended in phosphate buffered saline and cytospins were stained with May-Grünwald-Giemsa. At least 200 non-squamous cells were separately counted by an investigator blinded to any personal data. Samples with contamination of >80% squamous cells were excluded from analyses.

Statistical Analysis

Inflammatory cell counts in sputum and blood were log-transformed to normalize their distribution. We investigated the effect of BMI as a discrete variable, that is, BMI ≥30 kg/m² (obese) vs BMI <25 kg/m² (lean), and as a continuous variable. To test for differences in age, gender, smoking status and inhaled corticosteroid use at baseline between obese and nonobese patients, we used Fisher's exact tests for categorical variables and Mann-Whitney U tests for continuous variables. To test for baseline differences in lung function, bronchial hyper-responsiveness and inflammatory cells, we performed a linear regression analysis with

the presence of obesity as an independent variable and age and gender as covariates. Effects of increasing BMI on baseline values of lung function, bronchial hyper-responsiveness and inflammatory cells were performed by similar regression analyses with BMI (continuous) as an independent variable. To test for differences in a variable after treatment, we performed a linear regression analysis on the change in this variable with the presence of obesity or continuous BMI as an independent variable and age, gender, type of treatment (inhaled or oral corticosteroids) as covariates. In all analyses of treatment response, we also included the baseline value of the variable as a covariate, to correct for baseline differences. To assess if our results would be different when using other cutoff points for BMI, we repeated our analyses with overweight patients, thus obese (BMI ≥30 kg/m²) and nonobese patients (BMI <30 kg/m²). Also, we repeated our analyses only in men or women and without current-smokers, to investigate if gender or smoking status would affect our results. We corrected for age, gender, and height in all analysis in which absolute values of FEV_1 were used. We did not correct for age and gender in regression analyses in which FEV_1 %predicted was used, because the predicted value already includes these covariates. A p-value of ≤0.05 was considered statistically significant.

RESULTS

Baseline differences in obese and lean asthma patients

We included 423 asthma patients in our study. Median BMI was 24.9 kg/m² (range 16.9 – 44.5 kg/m²). A total of 63 patients were obese, 147 patients were overweight and 213 patients were lean. Obese patients were older than lean patients (45 vs 29 years respectively, p<0.001, table 2), and obese patients were more frequently female (65%) than lean patients (51%). The proportion of patients using inhaled corticosteroids at baseline was similar in the obese and lean patients. Smoking status was also similar in the two groups.

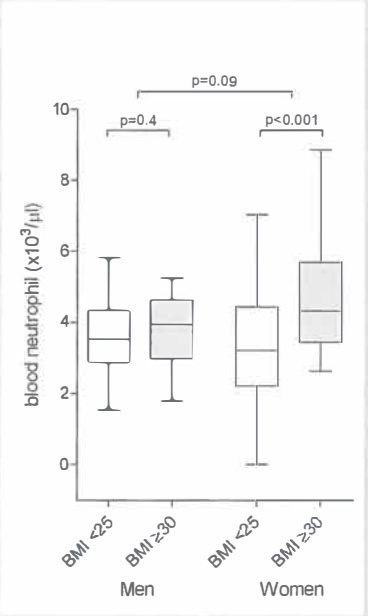
Clinical variables such as FEV_1, PC_{20} methacholine / histamine and PC_{20} AMP did not differ significantly between obese and lean patients at baseline. We found a positive association between BMI and FEV_1 %predicted, with an increase of 0.5% in FEV_1 for every kg/m² increase of BMI (p=0.02, **table 3**). Obese asthma patients had higher blood neutrophil cell counts (4.2 vs 3.4 x 10^3/μL, p<0.001) than lean asthma patients. There was also a trend for higher percentages of sputum neutrophils in obese patients (52% vs 36%, p=0.095), whereas values for eosinophils were similar between obese and lean patients. BMI was positively correlated to blood neutrophils (b=0.015, p<0.001). The increased number of blood neutrophils was only present in female, but not in male obese patients (4.3 vs 3.2 x 10^3/μL in female obese vs female lean patients and 4.0 vs 3.5 x 10^3/μL in male obese vs male lean patients, **figure 1** and supplementary table 1). Furthermore, there was a trend for an interaction between obesity and gender on blood neutrophil counts (p=0.09).

Table 3: Associations between baseline characteristics and BMI

	b	p-value
FEV_1 pre bronchodilator (L)	0.012 n=359	0.2
FEV_1 %predicted pre bronchodilator (%)	0.487 n=359	0.02
PC_{20} methacholine/histamine (doubling concentrations)	0.034 n=278	0.3
PC_{20} AMP (doubling concentrations)	0.006 n=278	0.9
AMP dose response slope (%/mg/ml) *	-0.022 n=276	0.5
Blood eosinophils (10^3/µL) *	0.001 n=273	0.6
Sputum eosinophils (%) *	-0.011 n=239	0.5
Blood neutrophils (10^3/µL) *	0.015 n=273	<0.001
Sputum neutrophils (%) *	0.006 n=239	0.4

Linear regression with outcome parameter as dependent variable and BMI as independent variable and age and gender as covariates (except for FEV_1 % predicted); n (number of patients) is only given for variables that were not available in all individuals in the specific BMI group; AMP = adenosine 5'-monophosphate; b = regression coefficient; BMI = body mass index; FEV_1 = forced expiratory volume in 1 second; * variable log transformed.

Figure 1: Blood neutrophils in obese and lean patients by gender



Differences between obese and non-obese tested with linear regression with blood neutrophils (log-transformed) as dependent variable and BMI as independent variable and age and gender as covariates. Difference between men and women tested with linear regression with an interaction term for gender and obesity; BMI = body mass index.

Table 4: Changes with corticosteroid treatment in obese (BMI ≥30 kg/m2) and lean (BMI <25 kg/m2) patients* (option 2)

	BMI <25 (n=70)			BMI ≥30 (n=19)			p-value
	Before	After	Difference	Before	After	Difference	
Age (years)	25 (21 – 29)			38 (35 – 45)			
Males (n, %)	20 (29)			7 (37)			
Use of ICS at baseline (n, %)	1 (1)			2 (11)			
Current-smoking (n, %)	19 (29)			6 (32)			
Treatment (FP500/FP2000/P30)	21/ 21/ 28			8/ 7/ 4			
FEV_1 (mL)	2965 (2543 – 3420)	3180 (2850 – 3615)	220 (0 – 515)	2550 (2050 – 2750)	2600 (1930 – 3080)	50 (-80 – 300)	0.3
FEV_1 (%predicted)	82.1 (68.8 – 95.2)	88.7 (78.1 – 98.0)	6.3 (0.1 – 13.0)	77.9 (64.1 – 87.5)	77.5 (64.7 – 93.0)	1.7 (-2.8 – 9.0)	0.04
PC_{20} methacholine (doubling concentrations)	-1.7 (-2.9 – 0.3)	0.3 (-0.6 – 1.7)	4.2 (0.7 – 6.0)	-1.6 (-2.8 – 0.5)	-0.7 (-2.2 – 1.5)	1.4 (-0.2 – 3.0)	0.1
Methacholine dose response slope (%/mg/ml) #	3.83 (2.58 – 4.71)	2.72 (1.74 – 3.34)	-0.99 (-1.84 – -0.40)	3.83 (2.39 – 4.91)	3.17 (2.41 – 4.49)	-0.42 (-2.16 – -0.02)	0.06
PC_{20} AMP (doubling concentrations)	1.9 (0.0 – 3.7)	6.4 (3.5 – 9.3)	4.2 (0.7 – 6.0)	1.6 (0.0 – 6.1)	6.7 (0.4 – 9.3)	1.3 (0.0 – 6.6)	0.5
AMP dose response slope (%/mg/ml) #	1.47 (0.23 – 2.72)	-1.65 (-2.47 – 0.70)	-2.76 (-3.63 – -0.97)	1.18 (-1.30 – 2.77)	-1.46 (-2.62 – 2.61)	-0.66 (-3.87 – 0.79)	0.1
Blood eosinophils ($10^3/\mu L$)	0.34 (0.25 – 0.51)	0.20 (0.12 – 0.35)	-0.12 (-0.28 – -0.02)	0.27 (0.13 – 0.50)	0.20 (0.14 – 0.34)	-0.03 (-0.24 – -0.01)	0.6
Sputum eosinophils (%)	6.0 (2.2 – 12.6)	0.5 (0.0 – 2.3)	-4.3 (-11.3 – 1.0)	2.5 (0.5 – 7.5)	0.8 (0.2 – 2.2)	-0.7 (-4.3 – 0.0)	0.2
Blood neutrophils ($10^3/\mu L$)	3.42 (2.84 – 4.55)	4.04 (2.89 – 5.45)	0.57 (-0.28 – 1.38)	4.48 (3.77 – 6.34)	4.89 (4.03 – 5.62)	0.32 (-0.36 – 1.29)	0.02
Sputum neutrophils (%)	29.2 (21.0 – 45.1)	34.4 (18.5 – 48.4)	0.8 (-10.9 – 12.0)	52.2 (42.5 – 71.1)	45.8 (34.3 – 70.5)	-3.2 (-18.9 – 2.9)	0.8

*All values are presented as medians with interquartile range, unless stated otherwise; # Variable log transformed. Treatment induced differences between the two groups (BMI <25 kg/m² and BMI ≥30 kg/m²) tested by linear regression with change in outcome parameter as dependent variable and BMI as independent variable and age, gender (except FEV_1 % predicted) and type of treatment as covariates; AMP = adenosine 5'-monophosphate; BMI = body mass index; FEV_1 = forced expiratory volume in 1 second; FP500 = fluticasone propionate 500 µg/day; FP2000 = fluticasone propionate 2000 µg/day; P30 = prednisolone 30 mg/day .

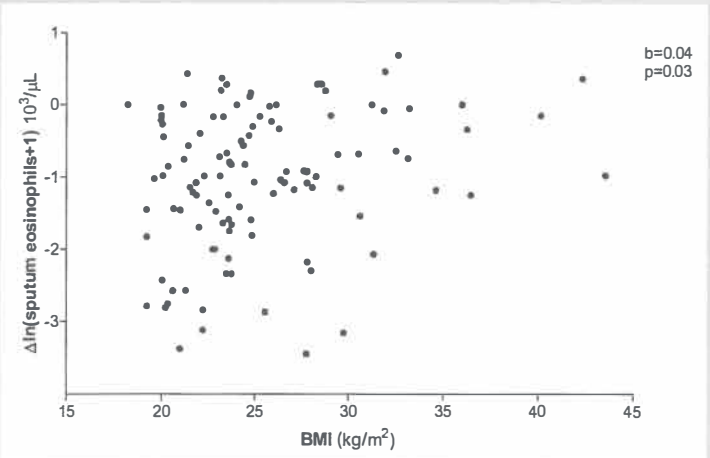
The results were similar when repeating our analyses also including overweight patients (supplementary table 2). Because smoking is known to affect neutrophilia, we also performed these analyses without the current-smokers and the results remained similar (supplementary table 3).

Response to corticosteroids

Forced expiratory volume in 1 s %predicted improved less in obese than lean asthma patients (median 1.7% vs 6.3%, p=0.04, **table 4**). Absolute FEV_1 appeared to also improve less in obese patients (50 mL vs 220 mL); however, this was not statistically significant. When BMI was analyzed as a continuous variable, FEV_1 %predicted also tended to improve less with increasing BMI (0.4% less improvement for each kg/m² increase in BMI, p=0.08, supplementary table 4).

Although no significant differences in corticosteroid-induced improvement in PC_{20} methacholine or PC_{20} AMP were found between obese and lean patients, there was a trend for less improvement in the methacholine dose response slope in obese patients (p=0.06, table 4). Blood neutrophil numbers increased significantly less with corticosteroids in obese than in lean patients. Furthermore, we found a smaller corticosteroid-induced reduction in the percentage of sputum eosinophils in obese than in lean asthmatics (-0.7% vs -4.3% respectively), but the difference was not statistically significant after correction for baseline values. When analyzing BMI as a continuous variable, the percentage sputum eosinophils improved less with increasing BMI (b=0.04, p=0.03, figure 2). In the analyses also including overweight patients, the results were similar (supplementary table 5). In the analyses without current-smokers, the smaller improvement in FEV_1 %predicted in obese patients was no longer present (supplementary table 6). Our sample size was not large enough to test for gender differences in corticosteroid treatment response.

Figure 2: Association between change in sputum eosinophils with corticosteroids treatment and BMI



Linear regression with change in sputum eosinophils with corticosteroid treatment (log-transformed) as dependent variable and BMI as independent variable and age, gender and type of treatment as covariates; b = regression coefficient; BMI = body mass index.

DISCUSSION

The results of our study show that the severity of airway obstruction and bronchial hyperresponsiveness is similar in obese and lean asthma patients. Interestingly, obese asthma patients have a higher level of neutrophilic inflammation, as reflected by both a higher percentage of sputum neutrophils and increased blood neutrophil counts. This increase in neutrophils is only seen in female, obese asthmatics and not in male, obese asthmatics. Finally, obese asthma patients have a blunted corticosteroid treatment response compared to lean patients.

Our finding of a higher level of neutrophilic inflammation in sputum and blood in obese asthma patients may help to explain the reduced corticosteroid treatment response in obese asthma patients.[30–32] In our study, a higher percentage of sputum neutrophils at baseline was also correlated with a lower improvement in FEV_1 %predicted (r=-0.22, p=0.007, supplementary figure 2).

In agreement with our findings, Scott et al. also found that obese asthma patients have a higher percentage of sputum neutrophils than nonobese patients and that BMI and sputum neutrophils are positively correlated in females.[33] The findings by Haldar and colleagues further strengthen the notion that obesity is associated with neutrophilic inflammation and gender.[17] They identified an asthma phenotype that was characterized by obesity and non-eosinophilic inflammation. These patients were mostly female, had high percentages of sputum neutrophils, low percentages of sputum eosinophils and a high level of symptoms. A possible explanation for the increased neutrophilic inflammation in obese asthma patients may be that adipose tissue produces several pro-inflammatory mediators, such as leptin, TNFα, and IL-6, also called adipokines.[13,34] This chronic low-grade systemic inflammation, as reflected by leukocytosis [10,16] and increased serum levels of C-reactive protein,[11,12] may theoretically affect local inflammation in asthmatic airways, leading to more neutrophilic instead of eosinophilic inflammation. The question then is how these increased levels of adipokines induce neutrophilia in asthma. Monocytes release TNFα after stimulation with leptin, which then activates human neutrophils.[35] TNFα could also be responsible for the recruitment of neutrophils to the airways, because increased expression of genes from the TNFα pathway is associated with increased neutrophilic inflammation in induced sputum of asthma patients.[36] We observed that the neutrophilic inflammation is increased primarily in women. This may be explained by the difference in body composition between men and women, women having mostly subcutaneous adipose tissue which is more metabolic active than intra-abdominal adipose tissue.[37] For instance, it secretes two to three times more leptin than intra-abdominal adipose tissue.[38] This leads to higher levels of plasma leptin in obese women than in men, which in turn may lead to the increased neutrophilic inflammation.[39]

We found a smaller FEV_1 %predicted improvement in obese than in lean asthma patients after 2 weeks of treatment with corticosteroids. A reduced corticosteroid treatment response in obese asthmatics has been found previously. Peters-Golden et al. showed that obese asthma patients have a lower number of days with total- or well-controlled asthma than

nonobese patients after treatment with beclomethasone.[5] Furthermore, obese patients are less likely to achieve well-controlled asthma after a 12-week treatment with fluticasone, or fluticasone and salmeterol.[6] Importantly, all abovementioned studies observed less asthma control in obese patients, yet not less improvement in FEV_1. Two other studies showed a lower improvement in FEV_1 in obese than in lean patients after treatment with inhaled corticosteroids and a long-acting β-agonist.[7,9] Together, the balance of evidence suggests that obese asthma patients respond less well to treatment with corticosteroids with regard to improvement of asthma control and lung function.

Obese and lean asthmatics had comparable FEV_1 %predicted levels at baseline in our study. The effect of obesity on lung function in asthma has been a matter of controversy. An association between obesity and lower FEV_1 has been observed in some studies [9,20,21,40] but not in others.[5,7,8,18,19] A possible explanation for these discrepant observations could be that inhaled corticosteroids were discontinued prior to inclusion in most studies that did not show a difference in FEV_1 in obese versus nonobese asthmatics,[5,7,19] whereas this was not the case in those studies that did find a lower FEV_1 in obese asthma patients.[9,20,21,40] Based on our findings, we hypothesize that the lower FEV_1 in obese asthma patients in those studies that allowed continuation of corticosteroids, actually reflects a lack of corticosteroid treatment response in obese compared to nonobese asthmatics.

The strength of our study is that we were able to investigated obese and lean asthma patients who were extensively characterized before and after corticosteroid treatment, including lung function, bronchial hyperresponsiveness to both a direct and indirect stimulus, and sputum induction. However, there are also some limitations. Our study was a pooled, post hoc analysis of clinical studies, and patients were treated with different types of corticosteroids, that is, oral and inhaled. These studies were not designed to investigate differences between obese and lean patients, and obese asthma patients were somewhat older and more often female. For this reason, we corrected for age, gender and corticosteroid type (i.e. oral or inhaled corticosteroids) in all our analyses. Finally, we did not include healthy controls in our study. It remains an open question whether the increased neutrophilic inflammation in blood and sputum is also present in healthy obese subjects or whether this is specific for obese asthma patients.

In conclusion, the results of our study show that obese asthma patients have a distinct phenotype of asthma that is characterized by a higher level of neutrophilic inflammation in sputum and blood. Especially, obese female asthma patient show this increased neutrophilic inflammation. The increased neutrophilic inflammation may help to explain why obese asthma patients respond less to corticosteroid treatment.

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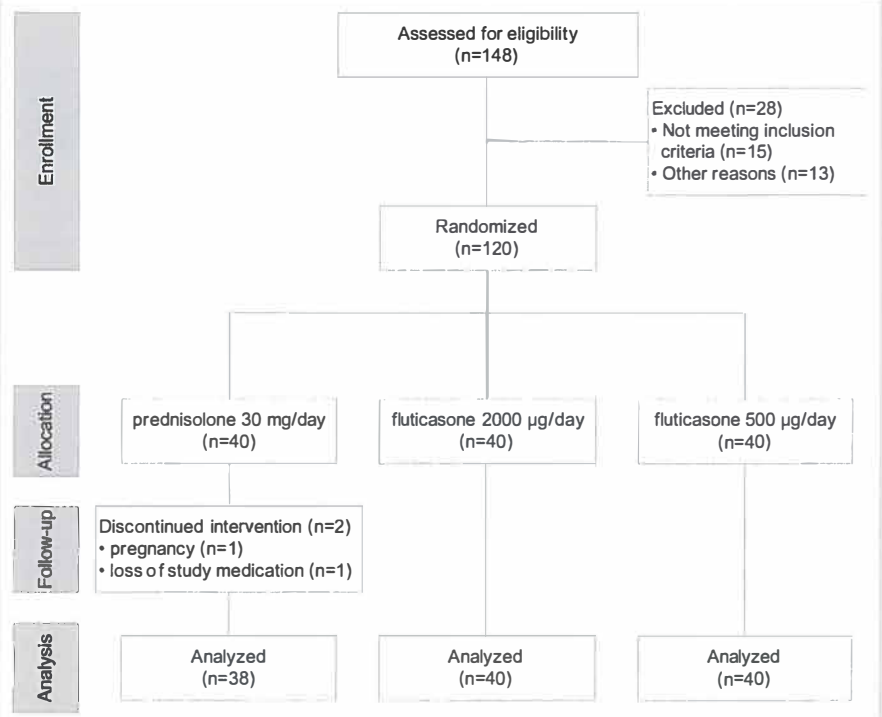
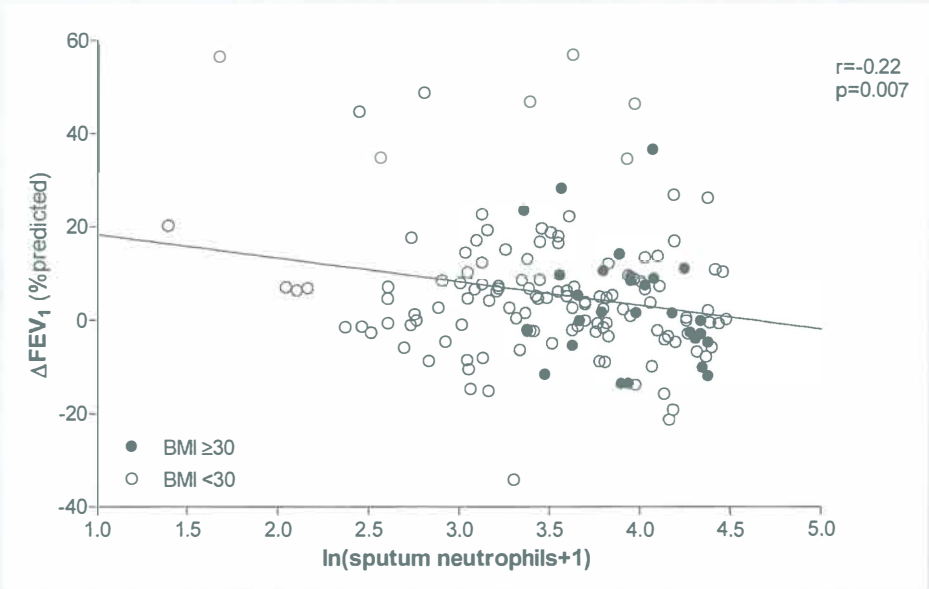
APPENDIX: STUDY DESIGN OF STUDY 1

Study 1 was a 2-week, double-blind, 3 arm parallel group, double dummy study. Patients, 18 to 56 years old, with a diagnosis of asthma were included if they met the following criteria: a PC_{20} methacholine ≤8 mg/ml, at least one positive skin test out of 17 aero-allergens, reversibility to β_2-agonist ≥9% of the predicted FEV_1 and ability to expectorate sputum after hypertonic saline inhalation. The study took place at the out-patient clinic of the University Medical Center Groningen. Inhaled corticosteroids (ICS) were tapered and when possible discontinued completely at least 3 weeks prior to the randomization visit. When patients experienced a worsening of their asthma before complete discontinuation of ICS for 3 weeks, they were asked to visit the hospital to be earlier randomized for inclusion. Patients experiencing an exacerbations of their asthma necessitating a prednisolone course were not randomized. Randomization was performed by a computerized minimization method with stratification according to age, previous dose of ICS, FEV_1 %predicted, reversibility after β_2-agonist, smoking status, serum IgE and PC_{20} methacholine. Patients were treated with either oral prednisolone (30 mg/day), fluticasone propionate 2000 µg/day or fluticasone propionate 500 µg/day, both via Diskhaler dry powder inhalation.

Supplementary figure 1: Flow chart of study 1



Supplementary figure 2: Monovariate correlation between baseline sputum neutrophils and change in FEV_1 %predicted with corticosteroid treatment



Overall Pearson correlation = -0.22, p=0.007; BMI <30 kg/m2 Pearson correlation = -0.17, p=0.06; BMI ≥30 kg/m2 Pearson correlation = -0.28, p=0.14; BMI = body mass index.

Supplementary table 1: Baseline characteristics of obese (BMI ≥30 kg/m2) and lean (BMI <25 kg/m2) patients in men and women*

	Men			Women		
	BMI <25 (n=105)	BMI ≥30 (n=22)	p-value	BMI <25 (n=108)	BMI ≥30 (n=41)	p-value
Age (years)	35 (25 – 48)	47 (35 – 52)	0.01	26 (22 -34)	45 (37 – 53)	<0.001
Use of ICS at baseline (n, %)	11 (11)	5 (23)	0.2	8 (7)	7 (17)	0.1
Current-smoking (n, %)	20 (20) n=102	3 (14)	0.8	22 (21)	7 (17)	0.7
FEV_1 pre bronchodilator (L)	2.9 (2.2 – 3.7) n=104	2.6 (2.0 – 3.5)	0.4	2.7 (2.1 – 3.3)	2.4 (2.0)	0.2
FEV_1 % predicted pre bronchodilator	70 (56 – 81) n=104	67 (49 – 86)	0.5	85 (67 – 97)	88 (72 – 100)	0.2
PC_{20} methacholine/ histamine (mg/ml) £	0.24 (0.02 – 7.20) n=78	0.31 (0.02 – 5.43) n=13	0.6	0.27 (0.02 – 7.31) n=88	0.38 (0.03 – 5.74) n=20	0.9
PC_{20} AMP (mg/ml) £	10.1 (0.02 – 640) n=49	13.2 (0.02 – 640) n=16	0.8	9.6 (0.02 – 640) n=75	15.2 (0.02 – 640) n=34	0.7
AMP dose response slope (%/ mg/ml)	1.44 (0.25 – 7.97) n=49	1.02 (0.03 – 10.83) n=15	0.7	2.72 (0.28 – 8.42) n=75	0.90 (0.06 – 9.37) n=34	0.8
Blood eosinophils (10^3/µL)	0.27 (0.22 – 0.46) n=49	0.25 (0.18 – 0.36) n=16	0.5	0.29 (0.15 – 0.43) n=73	0.18 (0.12 – 0.30) n=33	0.6
Sputum eosinophils (%)	3.4 (1.1 – 13.7) n=46	1.4 (0.2 – 6.7) n=12	0.4	3.3 (0.7 – 7.3) n=69	0.5 (0.2 – 3.1) n=33	0.4
Blood neutrophils (10^3/µL)	3.5 (2.9 – 4.3) n=49	4.0 (3.0 – 4.6) n=16	0.4	3.2 (2.2 – 4.4) n=73	4.3 (3.4 – 5.7) n=33	<0.001
Sputum neutrophils (%)	46 (25 – 65) n=46	50 (38 – 68) n=12	0.6	32 (21 – 55) n=69	53 (40 – 71) n=28	0.02

*All values are presented as median with interquartile range unless stated otherwise; £ geometric mean with range; n (number of patients) is only given for variables that were not available in all individuals in the specific BMI group; AMP = adenosine 5'-monophosphate; BMI = body mass index; FEV_1 = forced expiratory volume in 1 second; ICS = inhaled corticosteroids.

Supplementary table 2: Baseline characteristics for obese and non-obese patients*

	BMI <30 (n=360)	BMI ≥30 (n=63)	p-value
Age (years)	35 (25 – 48)	45 (36 – 52)	<0.001 #
Males (n, %)	181 (50)	22 (35)	0.03 ‡
Use of ICS at baseline (n, %)	43 (12)	12 (19)	0.2 ‡
Current-smoking (n, %)	71 (20) n=350	10 (16)	0.5 ‡
FEV_1 pre bronchodilator (L)	2.8 (2.2 – 3.5) n=359	2.5 (2.0 – 3.0)	0.5
FEV_1 %predicted pre bronchodilator	79 (65 – 93) n=359	84 (67 – 98)	0.7
PC_{20} methacholine/ histamine (mg/ml) £	0.27 (0.01 – 16) n=245	0.35 (0.02 – 5.74) n=33	0.5
PC_{20} AMP (mg/ml) £	16.9 (0.02 – 640) n=228	14.5 (0.02 – 640) n=50	
AMP dose response slope (%/mg/ml)	1.20 (0.13 – 5.34) n=227	1.02 (0.04 – 9.77) n=49	0.5
Blood eosinophils (10^3/μL)	0.22 (0.14 – 0.39) n=223	0.21 (0.13 – 0.33) n=49	0.6
Sputum eosinophils (%)	2.5 (0.7 – 6.8) n=199	0.5 (0.2 – 4.9) n=40	0.2
Blood neutrophils (10^3/μL)	3.3 (2.7 – 4.3) n=224	4.2 (3.3 – 5.0) n=49	<0.001
Sputum neutrophils (%)	40 (24 – 63) n=199	52 (39 – 70) n=40	0.04

*All values are presented as median with interquartile range unless stated otherwise; £ geometric mean with range; # tested by Mann-Whitney U test; ‡ tested by Fisher's exact test; all other variables tested by linear regression analysis with the presence of obesity as an independent variable and age and gender as covariates (except for FEV_1 % predicted); n (number of patients) is only given for variables that were not available in all individuals in the specific BMI group; AMP = adenosine 5'-monophosphate; BMI = body mass index; FEV_1 = forced expiratory volume in 1 second; ICS = inhaled corticosteroids.

Supplementary table 3: Baseline characteristics of obese (BMI ≥30 kg/m2) and lean (BMI <25 kg/m2) patients without current-smokers*

	BMI <25 (n=165)	BMI ≥30 (n=53)	p-value
Age (years)	30 (24 – 44)	45 (35 – 53)	**<0.001** #
Males (n, %)	82 (50)	19 (36)	0.08 ‡
Use of ICS at baseline (n, %)	18 (11)	12 (23)	**0.04** ‡
FEV_1 pre bronchodilator (L)	2.8 (2.1 – 3.5) n=164	2.4 (2.0 – 3.0)	0.9
FEV_1 %predicted pre bronchodilator	76 (61 – 92) n=164	84 (66 – 98)	0.3
PC_{20} methacholine/ histamine (mg/ml) £	0.25 (0.02 – 7.31) n=130	0.31 (0.02 – 5.74) n=26	0.7
PC_{20} AMP (mg/ml) £	10.9 (0.02 – 640) n=89	11.7 (0.02 – 640) n=41	0.5
AMP dose response slope (%/mg/ml)	2.19 (0.25 – 6.56) n=89	1.01 (0.04 – 11.31) n=40	0.8
Blood eosinophils (10^3/μL)	0.26 (0.15 – 0.42) n=88	0.24 (0.12 – 0.36) n=40	0.7
Sputum eosinophils (%)	2.8 (1.0 – 7.7) n=84	0.5 (0.2 – 5.2) n=32	0.7
Blood neutrophils (10^3/μL)	3.2 (2.4 – 4.1) n=88	4.0 (3.3 – 4.8) n=40	0.001
Sputum neutrophils (%)	34 (21 – 57) n=84	52 (38 – 68) n=32	0.1

*All values are presented as median with interquartile range unless stated otherwise; £ geometric mean with range; # tested by Mann-Whitney U test; ‡ tested by Fisher's exact test; all other variables tested by linear regression analysis with the presence of obesity as an independent variable and age and gender as covariates (except for FEV1 % predicted); n (number of patients) is only given for variables that were not available in all individuals in the specific BMI group; AMP = adenosine 5'-monophosphate; BMI = body mass index; FEV1 = forced expiratory volume in 1 second; ICS = inhaled corticosteroids.

Supplementary table 4: Associations between corticosteroid treatment response and BMI

	b	p-value
ΔFEV$_1$ (mL)	-5.121 n=115	0.6
ΔFEV$_1$ (% predicted)	-0.405 n=114	0.08
ΔPC$_{20}$ methacholine/histamine (doubling concentrations)	-0.036 n=110	0.3
ΔMethacholine dose response slope (%/mg/ml) *	0.033 n=105	0.2
ΔPC$_{20}$ AMP (doubling concentrations)	-0.045 n=107	0.5
ΔAMP dose response slope (%/mg/ml)	0.044 n=104	0.2
ΔBlood eosinophils (10^3/μL) *	0.005 n=106	0.99
ΔSputum eosinophils (%) *	0.042 n=110	0.03
ΔBlood neutrophils (10^3/μL) *	0.011 n=108	0.08
ΔSputum neutrophils (%) *	0.011 n=111	0.4

Linear regression with change in outcome parameter as dependent variable and BMI as independent variable and age, gender (except FEV$_1$ % predicted) and type of treatment as covariates; N (number of patients) is only given for variables that were not available in all individuals in the specific BMI group; AMP = adenosine 5'-monophosphate; b = regression coefficients; BMI = body mass index; FEV$_1$ = forced expiratory volume in 1 second; * variable log transformed.

Supplementary table 5: Changes with corticosteroid treatment in obese and non-obese patients *

Type of treatment	BMI <30 (n=99)	BMI ≥30 (n=19)	p-value
fluticasone 500 µg/day (DPI), n(%)	32 (32)	8 (42)	
fluticasone 2000 µg/day (DPI), n(%)	33 (33)	7 (37)	
prednisolone 30 mg/day, n(%)	34 (35)	4 (21)	
ΔFEV_1 (mL)	210 (-15 – 560) n=96	50 (-80 – 300)	0.3
ΔFEV_1 (% predicted)	6.2 (-0.5 – 13.2) n=95	1.7 (-2.8 – 9.0)	0.052
ΔPC_{20} methacholine/ histamine (doubling concentrations)	1.5 (0.6 – 2.4) n=93	1.4 (-0.2 – 3.0) n=17	0.2
ΔMethacholine dose response slope (%/mg/ml) #	-0.99 (-1.61 – -0.42) n=90	-0.42 (-02.16 – -0.02 n=15	0.07
ΔPC_{20} AMP (doubling concentrations)	3.3 (0.6 – 5.6) n=90	1.3 (0.0 – 6.6) n=17	0.6
ΔAMP dose response slope (%/mg/ml) #	-2.22 (-3.47 – -0.49) n=87	-0.66 (-3.87 – 0.79) n=16	0.1
ΔBlood eosinophils (10^3/µL)	-0.11 (-0.27 – 0.00) n=87	-0.03 (-0.24 – -0.01) n=19	0.5
ΔSputum eosinophils (%)	-3.8 (-11.3 – 1.3) n=93	-0.7 (-4.3 – 0.0) n=17	0.1
ΔBlood neutrophils (10^3/µL)	0.58 (-0.26 – 1.18) n=89	0.32 (-0.36 – 1.29) n=19	0.046
ΔSputum neutrophils (%)	0.8 (-11.2 – 13.4) n=94	-3.2 (-18.9 – 2.9) n=17	0.8

*All values are presented as median with interquartile range unless stated otherwise; # Variable log transformed. Differences testes by linear regression with change in outcome parameter as dependent variable and BMI as independent variable and age, gender (except FEV_1 % predicted) and type of treatment as covariates; n (number of patients) is only given for variables that were not available in all individuals in the specific BMI group; AMP = adenosine 5'-monophosphate; BMI = body mass index; FEV_1 = forced expiratory volume in 1 second; ICS = inhaled corticosteroids.

Supplementary table 6: Changes with corticosteroid treatment in obese (BMI ≥30 kg/m2) and lean(BMI <25 kg/m2) patients without smokers *

Type of treatment (n)	BMI <25 (n=47)	BMI ≥30 (n=13)	p-value
fluticasone 500 µg/day (DPI), n(%)	17 (36)	6 (46)	
fluticasone 2000 µg/day (DPI), n(%)	13 (28)	4 (31)	
prednisolone 30 mg/day, n(%)	17 (36)	3 (23)	
ΔFEV_1 (mL)	220 (0 – 450)	220 (-25 – 495)	0.5
ΔFEV_1 (% predicted)	5.9 (0.4 – 16.6) n=46	5.9 (0.8 – 12.7)	0.3
ΔPC_{20} methacholine/ histamine (doubling concentrations)	1.5 (0.6 – 2.4) n=46	1.5 (0.2 – 3.5) n=12	0.3
ΔMethacholine dose response slope (%/mg/ml) #	-0.99 (-1.83 – -0.41) n=46	-0.80 (-2.53 – -0.02) n=11	0.3
ΔPC_{20} AMP (doubling concentrations)	4.6 (0.7 – 6.0) n=45	3.2 (0.0 – 7.7) n=12	0.9
ΔAMP dose response slope (%/mg/ml) #	-3.16 (-3.67 – -1.19) n=44	-1.38 (-4.37 – 2.24) n=11	0.3
ΔBlood eosinophils (10^3/µL)	-0.12 (-0.33 – 0.00) n=42	-0.08 (-0.28 – 0.00)	0.5
ΔSputum eosinophils (%)	-3.8 (-11.3 – 1.1) n=45	-1.2 (-5.7 – 0.2) n=11	0.5
ΔBlood neutrophils (10^3/µL)	0.56 (-0.27 – 1.10) n=42	0.32 (-0.71 – 1.10) n=113	0.03
ΔSputum neutrophils (%)	0.9 (-5.8 – 10.8) n=46	-4.7 (-23.7 – 2.0) n=11	0.5

*All values are presented as median with interquartile range unless stated otherwise; # Variable log transformed. Differences testes by linear regression with change in outcome parameter as dependent variable and BMI as independent variable and age, gender (except FEV_1 % predicted) and type of treatment as covariates; N (number of patients) is only given for variables that were not available in all individuals in the specific BMI group; BMI = body mass index; ICS = inhaled corticosteroids; FEV_1 = forced expiratory volume in 1 second; AMP = adenosine 5'-monophosphate.

CHAPTER 5

Skin-blanching is Associated with FEV_1, Allergy, Age and Gender in Asthma Families

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ABSTRACT

Background: Inhaled glucocorticosteroids reduce airway inflammation in asthma patients, thereby improving lung function and reducing airway hyperresponsiveness and symptoms. The response to glucocorticosteroids can be measured with the glucocorticosteroid skin-blanching test. We investigated if asthmatics have a lower skin-blanching response to glucocorticosteroids than non-asthmatic subjects and if asthmatics with airway obstruction have lower skin-blanching response than those without obstruction. Finally, we assessed which clinical and inflammatory parameters influence the variability in skin-blanching response.

Methods: We evaluated the skin-blanching response to topical budesonide in a large group of 315 well-characterized asthmatics and their relatives (asthma n=114, healthy n=140, other= 61).

Results: The skin-blanching scores of the asthma probands and their healthy spouses were not significantly different. The skin-blanching score of patients with FEV_1 <80% predicted was lower than of patients without obstruction. Lower skin-blanching score was significantly associated with lower FEV_1 %predicted, higher age, female gender, absence of allergy and summer season, but not with use of inhaled or oral glucocorticosteroids or packyears smoking.

Conclusions: Asthmatics do not have lower skin-blanching response to glucocorticosteroids than healthy subjects. Furthermore, lower skin-blanching response to glucocorticosteroids is associated with lower FEV_1, female gender, higher age and the absence of allergy.

INTRODUCTION

Asthma is a common respiratory disease, characterized by airway hyperresponsiveness and accompanied by intermittent airway obstruction and respiratory symptoms. The underlying inflammatory process in asthma is effectively treated by inhaled glucocorticosteroids.[1–3] However, a subset of asthma patients has a reduced response to glucocorticosteroids. This reduced response can lead to worse asthma control and lower lung function. There are even patients who are completely resistant to glucocorticosteroids. These patients account for a large percentage of the burden of asthma morbidity, which greatly increases health care costs.[4]

A way to test for responsiveness to glucocorticosteroids is the glucocorticosteroid skin-blanching test, also known as the McKenzie skin-blanching test.[5] Thus far, only two studies have compared skin-blanching response of asthma patients to healthy controls and their results have been conflicting. In the first study, asthma patients had a lower skin-blanching response than healthy controls.[6] In this study, the asthma patients had marked airway obstruction (mean FEV_1 63% of predicted). In another study healthy controls and asthma patients had similar skin-blanching response.[7] However, the latter study showed that a subgroup of glucocorticosteroid resistant asthma patients who had more severe airway obstruction demonstrated reduced skin-blanching response. Taken together, it remains unclear whether a lower skin-blanching response is associated with the presence and severity of asthma.

There is considerable variability in glucocorticosteroid response between individuals, and it is unclear which variables determine the variation of the skin-blanching response to glucocorticosteroids between individuals.[8] In this study we investigated the skin-blanching response in patients with asthma (probands) and their families. Our primary research question was whether asthma patients have a lower skin-blanching response to glucocorticosteroids than non-asthmatic subjects. Secondary, we investigated if asthma patients with airway obstruction have a lower skin-blanching response than asthma patients without airway obstruction and, which clinical and inflammatory parameters are associated with the variability in skin-blanching response.

METHODS AND MATERIALS

Study population

Between 1962 and 1975, asthma patient in adolescence or early adulthood were admitted to a local asthma referral center.[9] From this population, asthma patients were included, based on the following criteria: age <45 years, bronchial hyperresponsiveness to histamine and clinical symptoms of asthma according to current criteria of the American Thoracic Society. This cohort of asthma patients, also called probands, has been followed for 25 – 30

years and participated with their family members (spouse, children, children's spouses and grandchildren) in a family study on the genetics of asthma between 1991 and 1994. The first 93 families (n=499) were asked to participate in a second evaluation between 1997 and 1999, which included the glucocorticosteroid skin-blanching test. The Medical Ethics Committee of the University Hospital Groningen approved the study; all participants provided written informed consent. For subjects <18 years written informed consent was provided by a parent/guardian.

Clinical evaluation

Phenotyping protocol family visit 1991 - 1994

Data on respiratory symptoms, allergic status, use of (glucocorticosteroid) medication and smoking were obtained by a modified version of the British Medical Research Council (MRC) questionnaire.[10] For children younger than 16 years of age, the mother was asked to complete an extended respiratory symptom questionnaire. The forced expiratory volume in 1 second (FEV_1) was measured using a water-sealed spirometer (Lode Spirograph type DL, Lode b.v., Groningen, The Netherlands). A subject was considered hyperresponsive to histamine if the provocative concentration producing a 20% fall (PC_{20} histamine) in FEV_1 was ≤32 mg/mL. In subjects older than 12 years intracutaneous tests with 16 common aeroallergens were performed. In children younger than 12 years, a skin prick test was performed with 10 allergens. Subjects with at least one positive skin test were considered to be allergic.

Phenotyping protocol second family visit 1997 – 1999

During the second family visit, questionnaires and spirometry were performed as described above. Additional phenotyping included the glucocorticosteroid skin-blanching test, measurements of blood eosinophils, blood cortisol and Immunoglobulin E (IgE) (enzyme-linked fluorescence assay, Mini Vidas, Biomerieux) and bronchial hyperresponsiveness to adenosine-5-monophosphate (AMP). A subject was considered hyperresponsive to AMP if the cumulative provocative dosage producing a 20% fall (PD_{20} AMP) in FEV_1 was ≤32 mg. The glucocorticosteroid skin-blanching test was performed by application of budesonide on the skin of the volar side of the forearm using a protocol adapted from Brown *et al.* [7] Briefly, budesonide was dissolved in 96% ethanol to concentrations from 0.3 μg/mL to 1000 μg/mL (0.3, 1, 3.3, 10, 33.3, 100, 333, 1000 μg/mL). Test sites of 2 cm diameter were outlined by double-sided adhesive tape. The eight test concentrations of budesonide were randomly applied to the skin, 10 mL to each site. After evaporation of the diluent, the sites were covered with a plastic film. After 6 h of exposure the plastic film and adhesive tape were removed. The degree of skin-blanching was assessed 1 h later after resolution of any tape-related erythema. The skin-blanching score was assessed under standard lighting conditions (with no natural light) by trained observers, blinded to the distribution of concentrations. Blanching was graded according to a 7-point scale, varying from 0 (no blanching), 0.5, 1, 1.5, 2,

2.5 to 3 (intense blanching).[11] A pilot experiment showed high agreement between observers (weighted Kappa = 0.88, Fleiss Cohen).

Statistical methods

The presence of asthma was based on an algorithm as described previously, which incorporates bronchial hyperresponsiveness to histamine, respiratory symptoms (MRC questionnaire), smoking, airway obstruction and bronchodilator response. [9] Subjects were divided into three categories: 'asthma', 'healthy' (no clinical evidence of asthma or COPD) and 'other' (COPD or unclassifiable airway disease). We used the skin-blanching score to assess the skin-blanching response. This score was calculated as the mean skin-blanching score over the total of all concentrations. To approximate a normal distribution, the mean skin-blanching score was log-transformed. To test for differences in skin-blanching between asthma patients and healthy subjects, we compared the skin-blanching score of asthma probands and their healthy spouses with Student's t-test. We used only the probands and their spouses for this analysis, since these groups are genetically independent. Sample size calculations were based on the findings of Livingston and colleagues.[6] To detect a difference of 0.26 in the mean blanching score between probands and healthy spouses, with a standard deviation of 0.36, an alpha of 0.05 and beta of 0.9, we needed 40 subjects per group. To test which clinical and cellular variables were associated with the skin-blanching response, we performed a regression analysis for skin-blanching score. All regression analyses were performed using linear mixed models, with family as random factor. This method corrects for a possible dependency between subjects from the same family. First we examined univariate models. The pool of variables was chosen based on available literature. Variables with a p-value of 0.10 or less in the univariate model were entered into the multivariate analysis. Age, gender, smoking and asthma status were included in the multivariate model irrespective of the results of the univariate analyses. All analyses were performed using SPSS statistical software version 16.

RESULTS

Members of 71 out of 93 invited families were willing to participate. These 71 families consisted of 329 individuals: 66 probands, 67 spouses, 165 children, 12 children's spouses and 19 grandchildren. The results from the skin-blanching test were available for analysis in 315 of the 329 participating subjects. It was not possible to classify one subject because of a missing bronchial hyperresponsiveness measurement. Of the 314 remaining subjects, 114 were classified as 'asthma', 140 as 'healthy' (no asthma or COPD) and 60 as 'other' (10 COPD and 50 unclassifiable). The clinical characteristics of the 315 participating individuals are outlined in **table 1**.

Table 1: Subject characteristics

	All n=315	Asthma n=114	Healthy § n=140
Age (yrs)	41 (15)	46 (16)	39 (14)
Male (n, %)	164 (52)	66 (58)	73 (52)
Mean skin-blanching score	0.8 (0.5)	0.8 (0.5)	0.8 (0.4)
Never-smokers (n, %)	133 (42)	49 (43)	59 (42)
Ex-smokers (n, %)	70 (22)	31 (27)	25 (18)
Current-smokers (n, %)	111 (35)	34 (29)	55 (40)
Packyears	6.1 (9.8)	6.8 (12.2)	5.8 (8.5)
Use of glucocorticosteroids (n, %)	42 (13)	41 (35)	1 (0.7)
ICS only	37 (11)	36 (31)	1 (0.7)
ICS and oral corticosteroids	4 (1)	4 (3)	0
Oral corticosteroids only	1 (0.3)	1 (0.8)	0
Total IgE(kU/L) *	47 (0 – 6120)	88 (4 – 6120)	27 (0 – 1500)
Blood eosinophils (10^7/L) *	9 (1 – 121)	12 (1 – 121)	8 (1 – 61)
Blood cortisol (nmol/L) *	467 (38 – 1160)	437 (38 – 1121)	479 (209 – 1160)
Allergy (n, %) ‡	161 (51)	94 (83)	40 (29)
PD_{20} AMP ≤32 mg (n, %)	89 (31)	60 (64)	13 (9)
FEV_1 %predicted	96 (20)	83 (24)	105 (12)

Values are in means (SD), *geometric mean (range), or number (%); ‡ = ≥1 positive skin test;
§ no clinical evidence of asthma or COPD; FEV_1 = forced expiratory volume in 1 second;
PD_{20} AMP = cumulative dose of adenosine-5-monophosphate provoking a 20% decrease in FEV_1.

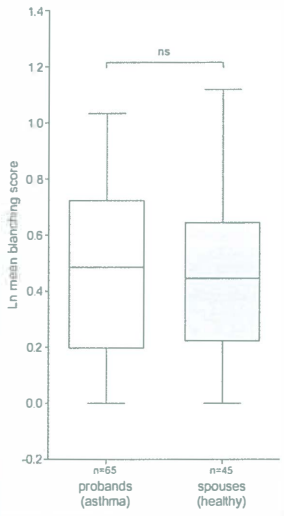
Skin-blanching response of asthma probands and healthy spouses

The skin-blanching scores of asthma probands and healthy spouses were not significantly different (p= 0.675, **figure 1**). However, the skin-blanching score of asthma probands with airway obstruction (FEV_1 <80% predicted) was lower than the skin-blanching score of asthma probands without obstruction (p=<0.001, figure 2). Probands with airway obstruction more often used glucocorticosteroids than probands without airway obstruction (23 / 32 *vs* 7 / 31) and they were older (58 *vs* 53 years) and less often allergic (23 / 31 *vs* 30 / 31). Therefore, we performed regression analysis to assess which variables are associated with the skin-blanching response. Moreover, the skin-blanching response to corticosteroid was not associated with decline in FEV_1 in 44 patients with sufficient data available (data not shown).

Predictors of skin-blanching response

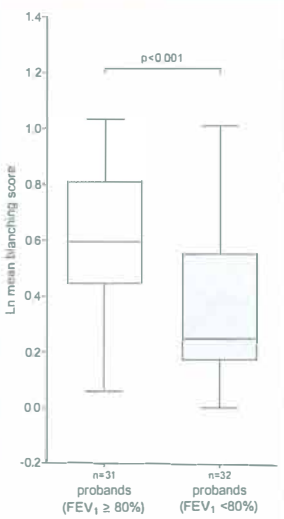
In the univariate analysis of all 315 subjects a lower skin-blanching score was associated with lower FEV_1, older age, absence of allergy, the use of glucocorticosteroids, more packyears smoked and a skin-blanching test performed in summer or fall (**table 2**). The skin-blanching score was not associated with gender, asthma status, bronchial hyperresponsiveness to AMP, total IgE, blood eosinophil number, cortisol, symptoms, or observer scoring the result. In the multivariate analysis (308 subjects) a lower skin-blanching score was independently

Figure 1: Skin-blanching score in asthma probands and healthy spouses.



FEV_1 = forced expiratory volume in 1 second; ns = not statistically significant.

Figure 2: Skin-blanching score in asthma probands with and without obstruction.



FEV_1 = forced expiratory volume in 1 second.

associated with lower FEV_1 ($p=0.012$), older age ($p<0.001$), female gender ($p=0.047$), absence of allergy ($p=0.0019$) and a skin-blanching test performed in summer rather than winter ($p=0.047$, **table 3**).

Table 2: Univariate analysis with skin-blanching score (log transformed) as outcome variable

Variable	b	SE	p-value
Age, per 10 years	-0.061	0.009	<0.001
Gender (male *vs* female)	0.038	0.028	0.177
Use of glucocorticosteroids (yes *vs* no)	-0.116	0.043	<0.007
FEV_1 %predicted, per 10%	0.029	0.007	<0.001
Allergy ‡	0.078	0.030	0.010
Packyears	-0.006	-0.006	<0.001
Season			
spring *vs* winter	-0.054	0.060	0.368
summer *vs* winter	-0.166	0.061	0.007
fall *vs* winter	-0.138	0.061	0.025
PD_{20} AMP ≤32 mg	0.027	0.033	0.414
Total IgE (kU/L)	-0.005	0.021	0.804
Blood eosinophils (10^7/L)	0.020	0.172	0.256
Blood cortisol (nmol/L)	0.130	0.086	0.131
Symptoms (MRC)	-0.010	0.011	0.405
Observer			
observer 1 *vs* 3	0.051	0.090	0.566
observer 2 *vs* 3	0.009	0.063	0.884
Asthma status			
asthma *vs* healthy	-0.058	0.041	0.164
unclassifiable *vs* healthy	-0.035	0.040	0.379

‡ = ≥1 positive skin test; b = regression coefficient; FEV_1 = forced expiratory volume in 1 second; PD_{20} AMP = Cumulative dose of adenosine-5-monophosphate provoking a 20% decrease in FEV_1; SE = standard error; Winter = December – February; Spring = March – May; Summer = June – August; Fall = September – November; MRC = Medical Research Council.

Table 3: Multivariate analysis with skin-blanching score (log transformed) as outcome variable

Variable	b	SE	p-value
Age, per 10 years	-0.047	0.011	<0.001
Gender (male *vs* female)	0.055	0.028	0.047
Use of glucocorticosteroids	0.007	0.053	0.897
FEV_1 %predicted, per 10%	0.024	0.009	0.012
Allergy ‡	0.083	0.032	0.010
Packyears	-0.001	0.002	0.406
Season			
spring *vs* winter	-0.005	0.055	0.924
summer *vs* winter	-0.112	0.056	0.047
fall *vs* winter	-0.062	0.056	0.276
Asthma status			
asthma *vs* healthy	-0.011	0.045	0.810
unclassifiable *vs* healthy	-0.032	0.038	0.394

‡ = ≥1 positive skin test; b = regression coefficient; FEV_1 = forced expiratory volume in 1 second; SE = standard error; Winter = December – February; Spring = March – May; Summer = June – August; Fall = September – November.

DISCUSSION

In this study, we provide evidence that patients with asthma do not have a lower skin-blanching response to glucocorticosteroids than non-asthmatic subjects. This is based on the observation that asthma probands and their healthy spouses had a similar skin-blanching score (figure 1). In addition, in our multivariate analysis asthma status was not associated with the skin-blanching score. Skin-blanching response does appear to be associated with level of airway obstruction, since probands with an FEV_1 <80% predicted (n=33) had lower skin-blanching scores than probands with normal lung function (n=31).Furthermore, lower skin-blanching score was significantly associated with lower FEV_1 in the complete study population. The other main finding of this study is that variability in skin-blanching response between individuals is associated with age, gender, allergy, FEV_1 and season. Importantly, the skin-blanching score was not associated with the use of glucocorticosteroids and smoking. Although the precise mechanisms of action of the glucocorticosteroid skin-blanching test are unknown, it provides an easy, direct assessment of glucocorticosteroid sensitivity, making it an interesting test for investigating glucocorticosteroid sensitivity in asthma.

The similar skin-blanching response of asthma patients and healthy subjects is consistent with a previous study, comparing steroid-sensitive asthma patients and controls.[7] However, it contrasts with another study in 75 asthma patients and 78 healthy controls. In this study, skin-blanching response to glucocorticosteroids was lower in asthma patients than in healthy controls.[6] However, those asthmatics had marked airway obstruction (mean FEV_1 63% of predicted) and this may have influenced the results, which fits with our observation that asthma probands with more severe airway obstruction (FEV_1 <80% predicted) had a lower skin-blanching response. Additionally, multivariate analysis revealed that a lower skin-blanching response was associated with lower FEV_1. These results suggest that, although skin-blanching response is similar in asthma patients in general and in non-asthmatic controls, it may be reduced in a subgroup of older patients who have non-allergic asthma and airway obstruction.

It is tempting to speculate that the airway obstruction in the subgroup of older non-allergic asthma patients with reduced skin-blanching response is associated with a lower treatment response to inhaled glucocorticosteroids, thus translating the response in the skin to the airways. However, this speculation is not supported by the results of a study in 22 patients with mild-to-moderate asthma.[11] In this study, the skin-blanching response to glucocorticosteroids was not associated with changes in serum cortisol or bronchial hyperresponsiveness after a 3-week treatment with inhaled glucocorticosteroids. However, the results of this study do not rule out an effect of lower treatment response either, since included patients may have had asthma of too mild severity (mean FEV_1 was 86% predicted) to show differences in treatment response. FeNO and sputum eosinophils are validated markers of airway inflammation which can be used for assessing the response to anti-inflammatory therapy in patients with asthma [12,13] and novel non-invasive markers of airway inflammation

are being characterized.[14] Future prospective studies to establish the relationships between skin-blanching response to glucocorticosteroids and lung inflammatory parameters should be undertaken.

Skin-blanching after topical application of glucocorticosteroids is caused by subdermal vasoconstriction. The mechanisms of this effect may include both non-genomic (i.e. direct inhibitory effects) and genomic (i.e. transcriptional) effects through binding to the glucocorticosteroid receptor. Studies in rabbit models have provided insight into non-genomic mechanisms of glucocorticosteroid action on vasoconstriction.[15,16] One of these mechanisms is via inhibitory effects on the enzyme organic cation transporter 3 (OCT3).[17] Inhibition of OCT3 by glucocorticosteroids increases noradrenalin at the α_1-adrenergic receptor, which then causes vasoconstriction. The rapid onset of the vasoconstrictive effect of inhaled glucocorticosteroids in the lung (i.e. 30 minutes) is consistent with a direct, non-genomic, effect.[18] However, the maximum effect 8 – 12 h after application and the duration of the vasoconstrictive effect (>24 h) suggests that additional mechanisms, like genomic effects, are likely important.[19,20] The genomic mechanisms may be binding to the glucocorticoid receptor (GR) and regulation of genes with glucocorticosteroid responsive elements (GRE´s) or recruitment of histone deacetylases.[21] An indication that regulation of genes with a GRE may be involved, is that screening of the Genomatix database showed a putative GRE in the promoter region of the *OCT3* gene (Huge gene name: *Solute carrier 22, member 3, SLC22A3*).[22] Further evidence for a role of GR's in skin-blanching response to glucocorticosteroids, was provided by a study of skin-blanching response in healthy volunteers with different genetic variants of the glucocorticosteroid receptor gene. A BclI restriction fragment length polymorphism (AA genotype) of the GR gene was associated with an increased skin-blanching response to glucocorticosteroids.[23] Finally, inhibition of the GR by a pharmacological inhibitor resulted in decreased skin-blanching response in healthy subjects, supporting the role of GR's in the skin-blanching response.[24] We found an increased skin-blanching response to glucocorticosteroids in individuals with positive skin tests to allergens, but not with elevated blood eosinophils or total serum IgE levels. This observation was surprising since asthma patients with higher levels of eosinophils or IgE have been reported to have a better glucocorticosteroid response.[25,26] The mechanism of increased skin-blanching response in allergic subjects is unknown. OCT3, which is likely to be involved in the skin-blanching response, may also play a role in allergy by impaired histamine clearance, since OCT3 is a transporter of histamine in rats.[17] Furthermore, OCT3 mRNA was down-regulated in a rabbit model of ovalbumin-induced allergic sensitization.[15]

In this study, several parameters were associated with the skin-blanching response to glucocorticosteroids. First, lower skin-blanching response was associated with increasing age. Little is known about glucocorticosteroid sensitivity and age. In one study, peripheral whole blood of older men showed less inhibition by dexamethasone of IL-6 production after stimulation with lipopolysaccharide than blood of younger men, which represents lower glucocorticoid sensitivity. [27] Another study showed that the number of GR's in the

hypothalamus decreases with increasing age.[28] However, it is not known if a similar effect is present in skin tissue. These and our studies suggest that with increasing age, glucocorticoid sensitivity decreases, however this has yet to be confirmed. Second, lower skin-blanching response was associated with lower lung function. No prior studies have described a relationship between glucocorticosteroid responsiveness and lung function in a general population. The causality of this association remains unclear. A possible explanation may be that subjects with a lower response to endogenous glucocorticosteroids have either less lung growth or a more rapid decline in lung function; however no data exists to support this claim. Third, lower skin-blanching response was associated with female gender. This finding is consistent with a study in which skin-blanching was measured with laser Doppler imaging and diffuse reflectance spectroscopy in healthy volunteers.[20] Gender differences were also found in a study in which asthma patients were treated with glucocorticosteroids. Treatment with inhaled corticosteroids significantly decreased the decline in FEV_1 in male, but not in female asthma patients.[29] These findings suggest that women have a reduced response to glucocorticosteroids. Female sex hormones may be involved in this reduced responsiveness, since estrogen has pro-inflammatory [30,31] and progesterone anti-inflammatory properties.[32,33] Finally, we found that the skin-blanching response was higher in winter compared to summer. This is in accordance with another study, which found the same association in healthy controls.[34] A possible explanation for the increased skin-blanching response is that vitamin D levels are increased in summer. Higher levels of vitamin D have been associated with increased glucocorticosteroid-response and higher lung function.[35] Another explanation may be that differences in skin tanning influences the reading of the test.

In conclusion, we have shown that asthma patients in our study population do not have a lower skin-blanching response to glucocorticosteroids than non-asthmatic subjects. When interpreting results from skin-blanching tests, the season should be taken into account, as the skin-blanching response differs between seasons. Finally, a lower skin-blanching response to glucocorticosteroids is associated with lower FEV_1, female gender, increased age and the absence of allergy.

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CHAPTER 6

Small Airways in Asthma: Their Independent Contribution to Severity of Hyperresponsiveness

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TO THE EDITOR,

Bronchial hyperresponsiveness (BHR), i.e. increased narrowing of the airways after exposure to non-allergic stimuli, is a hallmark of asthma. BHR is a risk factor for asthma development and, additionally, a marker of worse disease outcome in asthma.[1,2] It is generally acknowledged that obstruction of the large airways due to inflammation and remodeling contributes to more severe BHR. This is plausible, given that BHR is expressed as the concentration or dose of a stimulus that induces a 20% fall in forced expiratory volume in 1 s (FEV_1). Recent studies suggest that asthmatics with BHR have more severe small airways obstruction.[3,4] However, little is known about the converse, i.e. the association between small airways obstruction and severity of BHR. Our aim was to assess: 1) whether asthma patients with small airways obstruction express more severe BHR than those without small airways obstruction; and 2) whether small airways obstruction is associated with more severe BHR independently of FEV_1.

We analyzed data of patients with mild-to-moderate asthma from a previously published study on inhaled corticosteroids (ICS) in primary care.[5] All subjects underwent spirometry before and after 1 mg terbutaline, measuring FEV_1, forced vital capacity (FVC) and mean expiratory flow at 50% of FVC (MEF_{50}). BHR was assessed by a histamine challenge test, measuring the provoking dose causing a decrease of 20% in FEV_1 (PD_{20} histamine). All patients were hyperresponsive to histamine (PD_{20} < 9 mg). Small airways obstruction was defined as a MEF_{50} of less than or the same as the lower limit of normal (LLN); other small airways parameters, such as the mean expiratory flow between 25-75% of FVC (MEF_{25-75}), residual volume/total lung capacity and FVC/slow vital capacity, were not available in our database. We compared asthma patients with and without small airways obstruction using unpaired t-tests, Mann-Whitney U-tests or Chi-squared tests as appropriate. In addition, a multivariate linear regression analysis was performed to assess if MEF_{50} is independently associated with the PD_{20} histamine (log_2 transformed). We added FEV_1, age, gender, height and use of ICS as covariates.

Of the 94 patients, 34 had small airways obstruction (MEF_{50} ≤ LLN). **Table 1** shows that patients with small airways obstruction had more severe BHR (geometric mean PD_{20} histamine 0.2 *vs* 0.6 mg respectively, $p<0.01$). In addition, FEV_1, FVC and FEV_1/FVC values were significantly lower and reversibility values significantly higher in patients with small airways obstruction. Patients with small airways obstruction using ICS had a significantly higher daily dose of ICS than patients without small airways obstruction (800 *vs* 500 µg per day beclomethasone dipropionate equivalent). A lower MEF_{50} value was significantly associated with more severe BHR in the multivariate linear regression analysis, independently from FEV_1, age, gender, height and ICS use ($b=0.813$, $p=0.01$). We repeated this analysis with MEF_{50}/FVC instead of MEF_{50}, and with MEF_{50} %predicted and FEV_1 %predicted instead of the absolute values. These analyses showed similar results.

This study shows that asthma patients with small airways obstruction have more severe BHR independently of the level of FEV_1. Despite intensive research over the past 40 years,

Table 1: Differences between asthma patients with and without small airways obstructions (MEF_{50} ≤ LLN and MEF_{50} > LLN respectively)

	MEF_{50} > LLN (n=60)	MEF_{50} ≤ LLN (n=34)	p-value
Age (years)	43 (33 – 53)	47 (35 – 58)	0.22
Gender (males, n, %)	22 (37)	13 (38)	0.88
Smoking (n, %)			0.39
non-smoker	30 (51)	21 (62)	
ex-smoker	19 (32)	11 (32)	
occasional smoker	3 (5)	0 (0)	
habitual smoker	7 (12)	2 (6)	
Packyears ‡	3.0 (1.0 – 6.0)	4.0 (1.0 – 6.0)	0.96
ICS use (n, %)	54 (90)	31 (91)	0.85
ICS dose (BDP equivalent µg/day) *	500 (400 – 800)	800 (400 – 800)	<0.01
BMI (kg/m²)	26 (24 – 30)	26 (24 – 30)	0.69
PD_{20} histamine (mg) £	0.6 (0.1 – 4.2)	0.2 (0.1 – 2.7)	<0.01
FEV_1 (%predicted)	107.8 (99.9 – 113.2)	83.4 (70.9 – 89.5)	<0.01
FVC (%predicted)	115.0 (104.9 – 122.3)	103.8 (94.2 – 108.7)	<0.01
FEV_1/FVC (%)	79.4 (75.6 – 82.3)	68.5 (61.9 – 72.4)	<0.01
Reversibility (% of predicted)	4.9 (3.0 – 7.5)	8.4 (5.6 – 11.1)	<0.01
MEF_{50} (%predicted)	85.8 (66.9 – 100.6)	45.3 (38.9 – 51.1)	<0.01

Data are presented as median (interquartile range) or n (%), unless stated otherwise. Patients with small airways obstruction were defined as having a mean expiratory flow at 50% of forced vital capacity (MEF_{50}) that was less than or the same as the lower limit of normal (LLN). Patients without small airways obstruction were defined as having a MEF_{50} greater than the LLN. Data are presented as median (interquartile range) or n (%), unless stated otherwise. BDP = beclomethasone dipropionate, BHR = bronchial hyperresponsiveness, BMI = body mass index, FEV_1 = forced expiratory volume in 1 second, FVC = forced vital capacity; ICS = inhaled corticosteroids, PD_{20} = provoking dose causing a 20% decrease in FEV_1, ‡ = does not include non-smokers, * = only in patients using ICS, £ = data are presented as geometric mean (range).

the mechanisms underlying BHR have still not been clarified. It is known that the severity of BHR is related to airway wall inflammation in asthma. Other factors that influence BHR are structural changes of the airways such as deposition of connective tissue components, increased vessel formation and smooth muscle mass and edema, contributing to thickening and increased stiffness of the airways. These processes are known to occur in the large airways and there is now increasing evidence to suggest that they also occur in the small airways. Small airways obstruction, i.e. lower MEF_{25-75} values, has previously been reported to be present in children with BHR and in adults with mild asthma and BHR.[3,4] Another study showed that patients with hyperresponsiveness to mannitol have more severe small airways obstruction.[6] Together, these and our results together suggest that an unexplained part of BHR originates from the small airways.

A limitation of our study may be the use of MEF_{50} as a marker of small airways obstruction. Obstruction in the proximal airways may also lead to airflow limitation with reduced MEF_{50}. As the patients with a MEF_{50} ≤ LLN had a reduced FEV_1, we performed a multiple linear regression analysis in which we corrected for FEV_1. In this analysis, MEF_{50} was associated

with the severity of BHR independently of FEV_1. In addition, we performed our analysis with the MEF_{50}/FVC, since FVC is known to influence MEF_{50} levels, and with MEF_{50} %predicted and FEV_1 %predicted. The results remained similar, demonstrating the robustness of our results.

We found that small airways obstruction is associated with the severity of BHR in asthma, independently from FEV_1, and this may have important implications for asthma management. Treatment with large-particle ICS rapidly improves FEV_1 in asthma, an effect that remains stable thereafter. In contrast, improvement in BHR occurs gradually and ICS do not completely abolish the presence of BHR in most asthmatics, even after longstanding treatment.[7] This may have implications for asthma management; Sont *et al.* showed that ICS treatment tailored according to the severity of BHR improves the clinical outcome and airway remodeling of asthma.[8] In addition, targeting the small airways with small-particle ICS as currently available may be more effective in reducing BHR originating in the small airways. A recent study demonstrated that treatment with small-particle ICS is at least equally effective in achieving asthma control as large-particle ICS treatment at half the dose.[9] Moreover, we have recently shown that small-particle ICS improve BHR to AMP more than large-particle ICS, but only when the provocation was performed with small-particle AMP (1.74 *vs* 0.8 doubling doses respectively).[10] Taken together, our findings suggest that the currently applied provocation tests have important shortcomings for investigating BHR originating from the small airways. First, the read-out for airway obstruction during provocation is FEV_1, mainly a large airways parameter. Secondly, the DeVilbiss 646 (DeVilbiss Healthcare, Somerset, PA, USA) is the recommended nebulizer for provocation tests; this nebulizer produces relatively large particles of 3.7 µm.[10] Therefore, most particles will deposit in the large airways and few particles will actually reach the small airways. Thus, current provocation tests will underestimate the BHR of small airways. This is important, because BHR of the small airway may contribute to asthma symptoms and control in real life.

We conclude that the small airways contribute significantly to the severity of BHR, a contribution that is independent from the level of FEV_1. We hypothesize that the contribution of the small airways to the severity of BHR in asthma is much larger than has been considered to date. Performing studies with provocation tests with small airways measurements, e.g. impulse oscillometry, and small-particle stimuli will give better insights into BHR of the small airways. Further studies should show whether small-particle ICS improve BHR more rapidly and/or to a larger extent than large-particle ICS, with the ultimate benefit being to asthma patients in clinical practice.

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CHAPTER 7

Less Small Airway Dysfunction in Asymptomatic Bronchial Hyperresponsiveness than in Asthma

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Allergy (in press)

ABSTRACT

Background: Bronchial hyperresponsiveness (BHR) can be present in subjects without any respiratory symptoms. Little is known about the role of the small airways in asymptomatic subjects with BHR.

Methods: We investigated small airway function assessed by spirometry and impulse oscillometry, as well as Borg dyspnea scores at baseline and during a methacholine provocation test in 15 subjects with asymptomatic BHR, 15 asthma patients and 15 healthy controls.

Results: At baseline, small airway function (R_5-R_{20} and X_5) was comparable between subjects with asymptomatic BHR and healthy controls, whereas asthma patients showed small airway dysfunction as reflected by higher R_5-R_{20} and lower X_5 values. During methacholine provocation, small airway dysfunction was more severe in asthma patients than in subjects with asymptomatic BHR. Interestingly, a higher increase in small airway dysfunction during methacholine provocation was associated with a higher increase in Borg dyspnea scores in subjects with asymptomatic BHR, but not in asthma.

Conclusion: Subjects with asymptomatic BHR may experience fewer symptoms in daily life because they have less small airway dysfunction.

INTRODUCTION

Bronchial hyperresponsiveness (BHR) is defined as exaggerated airway narrowing in response to various non-specific stimuli such as fog, perfume or cold air. Although BHR is a hallmark of asthma, it has been reported that subjects without any respiratory symptoms may also exhibit BHR. In a review by Jansen *et al.*, a prevalence rate between 2.2% to 14.3% of this so-called 'asymptomatic BHR' was reported.[1] Asymptomatic BHR is associated with an increased risk to develop asthma later in life.[2–5]

The reason why some subjects do not have any respiratory symptoms, even though they do exhibit BHR is not clear, although several possible explanations have been investigated in the past decades, including presence and extent of airway inflammation, airway remodeling and decreased perception of symptoms.[6–9] Thus far, studies investigating asymptomatic BHR have focused mainly on the large airways. In recent years, there is increasing evidence that the small airways (i.e. those with an internal diameter < 2mm) are also involved in BHR and contribute importantly to the clinical expression of asthma.[10–15] Mansur *et al.* showed that small airway dysfunction during a methacholine provocation was associated with increased dyspnea perception in asthma patients.[16]

In line with these recently found associations between small airway dysfunction, BHR and asthma symptoms, we hypothesized that subjects with asymptomatic BHR have more small airways dysfunction than healthy control, but less small airways dysfunction than subjects with symptomatic BHR, i.e. patients with asthma. To investigate this, we performed a cross-sectional study measuring large and small airway function, both at baseline and during methacholine provocation, in subjects with asymptomatic BHR, patients with asthma and healthy controls.

METHODS

Study design

We performed a three-arm, cross-sectional, observational study. The three arms were: controls (subjects without BHR, no respiratory symptoms and no history of asthma or COPD), asymptomatic subjects with BHR (subjects with BHR, but without respiratory symptoms and no history of asthma or COPD) and asthma patients (subjects with BHR and a doctor's diagnosis of asthma). Subjects with asymptomatic BHR were available from a previous study aiming to obtain normal values of inflammatory variables in healthy individuals (the NORM study, NCT 00848406). During screening for the NORM study, these subjects, who were completely asymptomatic, showed BHR during a methacholine provocation test. For this reason, they were excluded from the NORM study. This observation led us to design the current study. We included subjects aged 18 to 65 years with a smoking history <10 packyears. BHR was defined as a provocative concentration of methacholine inducing a 20%

fall in FEV_1 (PC_{20}) ffi8 mg/ml. Absence of BHR was defined as a PC_{20} >16 mg/ml. Respiratory symptoms were assessed as in a previous study.[17] Briefly, asymptomatic subjects reported no symptoms of chronic cough or phlegm production, no shortness of breath when walking on level ground and no attacks of shortness of breath. Each subject was evaluated during 2 visits to our hospital. The study was approved by the Medical Ethics Committee of the University Medical Center Groningen and all subjects gave their written informed consent.

Pulmonary function tests

Spirometry was performed according to international guidelines before and after administering 400 µg salbutamol.[18] Forced expiratory volume in 1 second (FEV_1), forced vital capacity (FVC), FEV_1/FVC and forced expiratory flow at 50% and between 25-75% of FVC (FEF_{50} and FEF_{25-75} respectively) were obtained. Reversibility to salbutamol was expressed as the change in FEV_1 between the pre- and post-bronchodilator value as percentage of the predicted value. Impulse oscillometry (IOS) was used to measure the resistance at 5Hz (R_5), which reflects total airway resistance, resistance at 20Hz (R_{20}), which reflects the resistance in the large airways and the difference between R_5 and R_{20} (R_5-R_{20}), reflecting the resistance in the small airways. In addition, Reactance at 5 Hz (X_5) was measured, which reflects elastic properties of the small airways. Body plethysmography was used to measure total lung capacity (TLC), residual volume (RV) and airway resistance (R_{aw}).

Methacholine provocation with IOS and Borg dyspnea score

Methacholine provocation was performed according to the 2-minute tidal breathing method adapted from Crapo *et al.*[19] Subjects inhaled doubling concentrations of methacholine (0.03 to 16 mg/ml). After each inhalation, the FEV_1 was measured, impulse oscillometry was performed and the subjects were asked to score their perception of dyspnea by means of the modified Borg scale.[20] This scale ranges from 0 (no dyspnea) to 10 (maximal dyspnea). The challenge was discontinued when the FEV_1 had fallen by 20% or more from the pre-challenge level or when the highest concentration of methacholine had been administered. PC_{20} was calculated by linear interpolation between the last two data points of the logarithmic concentration-response curve.

Questionnaires

All subjects filled out the Dutch version of the bronchial hyperresponsiveness questionnaire (BHQ) and the asthma control questionnaire (ACQ). The BHQ consists of 15 questions about symptoms and 19 questions about provoking stimuli associated with bronchial hyperresponsiveness in the last 3 months (0: no BHR, 6: worst score). The ACQ is used to assess asthma control (0: best control, 6: worst control).[21,22]

Statistical analyses

Statistical analyses were performed using IBM SPSS Statistics, version 20. To analyse

changes during methacholine provocation between the 3 groups, we calculated the slopes of FEV_1, R_5-R_{20}, X_5, R_{20} and the Borg dyspnea score between the baseline measurement and the measurement at the last concentration of methacholine. A slope reflects the degree of change of a variable per mg/ml methacholine. In addition, to assess small airway dysfunction and dyspnea at the moment FEV_1 had fallen by 20% we calculated values at PC_{20} for R_5-R_{20}, X_5, R_{20} and the Borg dyspnea score in subjects with a positive provocation test, i.e. subjects with asymptomatic BHR and asthma patients. Values at PC_{20} were calculated by interpolating between the second-to-last and the last value, similar to the way the PC_{20} is calculated.[19] Baseline values, slopes and values at PC_{20} in the groups were compared by one-way analysis of variance (ANOVA) or Kruskall Wallis tests with post-hoc Holm's Bonferroni correction for multiple testing. To assess the association between changes in IOS parameters and change in dyspnea during a provocation test, we performed Spearman's correlation analyses.

RESULTS

Baseline characteristics

A total of 45 subjects were included in the study, i.e. 15 healthy controls, 15 subjects with asymptomatic BHR and 15 asthma patients. The baseline characteristics of the three groups are presented in **table 1**. The median age was 26 years in the control group, 24 years in the asymptomatic BHR group and 45 years in the asthma group. Body Mass Index (BMI) was significantly higher in asthma patients than in subjects with asymptomatic BHR and healthy controls. The PC_{20} methacholine was comparable between subjects with asymptomatic BHR and asthma.

There were no differences in small airway parameters between controls and subjects with asymptomatic BHR. Asthma patients had a significantly higher R_5-R_{20} and a lower X_5, i.e. more small airway dysfunction, than subjects with asymptomatic BHR and controls. Small airway airflow limitation reflected by FEF_{50} % predicted and FEF_{25-75} % predicted did not differ between the groups. Large airway parameters (FEV_1 % predicted and R_{20}) were comparable between the three groups. Scores on respiratory symptom questionnaires did not differ between controls and subjects with asymptomatic BHR, but were significantly higher in asthma patients. Since patients with asthma had a higher BMI than healthy controls and subjects with asymptomatic BHR, we investigated whether this would affect our main results. To this end, we also assessed the obese (BMI ≥30kg/m²) and non-obese (BMI <30kg/m²) asthma patients separately. This way, we found similar results, i.e. R_5-R_{20} values were higher in both obese and non-obese asthma patients compared to subjects with asymptomatic BHR ($p<0.001$ and $p=0.025$ respectively), whereas X_5-values were lower ($p=0.033$ and $p=0.010$ respectively).

Table 1: Baseline characteristics of the study population

	Controls (n=15)	Asymptomatic BHR (n=15)	Asthma (n=15)	p-value
Age (years)	26* (23–32)	24* (23–28)	45 (36-52)	0.005
Gender (female/male)	10/5	12/3	13/2	0.407
Smoking (n, %)				0.043
Current	5 (33)	8 (53)	2 (13)	
Ex	-	1 (7)	4 (27)	
Never	10 (67)	6 (40)	9 (60)	
Packyears	5.6 (1.9-8.3)	2.5 (1.3-8.7)	4.7 (2.8-8.9)	0.773
Family history of asthma (n, %)	4* (27)	5* (33)	13 (87)	0.002
Allergic rhinitis (n, %)	1 (7)*	3 (20)	9 (60)	0.008
Body Mass Index (kg/m²)#	21.6 (20.6-25.5)*	22.5 (20.8-26.6)*	30.9 (28.4-37.7)	<0.001
PC_{20} (mg/ml)§	-	1.9 (0.6-8.0)	1.4 (0.04-8.0)	1.000
FEV_1 (% predicted)#	106 (10)	103 (14)	101 (15)	0.566
FEV_1 reversibility (% predicted)	1.8 (0.8-4.1)	5.6 (3.0-8.4)	5.0 (1.1-9.4)	0.064
FEV_1/FVC (%)	82.6 (79.0-89.2)	82.7 (73.5-89.5)	76.5 (67.4-85.1)	0.110
FEF_{50} (% predicted)#	92 (18)	85 (23)	76 (29)	0.179
FEF_{50}/FVC ((l/s)/s)	0.97 (0.76-1.13)	0.91 (0.65-1.08)	0.74 (0.54-1.13)	0.346
FEF_{25-75} (% predicted)	95 (80–112)	83 (59–101)	69 (44–114)	0.231
FEF_{25-75}/FVC((l/s)/s)	0.83 (0.68-1.04)	0.78 (0.58-0.96)	0.62 (0.40-1.00)	0.242
RV/TLC (% predicted)	88 (74-97)	88 (77-99)	90 (82–110)	0.561
R_{aw} (% predicted)	80* (61-91)	73* (66-94)	120 (80–193)	0.007
R_5 (kPa/l/s)	0.32* (0.27–0.41)	0.35* (0.31–0.40)	0.54 (0.41–0.65)	<0.001
R_{20} (kPa/l/s)#	0.33 (0.08)	0.35 (0.09)	0.38 (0.06)	0.097
R_5-R_{20} (kPa/l/s)	0.00* (-0.01–0.01)	0.02* (-0.01–0.05)	0.12 (0.05–0.25)	<0.001
X_5 (kPa/l/s)	-0.07* (-0.09- -0.05)	-0.10* (-0.11- -0.06)	-0.16 (-0.23- -0.10)	<0.001
Questionnaires				
BHQ	0.0* (0.0–0.2)	0.2* (0.0–0.4)	2.4 (1.8–3.1)	<0.001
ACQ	0.0* (0.0–0.0)	0.0* (0.0–0.2)	0.7 (0.3–1.5)	<0.001

All values are presented as medians with interquartile ranges, unless stated otherwise; * Significantly different from asthma with Holm's Bonferroni correction; § Geometric mean with range; # Mean with standard deviation.

ACQ = asthma control questionnaire (0: best control, 6: worst control); BHR = bronchial hyperresponsiveness; BHQ = bronchial hyperresponsiveness questionnaire (0: no BHR, 6: worst score); FEV_1 = forced expiratory volume in 1 second; FEF_{50} = forced expiratory flow at 50% of the FVC; FEF_{25-75} = forced expiratory flow between 25 and 75% of the FVC; FVC = forced vital capacity; PC_{20} = provoking concentration causing a 20% fall in FEV_1; R_5 = resistance at 5 Hz; R_{20} = resistance at 20 Hz; R_{aw} = airway resistance; RV = residual volume; TLC = total lung capacity; X_5 = reactance at 5 Hz.

Small airways and dyspnea score during methacholine provocation

During methacholine provocation, small airway resistance as reflected by the slopes of R_5-R_{20} and X_5, increased to a higher extent in subjects with asymptomatic BHR and asthma than in controls **(Table 2)**. Subjects with asymptomatic BHR had significantly higher slopes of R_{20} than healthy controls. Furthermore, dyspnea increased significantly more both in subjects with asthma and asymptomatic BHR during the methacholine provocation test, i.e. they had a higher slope of the Borg dyspnea score, than in healthy controls. There were no significant differences in the slopes of FEV_1, R_5-R_{20}, R_{20}, X_5 and Borg dyspnea score between patients with asthma and subjects with asymptomatic BHR during methacholine provocation.

Table 2: Changes in variables during methacholine provocation

	Control (n=15)	Asymptomatic BHR (n=15)	Asthma (n=15)	p-value
Slope FEV_1 (l/mg/ml)	-0.02 (-0.03- -0.01)	-0.44 (-0.75- -0.18)*	-0.38 (-0.60- -0.09)*	<0.001
Slope R_5-R_{20} (kPa/l/s/mg/ml)	0.00 (0.00- 0.01)	0.06 (0.02–0.13)*	0.13 (0.04–0.56)*	<0.001
Slope X_5 (kPa/l/s/mg/ml)	0.00 (-0.01- 0.00)	-0.05 (-0.14- -0.02)*	-0.17 (-0.46- -0.04)*	<0.001
Slope R_{20} (kPa/l/s/mg/ml)	0.00 (0.00- 0.01)	0.02 (0.01- 0.04)*	0.01 (0.00- 0.05)	0.049
Slope Borg (score/mg/ml)	0.06 (0.00- 0.13)	1.00 (0.25-1.00)*	1.38 (0.53- 2.19)*	<0.001

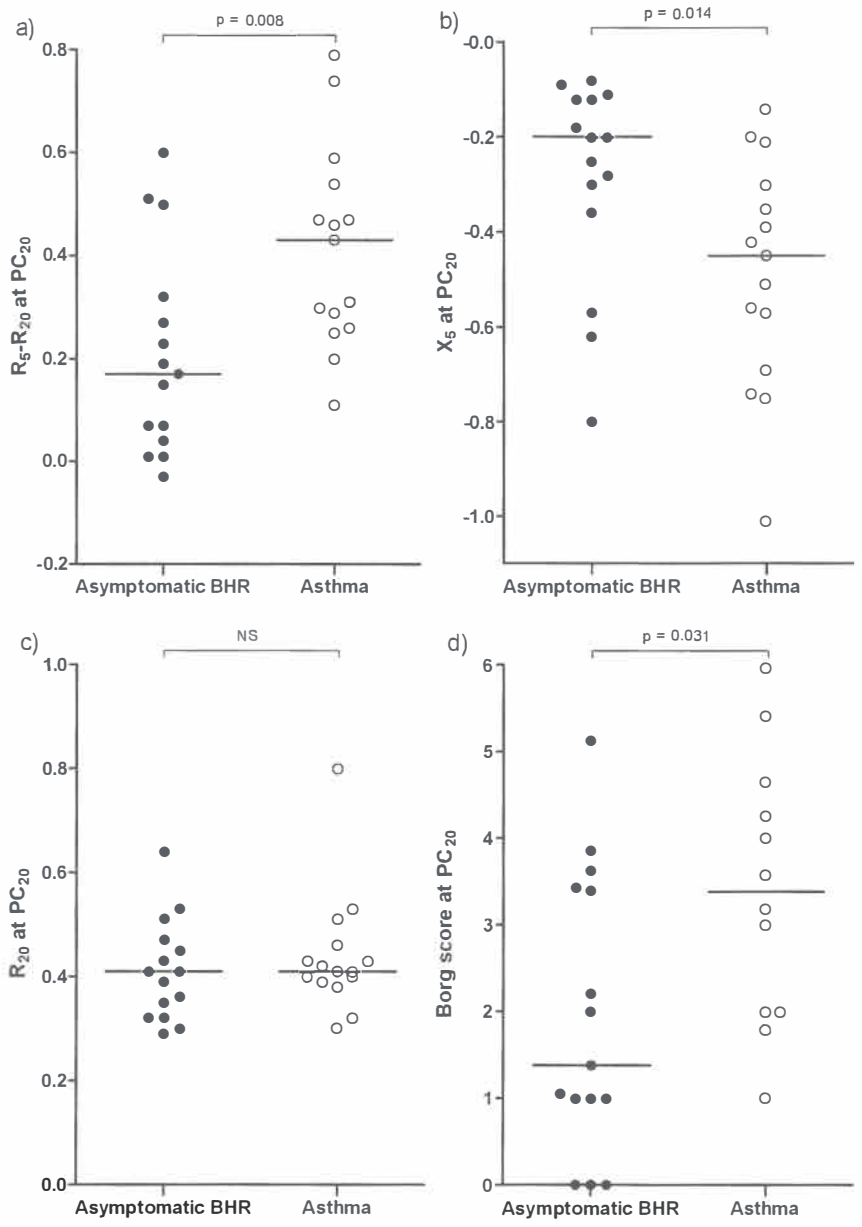
* Significantly different from controls ($p<0.05$ after Holm's Bonferroni correction); All values are presented as medians with interquartile ranges. BHR = bronchial hyperresponsiveness; FEV_1 = forced expiratory volume in 1 second; R_5 = resistance at 5 Hz; R_{20} = resistance at 20 Hz; X_5 = reactance at 5 Hz.

At the provocative concentration causing the FEV_1 to drop by 20% (i.e. PC_{20}), significantly higher values of R_5-R_{20} and lower values of X_5 were observed in asthma patients than in subjects with asymptomatic BHR (median R_5-R_{20} 0.43 *vs* 0.17 respectively, **Figure 1A**; median X_5 -0.45 *vs* -0.20 respectively, **Figure 1B**), suggesting that asthma patients have more small airway dysfunction than subjects with asymptomatic BHR at a similar fall in FEV_1. Interestingly, R_{20} did not differ between the groups at PC_{20} (median 0.41 in both groups, **Figure 1C**. At PC_{20}, all asthma patients and some subjects with asymptomatic BHR experienced dyspnea, but asthma patients experienced significantly more dyspnea than subjects with asymptomatic BHR (median Borg score 3.4 *vs* 1.4 respectively, **Figure 1D**).

Association between small airway dysfunction and dyspnea score

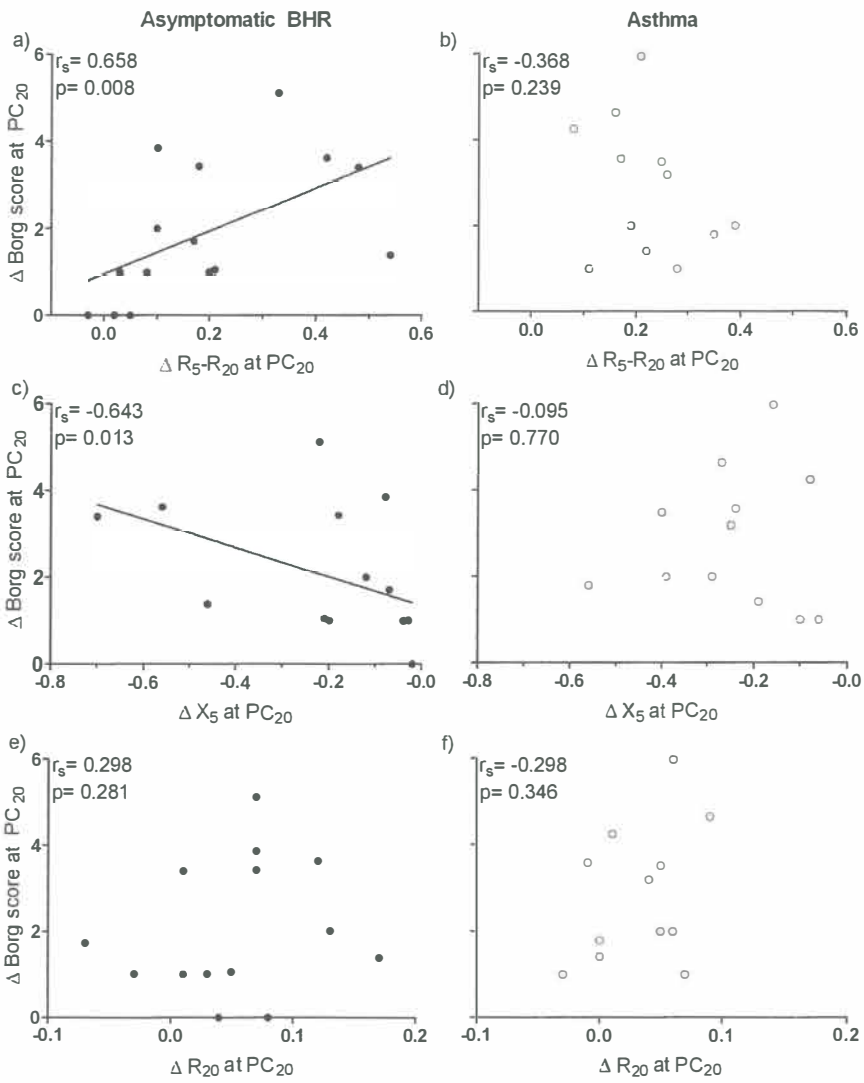
In asymptomatic BHR, the increase in Borg dyspnea score from baseline to PC_{20} (ΔBorg score) was significantly associated with the concomitant increase in small airway dysfunction as reflected by both the increase in R_5-R_{20} (ΔR_5-R_{20}) and the decrease in X_5 (ΔX_5) (Figure 2A-D). This was not the case in asthmatics. In contrast, an increase in large airway function, i.e. a change in R_{20} (ΔR_{20}), was not correlated with the ΔBorg dyspnea score neither in patients with asthma nor in individuals with asymptomatic BHR (Figure 2E-F).

Figure 1: IOS measurements and Borg dyspnea scores at PC_{20}



a) R_5-R_{20}, b) X_5, c) R_{20} and d) Borg dyspnea score for subjects with asymptomatic BHR (•) and asthma patients (o) at PC_{20}. The lines represent medians. NS = not significant; PC_{20} = provoking concentration causing a 20% fall in FEV_1; R_5 = resistance at 5 Hz, R_{20} = resistance at 20 Hz, X_5 = reactance at 5 Hz.

Figure 2: Correlation between the change in Borg dyspnea score (ΔBorg score) and change in IOS measurements at PC_{20} in asymptomatic BHR and asthma



a) ΔR_5-R_{20} asymptomatic in BHR, b) ΔR_5-R_{20} in asthma, c) ΔX_5 in asymptomatic BHR, d) ΔX_5 in asthma, e) ΔR_{20} in asymptomatic BHR, f) ΔR_{20} in asthma. r_s = Spearman's correlation coefficient; PC_{20} = provoking concentration causing a 20% fall in FEV_1; R_5 = resistance at 5 Hz, R_{20} = resistance at 20 Hz, X_5 = reactance at 5 Hz.

DISCUSSION

The present study shows that subjects with asymptomatic BHR have a similar level of small airway function at baseline compared to healthy controls, whereas asthma patients show small airway dysfunction. During methacholine provocation, small airway dysfunction increases more in subjects with asymptomatic BHR and asthma than in healthy controls. At a 20% fall in FEV_1, subjects with asymptomatic BHR have less small airway dysfunction than asthma patients. The increase in large airway dysfunction, as reflected by the change in R_{20}, is not associated with the increase in dyspnea during methacholine provocation either in subjects with asymptomatic BHR or patients with asthma. In contrast and of importance, a higher increase in small airway dysfunction during the provocation test associates with more worsening of dyspnea in subjects with asymptomatic BHR, whereas this is not the case in asthma patients.

We show that the level of small airway resistance at baseline is similar in subjects with asymptomatic BHR and in controls. This is in line with two other studies,[23,24] in which no baseline differences in small airway resistance and small airway inflammation between asymptomatic subjects with BHR and allergic rhinitis and controls were found. In contrast, we found an increase in small airway resistance in asthma patients at baseline. In line with this, Tufvesson *et al.* reported that the baseline level of exhaled alveolar nitric oxide, an indicator of small airway inflammation, was significantly higher in asthma patients than in subjects with asymptomatic BHR or controls. We extended the findings of previous studies by additionally analyzing changes in small airway function during methacholine provocation. Interestingly, we found that small airway dysfunction, as reflected by the slopes of R_5-R_{20} and X_5, increased more in subjects with asymptomatic BHR and asthma than in healthy controls. These results are in line with those of Aronsson *et al.*, showing that small airway resistance (i.e. the slope of R_5-R_{20}) during methacholine provocation increased the most in subjects with allergic rhinitis and asthma, followed by asymptomatic subjects with allergic rhinitis and BHR and finally subjects with allergic rhinitis without BHR.[23] Thus, a methacholine provocation test induces small airway dysfunction not only in patients with asthma, but also in subjects with asymptomatic BHR.

Besides the change of small airway parameters during methacholine provocation, we also investigated the level of small airway dysfunction at a 20% fall in FEV_1, i.e. at PC_{20}. At PC_{20}, we observed a higher degree of small airway dysfunction, i.e. a higher R_5-R_{20} and lower X_5, in patients with asthma than in subjects with asymptomatic BHR. Of interest, large airway resistance (R_{20}) increased only slightly during provocation and did not differ between asthma patients and subjects with asymptomatic BHR at PC_{20}. Next, we investigated the perception of dyspnea during methacholine provocation. As could be expected, Borg dyspnea scores were significantly lower in subjects with asymptomatic BHR than in asthma patients at PC_{20}.[9,25–27] Nevertheless, some subjects with asymptomatic BHR did experience an important increase in their Borg dyspnea score at the time their FEV_1 had dropped by

≥20%. This was a remarkable finding because these subjects were considered to be asymptomatic in their daily lives. However, it is in line with previous studies that also found subjects with asymptomatic BHR to report symptoms during a provocation test, albeit to a smaller extent than patients with asthma.[25,26] A possible explanation for this observation may be that subjects participating in a study like ours are more attentive than usual to report dyspnea during a provocation test. Alternatively, it could be argued that these subjects may not encounter stimuli in their daily lives that cause bronchoconstriction as severe as occurs during a methacholine provocation. In this study they were explicitly asked if they experienced dyspnea, and possibly they would not have considered this sensation as dyspnea in their daily lives. Another possible explanation could be that subjects with asymptomatic BHR are usually not or only minimally exposed to stimuli and therefore do not experience dyspnea in their daily lives. Interestingly, the increase in dyspnea during provocation (ΔBorg score at PC_{20}) in subject with asymptomatic BHR was significantly associated with the increase in small airway dysfunction (ΔR_5-R_{20} and ΔX_5, Figure 2A), supporting the hypothesis that the dyspnea sensation is influenced by the small airways. In contrast to our expectations, we did not find an association between the ΔBorg score and the increase in small airway dysfunction in asthma patients. It is possible that no association was found in the asthma patients due to the limited size of this group in our study, as an association between dyspnea and small airway dysfunction during provocation in asthma patients has been found previously.[16]

A strength of our study was the comparison between subjects with asymptomatic BHR and both controls and asthma patients. By comparing these three groups on several parameters, we not only obtained a broad overview of subjects with asymptomatic BHR alone, but were also able to put these results in perspective with respect to the two other groups. A limitation of the study is the relatively small size of the study population which may have limited some variables to show a significant difference between the groups. Interestingly, subjects with asymptomatic BHR were frequently female, although female prevalence did not significantly differ between the three groups. This is in line with several studies showing that BHR is more common and more severe in women than in men and this may also be the case for asymptomatic BHR.[28,29] Furthermore, asthma patients had a higher BMI than healthy controls and subjects with asymptomatic BHR. It has been reported that obese asthmatics have higher airway resistance and lower airway reactance than non-obese asthma patients.[30] To investigate whether the higher BMI in asthma patients may have influenced our results we analyzed the small airway resistance in obese and non-obese asthma patients separately. We found that both in obese and non-obese asthma patients, small airway dysfunction was higher than in subjects with asymptomatic BHR. Based on this outcome, we think it unlikely that the higher BMI in asthma patients will have influenced our results. Finally, we provoked the airways with relatively large-particle methacholine, which will deposit mostly in the large airways. It would be interestingly, to provoke also the small airways with small-particle methacholine or other stimuli like AMP, as has been done previously in the study of Cohen *et al*.[31]

In conclusion, our study shows that baseline small airway function of subjects with asymptomatic BHR is comparable to that of healthy controls, whereas asthma patients show small airway dysfunction. However, during provocation the small airways of subjects with asymptomatic BHR and those of asthma patients respond similarly. This results in significantly more small airway dysfunction at a 20% fall in FEV_1 in asthma patients than in subjects with asymptomatic BHR. We speculate that subjects with asymptomatic BHR experience less symptoms in their daily lives because they have less small airway dysfunction.

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CHAPTER 8

Airway Wall Thickness on HRCT Scans Decreases with Age and Increases with Smoking

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ABSTRACT

Purpose: To investigate if age, gender and smoking are associated with airway wall thickness (AWT) measured by high resolution computed tomography (HRCT) and if higher AWT is associated with lower levels of pulmonary function in healthy current- and never-smokers with a wide age range.

Material and Methods: The study was approved by the local medical ethics committee (METc 2007/007) and all subject signed informed consent. HRCT scans were performed in 99 subjects (48 never- and 51 current-smokers, median age 39 years [IQR 22–54], 57% males). The AWT at an internal perimeter of 10 mm (AWT Pi10) was calculated as an overall measurement of AWT, based on all measurements throughout the lungs. Extensive pulmonary function testing was performed in all subjects.

Results: Higher age was associated with a lower AWT Pi10 (b=-0.003, p<0.001). Current-smokers had a higher AWT Pi10 than never-smokers (mean 0.49 mm *vs* 0.44 mm, p=0.022). In multivariate analysis, age and current-smoking were independently associated with AWT Pi10 (age b=-0.002, p<0.001, current-smoking b=0.041, p=0.021), whereas gender was not (b=0.011, p=0.552). Higher AWT Pi10 was associated with a lower FEV_1, FEV_1/FVC, FEF_{25-75} and higher R5, R20 and X5.

Conclusion: AWT decreases with higher age, possibly reflecting structural changes of the airways. Additionally, current-smokers have a higher AWT, possibly due to remodeling or inflammation. Finally, higher AWT is associated with a lower level of pulmonary function, even in this population of healthy subjects.

INTRODUCTION

Chronic inflammatory processes in the airways, like those occurring in asthma and chronic obstructive pulmonary disease (COPD), induce airway remodeling. This is associated with changes in the epithelium, reticular basement membrane and smooth muscle, leading to thickening of the airway walls.[1,2]

A non-invasive method to assess airway wall thickness (AWT) is computed tomography (CT). Using this technique, it has been shown that the airways walls of patients with COPD are significantly thicker than those of healthy non-smoking controls.[3] In addition, it has been shown that higher AWT is associated with more severe airflow obstruction in COPD.[3–10] Although these studies have provided important new insights, they also had some limitations. AWT was measured manually in some studies,[5,9] which is less accurate than automated measurements and more susceptible to interobserver variation.[11] In addition, many studies used transverse CT slices and were therefore only able to measure airways oriented perpendicular to this plane, which markedly limits the number of airways that can be measured.[4–6,8,9] Some studies have even measured the AWT in only one airway, for instance the apical bronchus of the right upper lobe.[6] Advances in multi-detector row CT scanners, development of multiplanar, three-dimensional segmentation of airways and automated measurements of airway walls now make it possible to measure AWT in multiple airways throughout the bronchial tree.

In addition, most previous studies included selective populations of older subjects and often only current- or ex-smokers. For this reason, there are limited data on the effect of age and smoking on the AWT. This may be important, since aging has been shown to affect remodeling and repair processes of lung parenchyma and similar processes are likely to occur in the airway walls as well.[12,13] Additionally, smoking is the main risk factor for the development of COPD. It causes airway inflammation and remodeling, which both influence AWT.[14–16]

This study investigates the effect of age and smoking on AWT in a group of well characterized healthy subjects with an automated software program, which measures many airways perpendicular to the airway direction throughout the lungs. The objectives of this study were to investigate if age, gender and smoking are associated with AWT measured by high resolution computed tomography (HRCT) and if higher AWT is associated with lower levels of pulmonary function in healthy current- and never-smokers with a wide age range.

METHODS AND MATERIALS

Study population

In this study, healthy never- and current-smokers were included. Subjects were included if they met the following criteria: normal spirometry, no bronchial hyperresponsiveness to methacholine and normal pulmonary health according to the physician. Spirometry was considered to be normal if the forced expiratory volume in 1 second (FEV_1) was ≥80 %predicted, the FEV_1/forced vital capacity (FVC) was greater than the lower limit of normal and reversibility to salbutamol was <10% of the predicted value. Never-smokers were defined as subjects who had not smoked during the last year, had never smoked for as long as 1 year, and had not smoked more than 0.5 packyears. The study was approved by the local medical ethics committee ((METc 2007/007) and all subject signed informed consent. The study was registered with clinicaltrials.gov (NCT00848406).

High Resolution CT scans

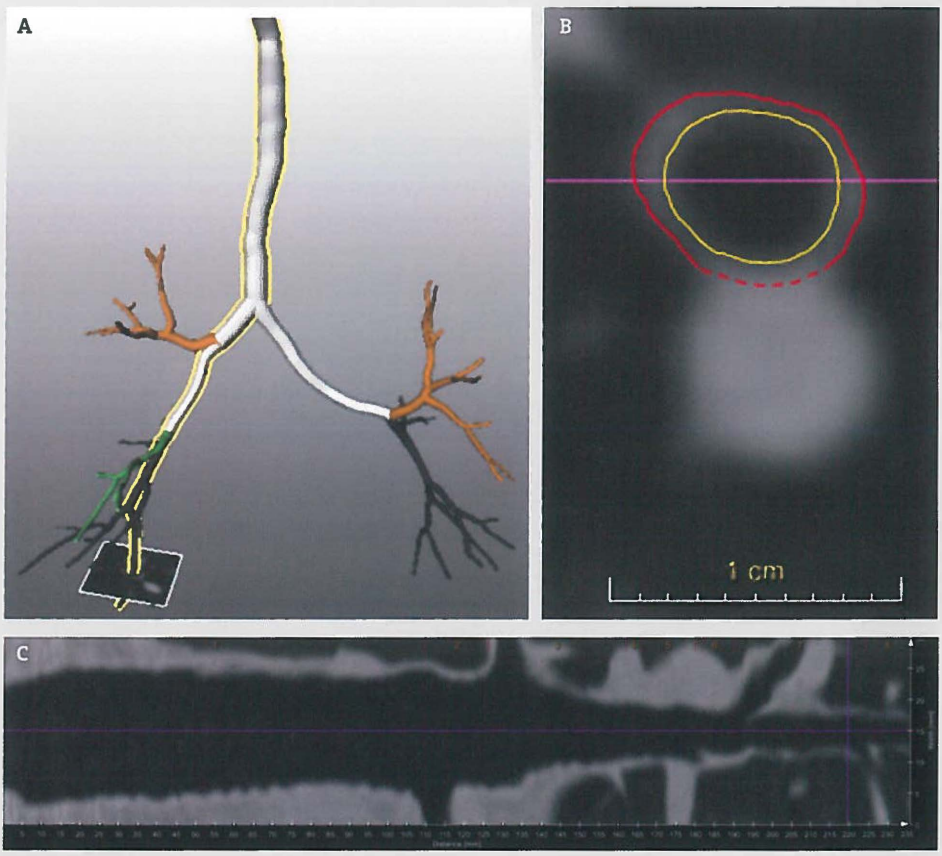
High resolution CT (HRCT) scans were performed using a 64-multidetector CT scanner (Somatom Definition, Siemens, Forchheim, Germany). Scans were performed at full inspiration. Scanning was performed with 20 mAs. The kV setting was determined by weight: 100 kV for subjects <60 kg, 120 kV for subjects ≥60 and <80 kg and 140 kV for subjects >80 kg. Acquired imaging data were reconstructed using a standard soft kernel (B30f), with 1.0 mm slice thickness and 0.7 mm increment. The CT radiation dose was approximately 0.8 mSv (100 kV) to 1.5 mSv (140 kV).

Airway measurements

Airway measurements were performed with the automated software program MeVis Airway Examiner 1.0 (Fraunhofer Institute for Medical Image Computing MEVIS, Bremen, Germany). This software is based on an algorithm presented by Weinheimer *et al.*[17] The software automatically extracts airway centerlines, re-samples images perpendicular to the airway direction at equally spaced locations along the centerline and detects inner and outer airway wall borders in these images **(figure 1)**. The outer wall border is detectable when no adjacent tissue with similar CT density is present. All HRCT scans were visually evaluated for appropriate segmentation and data from lobes that were incorrectly segmented were removed from further analyses. The software calculated AWT, airway wall area percentage (%AWA), i.e. airway wall area/total airway area *100, and the fraction of the airway that was detectable (assessed perimeter fraction, APF) for each location and reported the average AWT and %AWA per lung lobe for airways with predefined external diameters (3.5 mm, 4 mm, 4.5 mm, 5 mm, 6 mm, 8 mm and 10 mm) from all the separately measured airway locations. The software also reported the cumulative APF per lung lobe for each external diameter measured, i.e. the total number of airway perimeters measured, calculated by adding up the APF of separate airway locations. A higher cumulative APF reflects a higher

number of sampling points assessed. The mean AWT and %AWA per lung for each external diameter were calculated as the weighted average of the AWT and %AWA per lobe, with the cumulative APF per lobe as the weighting factor. As an overall measurement of AWT, the AWT at an internal perimeter of 10 mm (AWT Pi10) was estimated based on all measurements at the 7 different diameters.[18] The AWT Pi10 was calculated by plotting the square root of the average area of airway wall per lobe against the internal perimeter of these measurements. The resulting regression line was used to calculate the AWT Pi10.

Figure 1: Airway wall thickness analysis



A) The airway wall tree as extracted by the software program. The airway highlighted in yellow is stretched to a 2D picture in B). The grey square corresponds to the purple vertical line in B). This is a cross sectional image of the airway oriented perpendicular to the local centerline direction and given in more detail in C). The red line is the outer airway wall perimeter and the yellow line is the inner airway wall perimeter. Airway wall thickness (AWT) and wall area percentage (%AWA) are measured in areas of the continuous red line. Dashed parts of the outer perimeter are interpolated and not used for measurements. The assessed perimeter fraction (APF) is the fraction of the outer perimeter that has a continuous line.

Pulmonary function tests

Spirometry was performed before and after 400 µg salbutamol according to current ATS/ERS guidelines.[19,20] The following measurements were performed: FEV_1, reversibility of FEV_1, FVC and forced expiratory flow between 25 and 75% of FVC (FEF_{25-75}). Impulse oscillometry (IOS) was performed to measure resistance at 5 Hz (R5), resistance at 20 Hz (R20), the difference between R5 and R20 (R5-20) and reactance at 5 Hz (X5). Body plethysmography was performed to measure functional residual capacity (FRC), total lung capacity (TLC) and residual volume (RV). The diffusion capacity of the lung for carbon monoxide corrected for hemoglobin level (TLCOc) and TLCOc corrected for the alveolar volume (TLCOc/VA) were measured. Provocation tests were performed with methacholine (0.03 to 16 mg/ml), using a 2-minute tidal breathing method, adapted from Cockcroft *et al.*[21]

Statistical analysis

Differences in AWT Pi10, AWT and %AWA were tested with Student's t-test or analysis of variance (ANOVA), with post-hoc Holm's Bonferroni correction for multiple testing. To assess the effect of age on airway wall thickness, linear regressions with either AWT Pi10, AWT or %AWA as the outcome variable and age as the predictor variable were performed. To investigate whether age, gender and smoking status were independently associated with airway wall thickness, multivariate linear regression analyses with age, gender and smoking status as predictor variables were performed. To assess the effect of airway wall thickness on pulmonary function parameters, multivariate regression analyses with the pulmonary function parameter as outcome variable and AWT Pi10 as predictor variable with adjustment for age, gender and smoking status were performed. If height is part of the formula to calculate the predicted value of a pulmonary function parameter, height was also adjusted for. Similar analyses were performed with the AWT and %AWA transformed to the number of standard deviations (SD) from the mean at the different diameters. All statistical analyses were performed with IBM SPSS version 20 (IBM, Armonk, NY, USA). P-values <0.05 were considered statistically significant.

RESULTS

A total of 99 subjects were included. Their baseline characteristics are presented in **table 1**. More information about the measurements of AWT and %AWA at the different diameters can be found in the supplementary results.

Table 1: Baseline characteristics of the 99 subjects

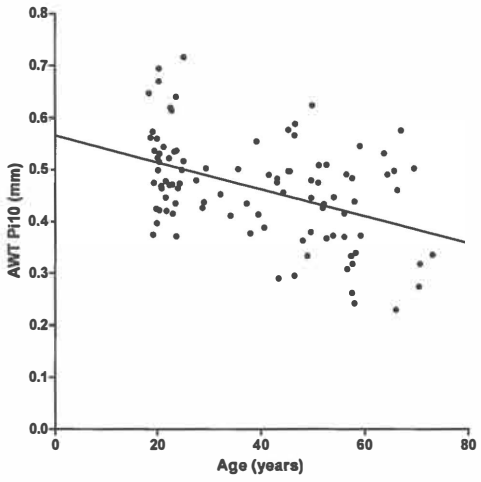
Age (years)	39 (22 – 54)
Males (n, %)	56 (57)
Current-smokers (n, %)	51 (52)
Cigarettes/day (n)*	15 (3 – 29)
Packyears (n)*	15 (10 – 20)
FEV_1 (%predicted)	105 (98 – 113)
FVC (%predicted)	113 (105 – 118)
FEV_1/FVC(%)	79 (76 – 84)
FEF_{25-75} (%predicted)	83 (71 – 99)
RV/TLC (%predicted)	86 (77 – 96)
TLCOc/VA (%predicted)	96 (88 – 104)
R5 (kPa/L/s)	0.30 (0.25 – 0.36)
R20 (kPa/L/s)	0.28 (0.22 – 0.33)
R5-20 (kPa/L/s)	0.02 (0.00 – 0.05)
X5 (kPa/L/s)	-0.07 (-0.10 – -0.06)

Values are presented as medians with interquartile ranges unless stated otherwise; * only in current-smokers; FEV_1 = forced expiratory volume in 1 second; FVC = forced vital capacity; FEF_{25-75} = forced expiratory flow between 25-75% of FVC; RV/TLC = residual volume / total lung capacity; TLCOc = diffusion capacity of the lung for carbon monoxide, corrected for hemoglobin level; VA = alveolar volume; R5 = resistance at 5 Hz; R20 = resistance at 20Hz; R5-20 = difference between R5 and R20; X5 = reactance at 5 Hz; AX = reactance area.

Effect of age, gender and smoking status on AWT Pi10, AWT and %AWA

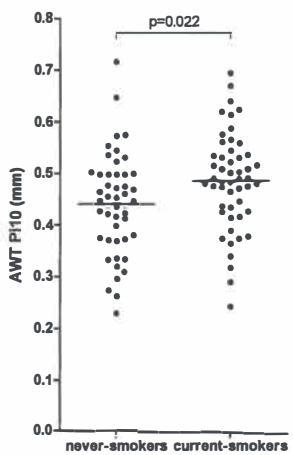
Higher age was associated with lower AWT, assessed by the overall measure of airway wall thickness, the AWT Pi10 (b=-0.003, p<0.001, **figure 2**). A trend was found towards a lower AWT Pi10 in men than in women (mean (SD) 0.45 (0.10) mm *vs* 0.48 (0.09) mm respectively, p=0.088). AWT Pi10 was significantly higher in current- than in never-smokers (mean (SD) 0.49 (0.09) mm and 0.44 (0.10) mm respectively, p=0.022, **figure 3**). AWT and %AWT were also higher in current- than never-smokers at 4 mm, 4.5 mm, 5 mm, 6 mm and 8 mm diameter, but not at 3.5 mm and 10 mm (supplementary tables 4 and 5). In multivariate linear regression analysis, both age and smoking status were significantly and independently associated with the AWT Pi10. AWT Pi10 was lower at higher age (b=-0.002, p<0.001) and higher in current-smokers (b=0.041, p=0.021). Gender was not independently associated with AWT Pi10 (b=0.011, p=0.552). No significant interaction between age and smoking status was found (p=0.45). In similar analyses, age was negatively associated with AWT and %AWA for all pre-defined diameters, except for 10 mm and current-smoking was associated with a higher AWT and %AWA at 4.5 mm, 5 mm and 6 mm diameter (supplementary tables 6 and 7).

Figure 2: Association between AWT Pi10 and age



b=-0.003, p<0.001; AWT Pi10 = airway wall thickness at an internal perimeter of 10 mm estimated from all measurements.

Figure 3: AWT Pi10 in never- and current-smokers



AWT Pi10 = airway wall thickness at an internal perimeter of 10 mm estimated from all measurements.

Association between airway wall thickness and pulmonary function

A higher AWT Pi10 was associated with lower FEV_1, FEF_{25-75}, FVC, FEV_1/FVC and X5 and higher R5 and R20, independently from age, gender, height and smoking status **(table 2)**. AWT Pi10 was not associated with RV/TLC or R5-20. Additionally, a higher AWT Pi10 was associated with higher TLCOc/VA. Similar associations were seen for AWT and %AWA at the different airway diameters (supplementary tables 8 and 9). The regression coefficients, representing the change in a pulmonary function test for every standard deviation change in AWT or %AWA, were very similar for the different diameters.

Table 2: Association between AWT Pi10 and pulmonary function parameters

	AWT Pi10	
	b	p-value
FEV_1 (L)	-1.697	0.001
FEF_{25-75} (L/s)	-3.215	0.001
FVC (L)	-1.288	0.047
FEV_1/FVC (%)	-13.358	0.018
RV/TLC (%)	3.202	0.468
TLCOc/VA (mmol/min/kPa/L)	0.431	0.029
R5 (kPa/L/s)	0.363	<0.001
R20 (kPa/L/s)	0.288	<0.001
R5-20 (kPa/L/s)	0.075	0.164
X5 (kPa/L/s)	-0.080	0.028

Linear regressions with pulmonary function parameters as outcome and airway wall thickness as predictor variable and age, gender, smoking status and height added as covariates; AWT Pi10 = airway wall thickness at an internal perimeter of 10 mm estimated from all measurements; FEV_1 = forced expiratory volume in 1 second; FVC = forced vital capacity; FEF_{25-75} = forced expiratory flow between 25-75% of FVC; RV/TLC = residual volume / total lung capacity; TLCOc = diffusion capacity of the lung for carbon monoxide, corrected for hemoglobin level; VA = alveolar volume; R5 = resistance at 5 Hz; R20 = resistance at 20Hz; R5-20 = difference between R5 and R20; X5 = reactance at 5 Hz.

DISCUSSION

The present study demonstrates that airway wall thickness (AWT) is lower in older subjects and higher in current- than in never-smokers, and that thicker airway walls are associated with lower levels of pulmonary function.

The effect of age on AWT has been previously described by Grydeland *et al.* in COPD patients and healthy smokers >40 years.[18] In this study, the estimated AWT at an internal perimeter of 10 mm (AWT Pi10) decreased with 0.003 mm per year in the healthy smokers, which is very similar to the results of this study (0.002 mm per year in the multivariate analysis). These findings are partly in agreement with a study by Zach *et al.*[22] In this study, age did not contribute to AWT Pi10 in 92 healthy subjects >45 years. However, a higher age was

associated with a lower %AWA. In the current study, these data were extended by including young subjects and non-smokers. This way, it was shown that airway walls become thinner from early adulthood on throughout life and that this process is independent from smoking.

Aging has profound influences on the lung. The elastic recoil of the lung decreases significantly with increasing age, due to alveolar enlargement without alveolar wall destruction, a condition that is also called 'senile lung'.[12] It has been suggested that remodeling of the extracellular matrix (ECM) in the lung parenchyma, with degradation of elastin and collagens, is the main factor in this aging process.[12,13] However, there is very limited knowledge of the effects of aging on changes in the airway walls. Animal studies suggest that aging of Clara cells, the progenitor cells of the airway epithelium, may impair airway regeneration, which could lead to thinning of the airway walls.[23] It is also possible that, similar to the parenchyma, ECM proteins are degraded in the airway walls with aging. ECM proteins also degrade with age in many other structures in the human body, for instance the skin.[24] Interestingly, in the aging skin, an altered function of fibroblasts has been observed, resulting in a decreased production of collagen and increased production of collagen-degrading matrix metalloproteinases. It is tempting to speculate that comparable changes may also occur in aging airway fibroblasts. Alternatively, inflammatory cells and other residential cells, like epithelial and smooth muscle cells, may have similar effects.

This study demonstrates that current-smokers have a higher AWT than never-smokers, independently from age. Donohue *et al*. did not find higher thickness of airway walls in current-smokers than in never- or ex-smokers in a large cohort of ex-, current- and never-smokers.[25] However, they did show that airway walls were thicker with more cigarette exposure, i.e. more packyears smoked and that current-smoking was associated with a narrower airway lumen. Thicker airway walls in current-smokers may be due to the effects of continuous smoke exposure on the epithelium, causing the epithelium to produce pro-inflammatory cytokines that induce remodeling.[14–16] In line with this, a study in 25 smokers and 14 non-smokers showed more goblet cell metaplasia, more smooth muscle hypertrophy and more inflammation in the walls of airways in current-smokers.[26] Thus, smoking may affect AWT in several different ways. It is not possible to distinguish between the different causes of airway wall thickening on HRCT scans.

An increase in AWT may be of clinical relevance, since it is associated with lower levels of pulmonary function, independent of age, gender, height and smoking status. Correlations between pulmonary function and wall thickness have previously been demonstrated in studies with COPD patients and older healthy controls.[3,5–9] In this study, associations between AWT and pulmonary function are demonstrated in a population of subjects who were specifically selected to be asymptomatic and have normal spirometry. Interestingly, AWT was associated not only with large airway parameters like the FEV_1, FVC and FEV_1/FVC, but also with small airway parameters like the FEF_{25-75} and X_5. This is remarkable since the small airways have an internal diameter <2 mm and are therefore too small to be measured on HRCT scans at the moment. Additionally, an increase of 1 SD in AWT in airways with

smaller diameters (e.g. 4 or 4.5 mm) or larger diameters (e.g. 6 or 8 mm) had similar effects on pulmonary function tests measuring large and small airways (supplemental tables 8 and 9). These results suggest that thickening of airway walls in current-smokers occurs not only in the airways that were measured, but throughout the lungs. Advances in ultra-high resolution imaging, like cone beam CT imaging, may provide more information about the AWT in the small airways in the future.[27,28] Unexpectedly, thicker airway walls were associated with a higher TLCOc/VA. As it is unlikely that subjects with thicker airway walls truly have a higher diffusion capacity, we hypothesize that the increased diffusion capacity in subjects with thicker airway walls most likely results from underestimation of the alveolar volume due to ventilation inhomogeneity.[29]

There are several strengths to this study. First, a software program that automatically detects the bronchial tree and measures AWT perpendicular to the airway direction was used. Using this method, one is able to measure a median of 146 airway perimeters (combined from partial airway perimeters) per patient. These measurements were performed at different airway diameters, ranging from 3.5 to 10 mm external diameter, in all lung lobes. Second, all HRCT scans were performed in the same center, with the same acquisition device, according to the same scanning protocol and with the same post-processing protocol. Finally, both older and younger subjects were included, and the latter group has rarely been evaluated in CT-studies, enabling the study of the effect of age and cigarette smoking on airway wall thickness independent of each other. Finally, all subjects were extensively phenotyped with pulmonary function tests, smoking history and medical history. There are also limitations to this study. As AWT was measured at pre-defined external airway parameters, it is not possible to determine if increases in AWT are due to actual thickening of the wall or due to reduction in the luminal diameter.

In conclusion, airway wall thickness measured with HRCT scans is a feasible technique to investigate anatomical changes of the airways. It has been proven in a group of well characterized healthy subjects that airway wall thickness decreases with increasing age, possibly reflecting structural changes of the airways during aging. Additionally, current-smokers have thicker airway walls than never-smokers, and this increased airway wall thickness is associated with lower levels of pulmonary function.

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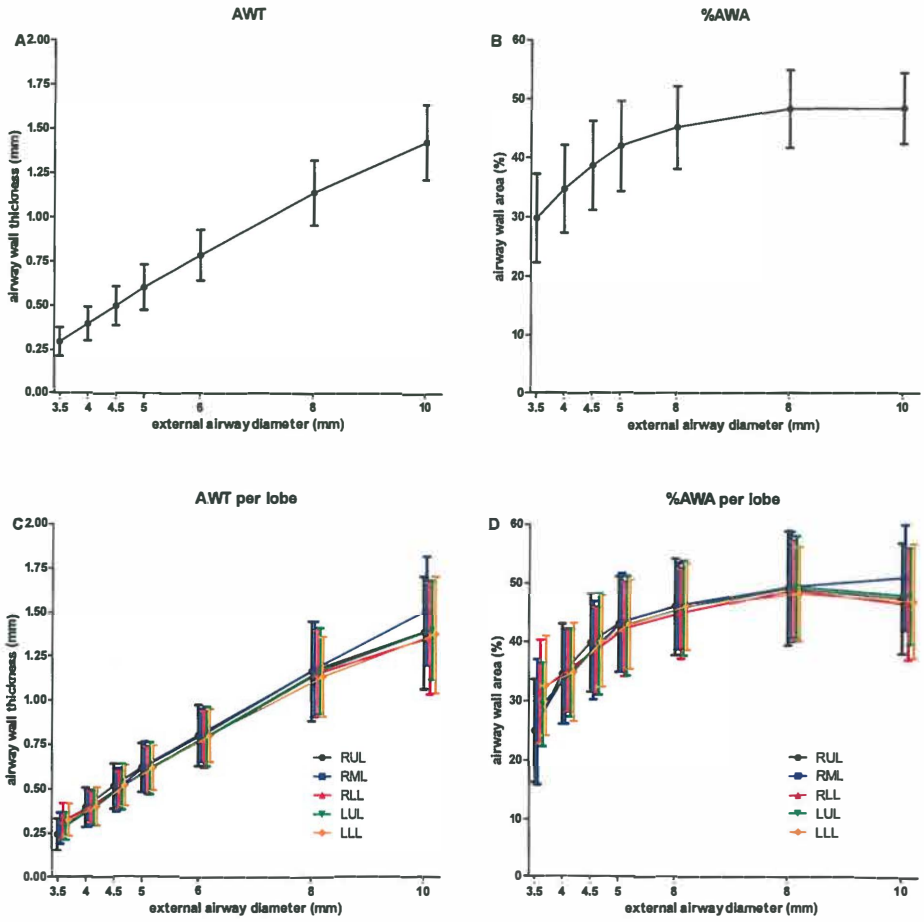
SUPPLEMENTARY RESULTS

The median cumulative assessed perimeter fractions (APF) per patient and per lung lobe are presented in online supplement table 1. The median cumulative APF per patient was 146 airways, reflecting that the AWT was measured on a total 146 airway perimeters. The cumulative APF was highest for airways with 5 mm external diameter. At this diameter the software measured a median of 55 airway perimeters. The software was able to measure wall thickness of all subjects between 4.5 and 6 mm. At smaller diameters there were less successful measurements (96 of 99 subjects at 4 mm and 58 of 99 subjects at 3.5 mm). Furthermore the number of airways measured became very limited with a cumulative APF of only 0.77 airways at 3.5 mm. Below 3.5 mm no airways were detected by the software. At higher diameters the APF also decreased to 1.31 airways at 10 mm. The lowest cumulative APF was observed in the right middle lobe, reflecting the smaller size of this lobe.

AWT and %AWA at different diameters and in different lobes

AWT increased linearly with increasing diameter from 0.30 mm at 3.5 mm external diameter to 1.42 mm at 10 mm (online supplementary figure 1a). The %AWA was 29.8% at 3.5 mm external diameter and increased to 45.1% at 6mm after this the %AWA reached a plateau around 48% (online supplementary figure 1b). The AWT and %AWA were comparable in all lobes for external diameters between 4 mm and 10 mm (online supplementary figures 1c and 1d and online supplement tables 2 and 3). At 3.5 mm the AWT and %AWA were significantly higher in the left lower lobe than in the right upper lobe.

Supplementary figure 1: AWT and %AWA at different external airway diameters



AWT = airway wall thickness; %AWA = airway wall area percentage.

Supplementary table 1: Cumulative APF at the different airway diameters per lung lobe

External perimeter	Total		RUL		RML		RLL		LUL		LLL	
	Median / IQR	n=	Median / IQR	n=	Median / IQR	n=	Median / IQR	n=	Median / IQR	n=	Median / IQR	n=
3.5 mm	0.8 (0.3 – 1.5)	58	0.7 (0.3 – 1.5)	20	0.5 (0.1 – 0.8)	15	0.4 (0.2 – 1.0)	33	0.6 (0.1 – 0.8)	18	0.3 (0.1 – 0.7)	27
4 mm	9.4 (4.3 – 17.4)	96	2.9 (1.0 – 5.3)	66	1.2 (0.7 – 2.5)	61	3.4 (1.5 – 6.5)	77	2.3 (1.0 – 4.3)	79	1.8 (1.0 – 3.7)	69
4.5 mm	41.0 (25.8 – 65.7)	99	9.1 (4.9 – 14.0)	95	4.0 (2.1 – 6.1)	84	13.1 (7.3 – 20.6)	91	10.4 (4.9 – 16.2)	91	9.4 (3.8 – 14.3)	88
5 mm	55.3 (39.9 – 74.1)	99	11.2 7.1 – 17.1)	97	4.2 (2.3 – 7.5)	95	15.7 (11.5 – 22.2)	92	14.4 (7.4 – 19.7)	93	13.2 (7.1 – 19.0)	88
6 mm	32.6 (22.8 – 46.8)	99	5.9 (3.2 – 9.8)	96	3.2 (1.8 – 4.9)	93	9.6 (5.6 – 15.0)	92	6.7 (4.1 – 9.7)	90	7.3 (4.0 – 12.6)	87
8 mm	5.3 (3.0 – 9.8)	98	0.9 (0.3 – 1.7)	69	0.6 (0.2 – 1.2)	75	1.9 (0.7 – 3.8)	81	1.0 (0.6 – 1.7)	80	1.7 (1.0 – 3.3)	85
10 mm	1.3 (0.6 – 2.7)	94	0.3 (0.2 – 0.6)	45	0.3 (0.1 – 0.5)	55	0.6 (0.2 – 1.3)	62	0.6 (0.2 – 1.5)	82	0.3 (0.2 – 0.7)	54
Total	146.1 (110.5 – 207.6)	99	33.1 (22.4 – 44.1)	97	13.3 (8.0 – 19.8)	99	44.8 (31.3 – 63.4)	96	33.3 (21.2 – 51.2)	98	30.8 (17.0 – 46.1)	99

APF = assessed perimeter fraction; RUL = right upper lobe; RML = right middle lobe; RLL = right lower lobe; LUL = left upper lobe; LLL = left lower lobe; IQR = interquartile range.

Supplementary table 2: Airway wall thickness per lobe

	Total			RUL			RML			RLL			LUL			LLL			p-value
	mean	SD	n=	mean	SD	n=	mean	SD	n=	mean	SD	n=	mean	SD	n=	mean	SD	n=	
AWT @3.5 mm	0.30	0.08	58	0.24	0.09	20	0.28	0.09	15	0.32	0.10	33	0.29	0.08	18	0.32*	0.09	27	0.026
AWT @4 mm	0.40	0.10	96	0.40	0.11	66	0.39	0.10	61	0.40	0.09	77	0.40	0.09	79	0.40	0.11	69	0.965
AWT @4.5 mm	0.50	0.11	99	0.52	0.13	95	0.49	0.12	84	0.49	0.11	91	0.51	0.13	91	0.52	0.11	88	0.433
AWT @5 mm	0.60	0.13	99	0.62	0.14	97	0.62	0.14	95	0.60	0.13	92	0.62	0.15	93	0.62	0.13	88	0.888
AWT @6 mm	0.78	0.14	99	0.80	0.17	96	0.80	0.15	93	0.78	0.16	92	0.79	0.17	90	0.80	0.15	97	0.852
AWT @8 mm	1.13	0.18	98	1.16	0.28	69	1.17	0.27	75	1.15	0.24	81	1.16	0.24	80	1.13	0.22	85	0.838
AWT @10 mm	1.42	0.21	94	1.38	0.32	45	1.50	0.31	55	1.35	0.32	62	1.40	0.28	54	1.37	0.33	82	0.070

* significantly different from RUL; RUL = right upper lobe; RML = right middle lobe; RLL = right lower lobe; LUL = left upper lobe; LLL = left lower lobe; SD = standard deviation.

Supplementary table 3: Airway wall area per lobe

	Total			RUL			RML			RLL			LUL			LLL			p-value
	mean	SD	n=	mean	SD	n=	mean	SD	n=	mean	SD	n=	mean	SD	n=	mean	SD	n=	
%AWA @3.5 mm	29.8	7.5	58	24.9	8.7	20	26.4	10.6	15	31.5	8.7	33	29.3	7.0	18	32.5*	8.4	27	0.017
%AWA @4 mm	34.7	7.4	96	34.6	8.5	66	34.1	8.0	61	35.1	7.0	77	34.7	7.4	79	34.8	8.3	69	0.961
%AWA @4.5 mm	38.7	7.5	99	39.8	8.3	95	38.5	8.3	84	38.5	7.2	91	39.6	8.6	91	40.0	7.7	88	0.565
%AWA @5 mm	42.0	7.7	99	43.0	8.2	97	43.2	8.4	95	42.1	8.1	92	42.7	8.5	93	42.9	7.6	88	0.912
%AWA @6 mm	45.1	7.0	99	45.9	8.3	96	46.0	7.4	93	44.7	7.7	92	45.7	8.1	90	45.9	7.4	97	0.806
%AWA @8 mm	48.3	6.6	98	49.1	9.7	69	49.3	9.3	75	48.7	8.5	81	49.3	8.6	80	48.1	8.1	85	0.883
%AWA @10 mm	48.4	6.0	94	47.3	9.4	45	50.8	9.0	55	46.2	9.5	62	47.7	8.2	54	46.7	9.7	82	0.070

p-value for ANOVA between the different lobes, followed by post-hoc testing with Holm's Bonferroni correction; * significantly different from RUL; RUL = right upper lobe; RML = right middle lobe; RLL = right lower lobe; LUL = left upper lobe; LLL = left lower lobe; %AWA = airway wall area percentage; SD = standard deviation.

Supplementary table 4: Airway wall thickness according to smoking status

	Never-smokers			Current-smokers			p-value
	mean	SD	n=	mean	SD	n=	
AWT @3.5 mm	0.28	0.09	33	0.31	0.07	25	0.219
AWT @4 mm	0.38	0.10	46	0.42	0.09	50	0.048
AWT @4.5 mm	0.47	0.11	48	0.52	0.10	51	0.032
AWT @5 mm	0.57	0.13	48	0.63	0.12	51	0.029
AWT @6 mm	0.75	0.14	48	0.82	0.13	51	0.013
AWT @8 mm	1.01	0.19	48	1.17	0.17	50	0.053
AWT @10 mm	1.39	0.23	47	1.44	0.20	47	0.250

AWT = airway wall thickness; SD = standard deviation.

Supplementary table 5: Airway wall area according to smoking status

	Never-smokers			Current-Smokers			p-value
	mean	SD	n=	mean	SD	n=	
AWT @3.5 mm	28.7	8.2	33	31.1	6.3	25	0.230
AWT @4 mm	33.2	7.8	46	36.1	6.8	50	0.055
AWT @4.5 mm	37.0	7.8	48	40.2	7.0	51	0.030
AWT @5 mm	40.2	8.0	48	43.6	7.0	51	0.023
AWT @6 mm	43.3	7.2	48	46.8	6.5	51	0.013
AWT @8 mm	46.9	7.0	48	49.6	5.9	50	0.041
AWT @10 mm	47.6	6.5	47	49.1	5.4	47	0.243

%AWA = airway wall area percentage; SD = standard deviation.

Supplementary table 6: Associations between airway wall thickness and age, gender and smoking status

	Age		Gender		Smoking Status	
	b	p-value	b	p-value	b	p-value
AWT @3.5 mm	-0.002	<0.001	0.013	0.497	0.029	0.129
AWT @4 mm	-0.003	<0.001	0.008	0.639	0.032	0.056
AWT @4.5 mm	-0.003	<0.001	0.004	0.833	0.043	0.028
AWT @5 mm	-0.003	<0.001	0.003	0.891	0.051	0.027
AWT @6 mm	-0.003	<0.001	0.027	0.319	0.065	0.013
AWT @8 mm	-0.003	0.005	0.017	0.652	0.067	0.059
AWT @10 mm	-0.001	0.615	-0.031	0.498	0.050	0.258

AWT = airway wall thickness; b = unstandardized regression coefficient.

Supplementary table 7: Associations between airway wall area and age, gender and smoking status

	Age		Gender		Smoking Status	
	b	p-value	b	p-value	b	p-value
%AWA @3.5 mm	-0.213	<0.001	1.482	0.419	2.623	0.137
%AWA @4 mm	-0.215	<0.001	0.686	0.615	2.422	0.066
%AWA @4.5 mm	-0.214	<0.001	0.408	0.764	2.924	0.026
%AWA @5 mm	-0.207	<0.001	0.306	0.828	3.158	0.021
%AWA @6 mm	-0.156	<0.001	1.412	0.286	3.219	0.012
%AWA @8 mm	-0.113	0.005	0.579	0.661	2.558	0.045
%AWA @10 mm	-0.023	0.556	-0.887	0.498	1.434	0.251

%AWA = airway wall area percentage; b = unstandardized regression coefficient.

Supplementary table 8: Association between pulmonary function parameters and airway wall thickness, independent of age, gender, smoking status and height

	FEV_1		FEF_{25-75}		FVC		FEV_1/FVC		RV/TLC	
	b	p-value	b	p-value	b	p-value	b	p-value	b	p-value
AWT @3.5 mm	-0.131	0.095	-0.219	0.100	-0.126	0.187	-0.796	0.271	0.522	0.403
AWT @4 mm	-0.129	0.016	-0.274	0.004	-0.078	0.240	-1.293	0.024	0.475	0.296
AWT @4.5 mm	-0.145	0.005	-0.292	0.002	-0.093	0.149	-1.408	0.011	0.242	0.585
AWT @5 mm	-0.141	0.005	-0.284	0.002	-0.087	0.168	-1.357	0.013	0.217	0.616
AWT @6 mm	-0.142	0.005	-0.284	0.002	-0.098	0.120	-1.357	0.040	0.180	0.674
AWT @8 mm	-0.120	0.013	-0.149	0.089	-0.105	0.077	-1.130	0.332	0.054	0.895
AWT @10 mm	-0.038	0.410	-0.043	0.605	-0.038	0.498	-0.511	0.873	-0.106	0.785

	TLCOc/VA		R5		R20		R5-20		X5	
	b	p-value	b	p-value	b	p-value	b	p-value	b	p-value
AWT @3.5 mm	0.026	0.284	0.017	0.150	0.012	0.177	0.005	0.433	-0.002	0.665
AWT @4 mm	0.045	0.022	0.034	0.001	0.024	0.004	0.010	0.066	-0.010	0.009
AWT @4.5 mm	0.039	0.046	0.033	0.001	0.026	0.001	0.007	0.207	-0.007	0.047
AWT @5 mm	0.041	0.031	0.036	<0.001	0.027	0.001	0.009	0.106	-0.008	0.020
AWT @6 mm	0.036	0.064	0.033	0.001	0.026	0.001	0.007	0.170	-0.009	0.007
AWT @8 mm	0.060	0.001	0.029	0.002	0.018	0.018	0.012	0.018	-0.009	0.005
AWT @10 mm	0.034	0.048	0.020	0.027	0.014	0.049	0.006	0.232	-0.005	0.177

Linear regressions with pulmonary function parameters as outcome and normalized airway wall thickness at different external diameters as predictor variable and age, gender, smoking status and height added as covariates; AWT = airway wall thickness; FEV_1 = forced expiratory volume in 1 second; FVC = forced vital capacity; FEF_{25-75} = forced expiratory flow between 25-75% of FVC; RV/TLC = residual volume / total lung capacity; TLCOc = transfer capacity of the lung for carbon monoxide, corrected for hemoglobin level; VA = alveolar volume; R5 = resistance at 5 Hz; R20 = resistance at 20Hz; R5-20 = difference between R5 and R20; X5 = reactance at 5 Hz; AX = reactance area, b = regression coefficient, it represents the change per SD change in predictor variable.

Supplementary table 9: Association between pulmonary function parameters and airway wall area, independent of age, gender, smoking status and height

	FEV_1		FEF_{25-75}		FVC		FEV_1/FVC		RV/TLC	
	b	p-value	b	p-value	b	p-value	b	p-value	b	p-value
%AWA @3.5 mm	-0.134	0.086	-0.238	0.073	-0.122	0.203	-0.078	0.193	0.470	0.450
%AWA @4 mm	-0.140	0.009	-0.286	0.003	-0.092	0.164	-0.938	0.024	0.494	0.276
%AWA @4.5 mm	-0.151	0.004	-0.304	0.001	-0.099	0.126	-1.296	0.011	0.249	0.576
%AWA @5 mm	-0.149	0.003	-0.295	0.001	-0.097	0.127	-1.427	0.013	0.215	0.623
%AWA @6 mm	-0.149	0.003	-0.294	0.001	-0.106	0.095	-1.376	0.034	0.203	0.636
%AWA @8 mm	-0.116	0.017	-0.147	0.096	-0.102	0.089	-1.173	0.348	0.021	0.959
%AWA @10 mm	-0.037	0.248	-0.042	0.615	-0.035	0.534	-0.496	.0844	-0.108	0.782

	TLCOc/VA		R5		R20		R5-20		X5	
	b	p-value	b	p-value	b	p-value	b	p-value	b	p-value
%AWA @3.5 mm	0.025	0.312	0.020	0.088	0.013	0.125	0.006	0.309	-0.004	0.508
%AWA @4 mm	0.047	0.017	0.035	0.001	0.025	0.002	0.010	0.063	-0.010	0.007
%AWA @4.5 mm	0.040	0.043	0.033	0.001	0.026	0.001	0.007	0.188	-0.007	0.048
%AWA @5 mm	0.042	0.030	0.036	<0.001	0.027	0.001	0.009	0.103	-0.008	0.021
%AWA @6 mm	0.036	0.060	0.034	0.001	0.027	0.001	0.007	0.167	-0.009	0.008
%AWA @8 mm	0.060	0.001	0.029	0.002	0.017	0.021	0.011	0.022	-0.009	0.006
%AWA @10 mm	0.033	0.057	0.021	0.023	0.015	0.042	0.006	0.235	-0.005	0.152

Linear regressions with pulmonary function parameters as outcome and normalized airway wall thickness at different external diameters as predictor variable and age, gender, smoking status and height added as covariates; %AWA = airway wall area percentage; FEV_1 = forced expiratory volume in 1 second; FVC = forced vital capacity; FEF_{25-75} = forced expiratory flow between 25-75% of FVC; RV/TLC = residual volume / total lung capacity; TLCOc = transfer capacity of the lung for carbon monoxide, corrected for hemoglobin level; VA = alveolar volume; R5 = resistance at 5 Hz; R20= resistance at 20Hz; R5-20 = difference between R5 and R20; X5 = reactance at 5 Hz; AX = reactance area, b = regression coefficient, it represents the change per SD change in predictor variable.

CHAPTER 9

Untargeted Lipidomic Analysis in COPD: Uncovering Sphingolipids

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ABSTRACT

Rationale: Cigarette smoke is the major risk factor in the development of chronic obstructive pulmonary disease (COPD). Lipidomics is a novel and emerging research field that may provide new insights in the origins of chronic inflammatory diseases like COPD.

Objective: This study investigates whether expression of the lung sputum lipidome is affected in COPD or by cigarette smoke exposure.

Methods: Lipid expression was investigated with LC-MS(/MS) in induced sputum comparing smokers with COPD, smokers without COPD, and never-smokers. Changes in lipid expression after 2-month smoking cessation were investigated in smokers with and without COPD. Cellular lipid expression was assessed in BEAS-2B cell lines after 6-month exposure to cigarette smoke extract (CSE).

Measurements and Main Results: Over 1500 lipid compounds were monitored in lung sputum. 168 sphingolipids, 36 phosphatidylethanolamine lipids and 5 tobacco-related compounds were significantly up-regulated in sputum of smokers with COPD compared to smokers without COPD and 1 neuraminic acid containing glycosphingolipid was down-regulated. Only 20 (glyco)sphingolipids and 6 tobacco-related compounds were up-regulated in smokers without COPD compared to never-smokers. 2-month smoking cessation reduced expression of 28 lipids in smokers with and without COPD. 6-month CSE exposure in BEAS-2B up-regulated 16 lipids and down-regulated 13.

Conclusions: This study demonstrates that lipids from the sphingolipid pathway are up-regulated in smokers with COPD compared to smokers without COPD. The findings are strengthened by the observation that long-term CSE exposure in BEAS-2B cells changes the expression of the sphingolipid pathway. Smoking cessation subsequently reduced lipid expression.

INTRODUCTION

Lipidomics is an emerging "omics" science, which is based on the analysis of all lipids present in a biological system known as the lipidome. The cellular lipidome consists of species that are of key importance to various cellular processes, including energy storage, membrane integrity and cellular signaling processes like, cell proliferation, metabolism and apoptosis induction.[1–3] The complex composition and function of the lipidome in various diseases is currently poorly understood. The crucial role of lipids in cell, tissue and organ physiology has been demonstrated by a large number of genetic studies and by the existence of many human diseases that involve the disruption of lipid metabolic enzymes and pathways.[2] Examples of such diseases are cancer, diabetes, neurodegenerative diseases, infections and inflammation.[4,5] To date, little is known about the role and the impact of the lipidome/lipid signaling in Chronic Obstructive Pulmonary Disease (COPD), a disease that affects millions worldwide and is associated with a high disease burden and mortality rate.

It is plausible that the lipidome plays a role in COPD since extracellular signals from (inflammatory) cytokines, being abundantly present in the lungs of COPD patients, are able to influence the activity of key lipid-modifying enzymes, such as phospholipases, sphingosine kinases and sphingomyelinases.[5] Lipids from the sphingolipid pathway, like ceramides, are major players in the induction of apoptosis and cellular senescence,[6,7] and free fatty acids can activate TLR4, thereby inducing similar inflammatory responses as lipopolysaccharides do.[8] An imbalance of major lipid signaling caused by cigarette smoke might result in lipid accumulation and/or alterations that are also harmful to the cell by compromising cellular integrity of various cell/organelle membranes. This together may contribute to disease progression seen in COPD by sustaining chronic inflammation.[5,8]

To assess whether the lipidome plays a role in COPD and is affected by smoking, we investigated induced sputum. This allowed us to collect epithelial lining fluid from the lower airways, an interesting compartment since the epithelium is the first line of defense against noxious particles such as cigarette smoke.

We investigated the effect of COPD on lipid expression in chronic smokers with and without COPD. The effect of smoking on lipid expression was furthermore assessed in smokers without COPD and never smokers without COPD and in a group of smokers with and without COPD before and after 2-month smoking cessation. Additionally, we investigated *in vitro* in whole cell homogenates of BEAS-2B cell lines how 6-month exposure to 2.5% and 10% cigarette smoke extract (CSE) changes lipid expression and Sphingomyelinase activity.

METHODS AND MATERIALS

Human studies

Induced sputum supernatant samples from 3 studies were analyzed (study-I: clinicaltrials.gov NCT00848406, study-II: Lo Tam *et al.*[9], study-III: Willemse *et al.*[10]). We included 19 smokers with COPD, 20 smokers without COPD and 14 never-smokers. Additionally, induced sputum samples were collected at baseline and after 2-month smoking cessation from 7 smokers with and 10 smokers without COPD in study III. All studies were approved by the University Medical Center Groningen medical ethical committee and all subjects provided written informed consent.

All COPD patients were current-smokers and had severity stage-II or stage-III according to the Global Initiative for Chronic Obstructive Lung Disease (GOLD).[11] Subjects without COPD were asymptomatic, had no history of pulmonary diseases and had normal spirometry (post-bronchodilator forced expiratory volume in 1 second (FEV_1) ≥80% predicted, post-bronchodilator FEV_1/forced expiratory volume (FVC) ≥ lower limit of normal, reversibility to 400 µg salbutamol <10% of the predicted FEV_1). Never-smokers are subjects who had not smoked in the last year, had never smoked for ≥1 year and had a total cigarette exposure <0.5 packyears.

Sputum induction

Sputum inductions were performed following standard protocol, and sputum processing was performed according to standard operating procedures (see supplementary methods). The sputum samples were centrifuged at 450 x*g* for 10 minutes at 4 °C. Supernatants were collected for analysis.

In vitro study

To study the effects of long-term cigarette smoke exposure on lung epithelial cells, BEAS-2B cells were cultivated in 6-well plates and were exposed for six months to (a) CSE 2.5% (n=5), (b) CSE 10% (n=5) or (c) CSE 0% (control, n=5). BEAS-2B cells were grown in RPMI 1640 (Lonza, Verviers, Belgium) supplemented with 15% heat-inactivated Fetal Bovine Serum (FBS), penicillin (100 U/ml) (Lonza, Verviers, Belgium) and streptomycin (100 µg/ml) (Lonza, Verviers, Belgium) on collagen-coated plates. Cells were grown to >90 % confluence and passaged by trypsin. CSE was prepared from filter-less research-grade cigarettes (3R4F, Tobacco Research Institute, University of Kentucky, Lexington, KY).[12]

Lipidomics analysis

A detailed description of the lipidomics analysis, which is based on high-resolution liquid chromatography and high-resolution quadrupole time-of-flight mass spectrometry (LC-QTOF-MS), is available in the supplementary methods.[13–15] In brief, lipids were extracted from sputum using liquid/liquid extraction incorporating methyl-tert-butylether (MTBE).

Extracts were analyzed on a 6530 Q-TOF MS system (Agilent Technologies, Walbronn, Germany) combined with a 1200 RRLC system (Agilent Technologies). Acquired data were processed in an untargeted manner using the MassHunter Qualitative Analysis software (Agilent Technologies) and converted to a statistically accessible data matrix in MassProfiler Professional (Agilent Technologies). Data was normalized using total lipid abundance, correcting for sputum dilution. Lipid identification was based on accurate mass, MS/MS fragmentation and chromatographic retention time. All experiments were quality controlled by analyzing quality control samples through the different analytical sequences.

Lipid Nomenclature

The nomenclature of the International Lipid Classification and Nomenclature Committee (ILCNC) has been used, i.e. "Comprehensive Classification System for Lipids".[16–18] Regarding glycosphingolipids, LC-MS cannot discriminate between galactose and glucose, or N-acetylglucosamine and N-acetylgalactosamine. Therefore, lipid nomenclature of these species contains hexose (Hex) instead of glucose or galactose.

Statistical analyses

Mann-Whitney-U test was used for cross-sectional studies and Wilcoxon signed-rank test for the smoking cessation study. Multiple testing corrections (Benjamini Hochberg false discovery rate) were performed.[19] A corrected p-value <0.05 was considered significantly different.

RESULTS

In vivo studies

Subjects characteristics

Smokers with COPD were older, more often male and had smoked more packyears than the smokers without COPD **(table 1)**. There were no significant differences in baseline characteristics between the smokers without COPD and never-smokers. Twelve COPD patients had COPD GOLD stage-II and 7 patients GOLD stage-III. For the smoking cessation study we included 10 smokers without COPD and 7 smokers with COPD. Their baseline characteristics are shown in **table 2.**

Table 1: Baseline characteristics of the comparison study subjects

	Never-smokers n=14	Smokers without COPD n=20	Smokers with COPD n=19
Study (study I / II / III)	14 / 0 / 0	15 / 0 / 5	0 / 4 / 15
Age (years	54 (23-58)	42 (20-51)	59 (54-65)
Gender (m/f)	6 / 8	10 / 10	16/3
Cigarettes / day	0	17 (11-24)	20 (12-25)
packyears	0	13 (3-25)	37 (31-46)
ICS use (y/n)	0 / 14	0 / 20	4/ 15
GOLD stage (II / III)			12 / 7
FEV_1 post bd (%predicted)	108 (103 - 111)	110 (102 - 117)	70 (52 -79)
FEV_1/FVC post bd(%)	82 (76 - 85)	83 (80 - 88)	56 (47 - 70)

Data are presented as medians with interquartile range or numbers; COPD = chronic obstructive pulmonary disease; ICS = inhaled corticosteroids; FEV_1 = forced expiratory volume in 1 second; FVC = forced vital capacity; bd = bronchodilator.

Table 2: Baseline characteristics of the smoking cessation study subjects

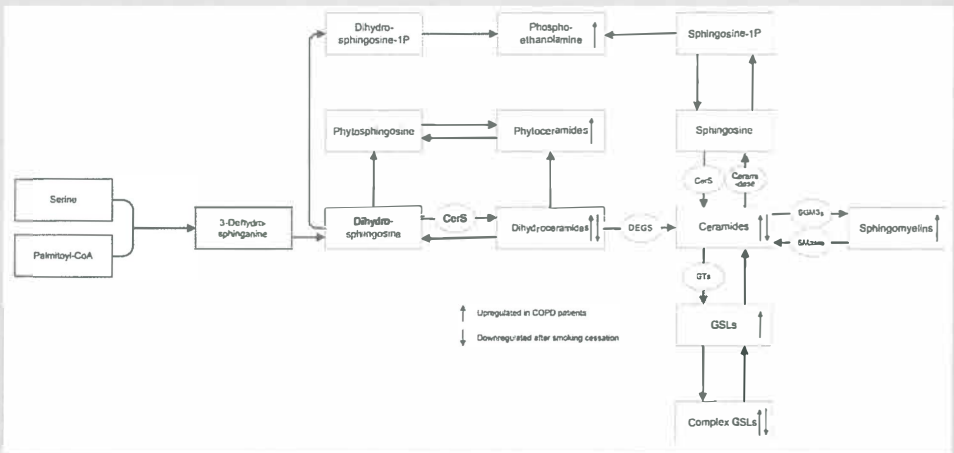
	Smokers without COPD n=10	Smokers with COPD n=7
Age (years)	50 (48 - 52)	63 (59 - 65)
Gender (m/f)	6 / 4	5 / 2
Cigarettes / day	25 (18 - 26)	20 (15 - 23)
packyears	22 (17 - 26)	40 (33 - 54)
FEV_1 post bd (%predicted)	112 (104 - 116)	51 (47 - 64)
FEV_1/FVC post bd (%)	83 (79 - 85)	50 (42 - 55)

COPD = chronic obstructive pulmonary disease; FEV_1 = forced expiratory volume in 1 second; FEV_1/FVC = FEV_1 / forced vital capacity; bd = bronchodilator.

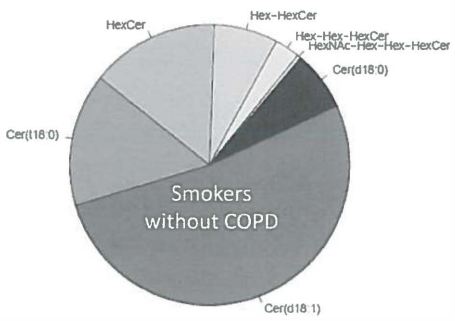
Differentially expressed lipids in smokers with and without COPD

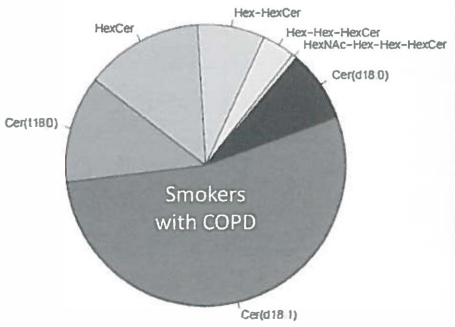
In general, a large portion of lipids from the sphingolipid pathway were up-regulated in smokers with COPD compared to smokers without COPD (supplementary table 1 and **figure 1**). 168 sphingolipids were significantly up-regulated (28 ceramides, 11 dihydroceramides, 19 phytoceramides, 36 sphingomyelins and 74 glycosphingolipids (GSLs)); whereas only 1 neuraminic acid containing glycosphingolipid was down-regulated. Although many individual lipid compounds showed a significant increase in COPD patients, the total lipid abundance distribution (supplementary figure 1) and overall sphingolipid distribution within a subject group did not differ drastically when we compared this distribution in smokers with COPD and without COPD **(figure 2)**. Remarkably, up-regulation of certain ceramide species (sphingosine ceramides, dihydrosphingosine ceramides and phytosphingosine ceramides) was dependent on (saturated) fatty acid chain length **(figure 3)**.

Figure 1: The sphingolipid pathway



CerS = ceramide synthases; DEGS = dihydroceramide desaturase; GTs = glycosyltransferases; SGMSs = sphingomyelin synthases; GSLs = glycosphingolipids.





Sphingolipid class	Smokers without COPD (%)	Smokers with COPD (%)
Cer(d18:0)	6.93 ± 3.09	8.29 ± 2.04
Cer(d18:1)	52.30 ± 21.77	53.71 ± 13.76
Cer(t18:0)	15.33 ± 5.51	12.32 ± 2.92
HexCer	14.80 ± 7.46	13.59 ± 5.05
Hex-HexCer	7.35 ± 6.23	7.78 ± 6.82
Hex-Hex-HexCer	2.89 ± 2.18	3.79 ± 1.84
HexNAc-Hex-Hex-HexCer	0.40 ± 0.40	0.53 ± 0.42

Figure 2: The distribution of sphingolipids in smokers with COPD and smokers without COPD. Peak areas of sphingolipid species (n = 215) were summed up per sphingolipid class for each individual sample. After normalization for sputum dilution, relative quantities for each sphingolipid class were calculated within each sample group (table denotes average percentage ± standard deviation). Sphingolipid abundance within the subject groups shows approximately equal distribution of all sphingolipid sub-classes. There is no significant shift in sphingolipid sub-classes between smokers with and without COPD. (Cer (d18:0) – dihydrosphingosine ceramides; Cer (d18:1) – sphingosine ceramides; Cer (t18:0) – phytosphingosine ceramides; Hex - glucose or galactose; HexNAc – N-acetylglucosamine or N-acetylgalactosamine)

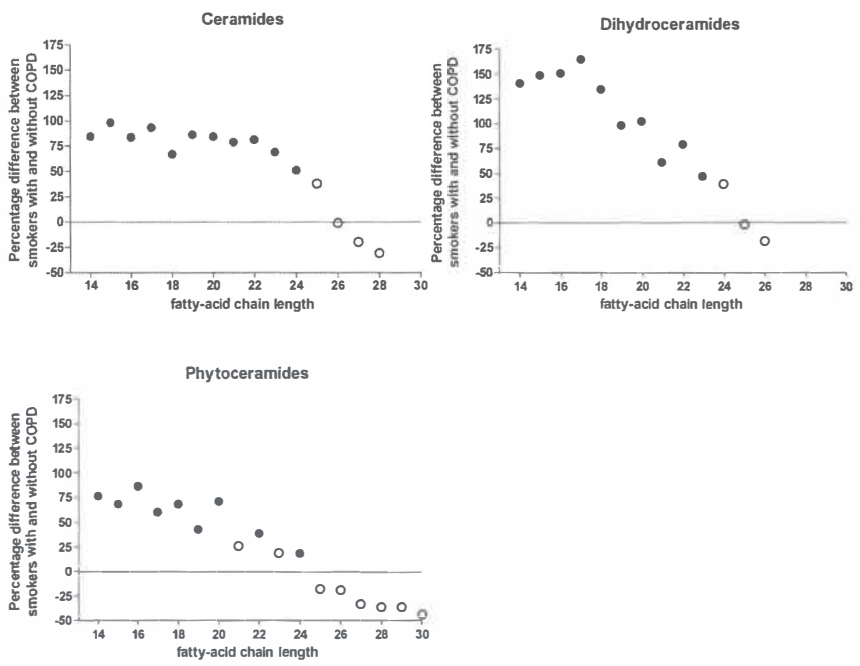


Figure 3: Up-regulation of different ceramide species (ceramides, dihydroceramides and phytoceramides) in smokers with COPD compared to smokers without COPD is dependent on the carbon number of the (saturated) fatty acid side chain of the ceramides (X-axis). A positive percentage means higher expression in smokers with COPD compared to smokers without COPD. A negative percentage means a lower expression in smokers with COPD compared to smokers without COPD (Y-axis). • = corrected p≤0.05; ○ = corrected p>0.05

The other major lipid class that was significantly up-regulated was the class of phosphatidylethanolamines or PEs, consisting of sub-classes acyl-phosphatidyl-ethanolamines (2) lyso-phosphatidylethanolamines (LysoPE,14) and plasmalogen-phosphat-idylethanolamines (pPE, 20) (supplementary table 1). The ratio LysoPE/LysoPC of species with identical fatty acid chain length and saturation shows a significant increase for several LysoPE compounds in COPD patients **(figure 4)**. Additionally, up-regulation of PEs was only found in compounds containing unsaturated fatty acids, the most being poly-unsaturated, i.e. showing 2 or more double bonds.

Differentially expressed lipids between smokers without COPD and never-smokers

A lower number of lipids were differentially expressed between smokers without COPD and never-smokers (n=28, supplementary table 2) than between smokers with and without COPD (n=210, supplementary table 1). Only 1 ceramide and 21 glycosphingolipids were significantly differentially regulated in smokers without COPD compared to never-smokers. Of interest, only two compounds were down-regulated and again, both belonging to the neuraminic acid

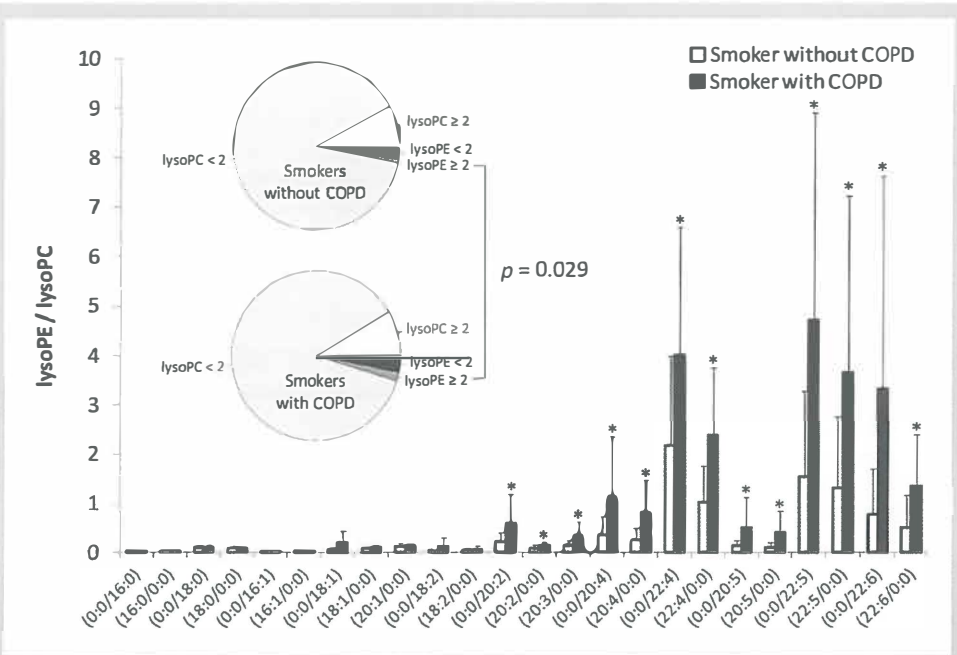


Figure 4: The lysoPE/lysoPC abundance ratio of species with identical fatty acid chain length and saturation in smokers with COPD (■) and smokers without COPD (□). LysoPE/lysoPC ratios from species containing fatty acid chains with 2 or more double bonds were significantly different ($p<0.05$). Pie charts indicate relative quantities of LysoPE and LysoPC, with less than 2 double bonds or 2 or more double bonds in smokers with COPD and smokers without COPD. LysoPE species with 2 or more double bonds were found up-regulated in smokers with COPD compared to smokers without COPD.

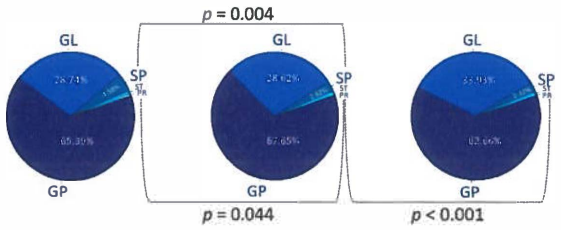
containing glycosphingolipid sub-class. This strongly suggests that neuraminic acid linked glycosphingolipid levels are down-regulated by cigarette smoke. The other 6 lipids that were differentially regulated are lipids that were almost exclusively found in smokers. These lipids are known tobacco components which include solanesol, its esters, solanesyl palmitate, solanesyl stearate, and its degradation products, bombiprenone, geranylfarnesylacetone, farnesylfarnesylacetone.[20] There was no significant difference found in the lysoPE/lysoPC ratios.

Lipid expression in different (positive and negative) electrospray ionization modes

In order to obtain a comprehensive lipidomics analysis, lipid extracts were analyzed both in positive (ESI+) and negative (ESI-) electrospray ionization, as some lipid species are only detected in one ionization mode (e.g. fatty acyls in ESI- and glycerolipids in ESI+).[13,14] An overview of the acquired ESI+ and ESI- lipid distribution in smokers with COPD, smokers without COPD and never-smokers is shown in **figure 5**. Sphingolipids are highly overexpressed in smokers with COPD compared to smokers without COPD, both in ESI+ as well as in ESI-

Sphingolipid overexpression in COPD (+ ESI)

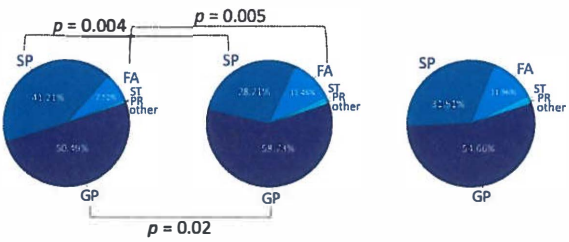
(A)



Lipid class	Smoker withCOPD	Smoker without COPD	Never-smoker
Glycerophospholipids	65.39 ± 13.25	67.65 ± 18.29	62.66 ± 22.58
Glycerolipids	28.74 ± 14.11	28.62 ± 18.38	33.93 ± 23.15
Sphingolipids	4.58 ± 2.34	2.62 ± 1.90	2.42 ± 1.01
Sterol lipids	0.85 ± 0.38	1.02 ± 0.63	0.98 ± 0.54
Prenol lipids	0.45 ± 0.52	0.09 ± 0.07	0.01 ± 0.00

Sphingolipid overexpression in COPD (- ESI)

(B)



Lipidclass	Smokers withCOPD	Smokers without COPD	Never-smokers
Glycerophospholipids	50.49 ± 8.82	58.73 ± 12.68	54.66 ± 11.59
Sphingolipids	41.21 ± 9.82	28.21 ± 12.68	31.51 ± 10.06
Fatty acyls	7.52 ± 3.38	11.46 ± 3.58	11.96 ± 4.26
Sterol lipids	0.56 ± 0.22	0.85 ± 0.61	1.20 ± 1.31
Other	0.23 ± 0.40	0.76 ± 1.53	0.67 ± 0.93

Figure 5: Lipid distribution pie charts measured in (A) positive and (B) negative ESI in smokers with COPD, smokers without COPD and never-smokers. Peak areas of all identified lipid species were summed up per lipid class for each individual sample, for both the analysis in positive and negative ESI mode. After normalization for sputum dilution, relative quantities for each lipid class were calculated within each sample group (table denotes average percentage ± standard deviation). Sphingolipids are highly overexpressed in smokers with COPD compared to smokers without COPD in positive ESI as well as negative ESI mode. Prenol lipids are differentially expressed in smokers with COPD vs. smokers without COPD and smokers without COPD vs. never-smokers (Figure 5A). Glycerophospholipids and fatty acids were differentially expressed in negative ESI mode in smokers with COPD vs. smokers without COPD (Figure 5B).

mode. Prenol lipids, the majority of which are tobacco compounds and only detected in positive ESI analysis, are differentially expressed in smokers with COPD *vs* smokers without COPD and smokers without COPD *vs* never-smokers (figure 5A). Glycerophospholipids and fatty acids are down-regulated in negative ESI- in smokers with COPD *vs* smokers without COPD, probably as a counterbalance for the increased sphingolipid level (figure 5B). This difference has been averaged on the individual lipid level, as individual fatty acid species or glycerophospholipid species (apart from the PEs) were not differentially expressed between smokers with COPD *vs* smokers without COPD.

Effects of smoking cessation

We analyzed induced sputa from 17 individuals (7 with and 10 without COPD) before and after 2 months of smoking cessation. Analyzed together, n=28 lipids were down-regulated after smoking cessation including 3 ceramides, 6 dihydroceramides, 17 GSLs (supplementary table 3). This effect was also observed for tobacco related compounds, solanesol and solanesyl palmitate.

In vitro studies

Long-term exposure of BEAS-2B cells to CSE

Because lung epithelial cells are the first cells in line that come in contact with cigarette smoke we hypothesized that these cells could be major contributors to differentially expressed lipid compounds observed in smokers with COPD. We utilized the lung epithelial cell line BEAS-2B and exposed the cells 6 months to 0% (control), 2.5% and 10% cigarette smoke extract (CSE). In contrast to lung sputum we used whole cell homogenates and all lipid compounds present in the lung epithelial cell line BEAS-2B are also found in lung sputum. Lipids expression levels were analyzed after 6 month of CSE exposure in BEAS-2B cells and no significant changes were observed when the cells were exposed to low concentration of CSE (2.5%). However, 15 sphingolipids tended to change (supplementary table 4): 2 ceramides, 1 dihydroceramide, 3 phytoceramides, 3 sphingomyelins, and 6 GSLs. The high concentration of CSE (10%) significantly altered the expression of 29 sphingolipids (supplementary table 5): 4 ceramides, 1 dihydroceramide, 3 phytoceramides, 9 sphingomyelins and 12 GSLs. 16 lipids were up-regulated and 13 were down-regulated. Of interest, in some subclasses lipids were entirely up-regulated or down-regulated and sometimes a subclass distributed in up- and down-regulated compounds. Almost all sphingomyelins were down-regulated (7 of 9) and same trend was visible for ceramides of equal chain length. Phytoceramides and lactotriaosylceramides were highly up-regulated (>3 fold). There was overlap in the results of the low and high concentration CSE exposure experiments. All lipids that showed an increasing or decreasing trend in the 2.5% CSE exposed cells are significantly differentially regulated in 10% CSE exposed cells in the same direction. Neuraminic acid containing

sphingolipids were down-regulated which correlates to our findings in smokers and COPD patients suggesting that cigarette smoke strongly influences neuraminic acid quantity.
Additionally, we investigated the effect of cigarette smoke on sphingomyelinase activity. The ratio of identical carbon chain length lipid compounds of ceramides and sphingomyelin were used to determine sphingomyelinase activity. A higher ratio means that more sphingomyelin is converted to ceramides, indicating a higher sphingomyelinase activity. The ceramide to sphingomyelin ratio for d18:1/16:0 was increased in both 2.5% and 10% CSE exposed BEAS-2B cells and the ratio of d18.1/17.0 and d18.1/18.0 were only significantly increased in BEAS-2B exposed to 10% CSE (figure 6).

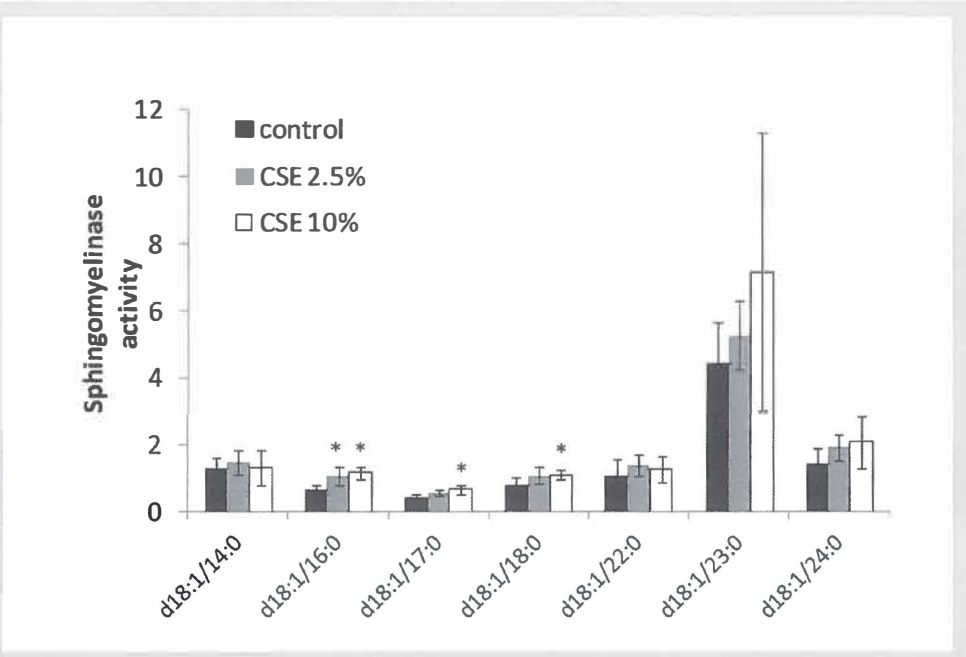


Figure 6: Sphingomyelinase activity in BEAS.2B cells. Sphingomyelinase activity can be calculated as the ratio between a ceramide and its related sphingomyelin, with identical sphingoid base and fatty acid chain, e.g. Cer(d18:1/16:0) and SM(d18:1/16:0). Sphingomyelinase activity was increased for d18:1/16:0 (2.5% and 10% CSE), d18.1/17.0 (10% CSE) and d18.1/18.0 (10% CSE) in CSE exposed BEAS.2B cells. AUC = area under the curve. * = $p<0.05$ in comparison with control

DISCUSSION

This study shows that lipid expression in induced sputum significantly differs between smokers with and without COPD. In smokers with COPD, 2 major classes of lipids (168 sphingolipids and 36 phosphatidylethanolamine lipids) are up-regulated compared to smokers without COPD. Only neuraminic acid containing sphingolipids are down-regulated in smokers with COPD as well as in smokers without COPD and BEAS-2B cells exposed to CSE. Importantly, in smokers without COPD only 20 (glyco-)sphingolipids are up-regulated compared to never-smokers. The modulatory expression of various lipids from 6-month *in vitro* exposure of BEAS-2B cells to cigarette smoke extract (CSE) supports the *in vivo* results in induced sputum from our patients. Together our findings suggest that the above described lipids may play a pivotal role in the cigarette smoke induced lung disease COPD.

There is increasing interest in the immunomodulatory properties of lipids, especially the sphingolipids. Sphingolipids can be formed *de novo* from serine and palmitoyl-CoA (figure 1). Particularly the ceramides are of interest as they form the central hub in the sphingolipid pathway.[6,7] Lipidomics can help with understanding the role of the sphingolipids in the development of COPD, since lipidomics allows you to investigate both the behavior of lipid classes and individual lipid species. In COPD, ceramides have previously been implicated in the development of emphysema in animal models.[21,22]. For the first time our study confirms these previous observations with *in vivo* results, since we found significant up-regulation of 28 ceramides in sputum from COPD patients, although there was no difference in overall ceramide distribution. Ceramides have been shown to act as second messengers involved in apoptosis and may exert their effect in a number of ways.[6,7,23] First, ceramides can directly inhibit pro-growth molecules like Akt and protein kinase C alpha (PKCα) and can activate pro-apoptotic molecules (JNKs, SAPKs, KSR, PP1, PP2A, PLA_2).[6,7,24–27] Second, ceramides can alter membrane permeability and induce the formation of membrane channels, which can lead to the release of pro-apoptotic proteins from mitochondria.[28,29] Finally, oxidative stress/inflammation has shown to induce ceramide generation which has been put forward as an important component of COPD development and progression.[30–32]. Our study shows that not only ceramides are up-regulated in COPD, but additionally 11 dihydroceramides and 19 phytoceramides. Unfortunately, little is known about the potential role of these lipids. Recent studies suggest that dihydroceramides and phytoceramides are involved in autophagy and may have (anti-)apoptotic effects.[33,34] Of interest, ceramides, dihydroceramides and phytoceramides with saturated fatty acid side chains showed an increase in COPD that was dependent on fatty acid chain length. Only ceramides with a fatty acid side chain from 14 up to 24 C-atoms were up-regulated in smokers with COPD in our study (figure 3). Their role in COPD development remains unclear. However, there are indications that lipids with alternate chain lengths have specific effects which are regulated by different enzymes.[35–38] Another finding of interest in our study is the up-regulation of glycosphingolipids (GSLs) that was present in both COPD patients and in BEAS-2B cells (only the neuraminic acid containing

sphingolipids were down-regulated) exposed to CSE. These sphingolipids, usually ceramides modified with one or more sugars, are important components of cell membranes. GSLs play an important role in the formation of glycoprotein micro-domains or `lipid rafts` and have been implicated to be involved in signal transduction, phagocytosis, mast cell degranulation and multidrug resistance.[39–44] To date no studies investigating the role of GSLs in COPD are available. However, the multidrug resistance pump protein (MRP1) has been shown to be involved in the synthesis of GSLs and we previously showed that single nucleotide polymorphisms in the *MRP1* gene are associated with COPD development and severity, strengthening the observed connection between GSLs and COPD.[45,46]

Phosphatidylethanolamines (PE) were another up-regulated class of lipids in smokers with COPD, compared to smokers without COPD, especially PE species containing polyunsaturated fatty acid side chains. LysoPEs are formed by cleaving of PEs by the enzyme PLA_2, forming lysoPEs and fatty acids, like arachidonic acid (AA).[47] AA can be further transformed to pro-inflammatory eicosanoids like leukotrienes and prostaglandins. Previous studies have shown that oxidative stress activates cytosolic Phospholipase 2 (PLA_{2j}), leading to the release of AA and increased levels of lysoPEs.[47] It is possible that lysoPEs are only a byproduct of AA production. Nevertheless, they do seem to be biologically active and contrary to the pro-inflammatory properties of AA, effects of lysoPEs are shown to be anti-inflammatory.[48] In addition, oxidative stress could directly give rise to chemical degradation of phospholipids to lysophospholipids.[49] This mechanism however is unlikely to be responsible for the higher expression of lysoPEs, as it can be expected that chemical degradation due to oxidative stress would also affect other lipid classes apart from phosphatidylethanolamines. To date, lysoPEs have not been associated with COPD.

Long-term *in vitro* exposure of BEAS-2B to cigarette smoke extract found lipidomic changes that can be identified specifically from bronchial epithelial cells, suggesting a clear role for cigarette smoke in inducing differential lipidomic profiles. 2.5% CSE exposure only showed a trend in elevated lipids. However, 10% CSE showed a significant change of 29 lipids (including all lipids shown in 2.5% CSE). Because this analysis was done on whole cell homogenates, we could see the effect of lipids that were down-regulated or up-regulated in lung epithelial cells exposed to CSE. This suggests that key components regulating lipid expression were influenced by the effect of cigarette smoke. This hypothesis was confirmed when we studied sphingomyelinase activity by determining the ratio of identical carbon chain length lipid distribution of ceramides and sphingomyelin. We showed that sphingomyelinase activity was increased for ceramides and sphingomyelins with a fatty acid chain length of 16 to 18 carbon atoms after CSE exposure (figure 6).

This study demonstrates the feasibility to use LC-MS with MS/MS in induced sputum and long-term CSE exposed BEAS-2B cells as a powerful tool to identify important lipid pathways in pulmonary disease. LC-MS with MS/MS is able to measure the expression of thousands of lipids in a very accurate way. However, the determination of the expression of lipids with a very low abundance, e.g. leukotrienes, remains difficult using an untargeted

platform. Additionally, it is not possible to distinguish glucose and galactose molecules in GSLs, hampering the exact identification of these lipids. Although over 1500 lipids have been identified, several (sphingo-)lipids that were differentially expressed in smokers with COPD have not yet been identified. Therefore, it is possible that important biological signals were missed. In this study, we used induced sputum to investigate the lipidome of the epithelial lining fluid of the lungs. The main benefit of using induced sputum to investigate the lipidome of epithelial lining fluid is that sputum induction is a relatively easy procedure, which is non-invasive and safe to perform, even in COPD patients with an exacerbation.[50,51] However, the use of induced sputum needs some points of attention. First, we normalized the expression of lipids by the total lipid expression in the sample. In this way we could identify differentially expressed lipids without the risk of identifying concentration differences. Second, the sputum sample was centrifuged and only the supernatant was used for analysis. This removes cells and large cellular debris of, most likely, various cells types and lipids that were detected were either excreted and/or released upon destruction of these cells. In perspective, the *in vitro* BEAS-2B experiments, whole cell homogenates of (solely) lung epithelial cells (which is the first barrier to come in contact with cigarette smoke) were used to study the direct influence of cigarette smoke on lung epithelial cell lipidome expression and regulation.

In conclusion, our findings show crucial changes in the lipidome of COPD patients, assessed in sputum of smokers with and without COPD, whereas smoking induced only minimal changes in the lipidome in smokers without COPD. Moreover, long-term CSE exposed BEAS-2B cells showed up-regulation of lipids belonging to the same class of lipids that were up-regulated in induced sputum of smokers with COPD. We speculate that smoking-induced changes in the lipidome may play a role in the induction and progression of COPD. This new field of research may lead to new drug targets and biomarkers for COPD.

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SUPPLEMENTARY METHODS

Sputum induction

Fifteen minutes after inhalation of 400 µg salbutamol, hypertonic saline (4.5% in the first two studies and 3%, 4%, and 5% in the third study) was nebulized with an ultrasonic nebulizer (Ultraneb, DeVilbiss, Somerset, PA, USA) and inhaled for 5 minutes in the first two studies and 7 minutes in the third study. The output of the nebulizer was calibrated at 1.5 ml/min. After each concentration, patients were encouraged to cough and expectorate sputum. Whole samples were processed according to the method of Fahy *et al.* with some modifications.[1] An equal volume of dithiothreitol 0.1% (Sputalysin 10%, Behring Diagnostics Inc, Sommervillle, NY, USA) was added to the weight of the sputum and after 15 minutes filtered through a nylon (48 µm) gauze. The sputum sample was centrifuged (10 min, 450g, 4° Celsius) and the supernatant was stored at -80°C.

Induced sputum lipid extraction

All samples were randomized before the lipid extraction. The lipid extraction method was based on the procedure described by Matyash et al.[2] 120 µL of induced lung sputum was transferred to a 2 mL Eppendorf tube and 300 µL of methanol was added. After vortexing for 10 seconds, 1000 µL of MTBE was added after which the sample was incubated for 1 hour at room temperature in a shaker. Subsequently, phase separation was induced by adding 260 µL of water. After 10 minutes incubation at room temperature, the extract was centrifuged for 10 minutes at 1000 *x g* thereby generating a lower lipophilic and upper hydrophilic phase separated by a protein layer. 1000 µL of the upper phase has been removed and dried in a centrifugal vacuum evaporator (miVac duoconcentrator, Genevac Lim., Ipswich, UK). The dried sample has been reconstituted in 40 µL methyl-tert-butylether/isopropanol 50/50 (v/v). An injection volume of 2 µL and 6 µL of this extract has been used for LC-MS analysis in positive ESI and negative ESI mode, respectively.

Airway epithelial (BEAS-2B) cell lipid extraction (in vitro study)

Methanol was used to extract the lipids by direct application onto the mono-layer cell culture. 200 µL aliquots of methanol extracts were transferred into 500 µL Eppendorf tubes and centrifuged at 15,000 rpm during 10 min. The resulting supernatant (185 µL) was transferred into a vial with insert, dried in centrifugal vacuum evaporator (miVac duoconcentrator, Genevac Lim., Ipswich, UK) and reconstituted in 37 µL chloroform/isopropanol 50/50 (v/v). 10 µL of this extract was injected into the LC-MS system.

Experimental setup, preparation and use of quality control (QC) samples

For each experiment, the use of QC samples allowed monitoring the repeatability and stability of the lipid measurement. Three independent analytical experiments have been performed: induced sputum analysis comparing current-smokers with COPD, current-smokers without

COPD and never-smokers (53 induced sputum samples, 34 QCs); induced sputum analysis comparing smokers with and without COPD before and after 2-month smoking cessation (34 induced sputum samples, 13 QCs); BEAS-2B cell lines after 6-month exposure to cigarette smoke extract (CSE) (15 cell extracts, 6 QCs).

To prepare QC samples within each experiment, 60 µL aliquots of the study samples were collected in a QC pool. This QC pool was then divided into 120 µL aliquots to obtain representative QC samples. QC samples were prepared simultaneously along with study samples and were analyzed throughout the LC-MS analysis sequences every five study samples. These samples did not contain any biological variability and can thus be considered as technical replicates. The relative standard deviation percent (RSD%) of all significantly up- or downregulated lipids has been calculated to demonstrate the measurement error of the lipidomics analytical platform.

Liquid chromatography-mass spectrometry

A 1200 RRLC system (Agilent Technologies, Waldbronn, Germany) was used for RP-LC measurements. Lipid extracts were analyzed on an XBridge BEH C18 Shield column (2.1 x 100 mm; 1.8 µm; Waters, Milford, MA, USA) placed in a Polaratherm 9000 series oven (Selerity Technologies, Salt Lake City, UT, USA) at 80°C. Elution was carried out with a multistep gradient of (A) 20 mM ammonium formate pH 5 and (B) methanol, starting from 50% B to 70% B in 5 minutes, followed by a gradient of 70–90% B in 30 minutes. Mobile phase B subsequently reached 100% in 0.1 minutes where it was maintained for an additional 5 minutes. The flow rate was 0.5 mL/min and the injection volume 6 µL for negative ESI and 2 µL for positive ESI measurements. The whole system was allowed to re-equilibrate under starting conditions for 15 min.

High-resolution accurate mass measurements were obtained on an Agilent 6530 Q-TOF mass spectrometer (Agilent Technologies) equipped with a Jetstream ESI source. The instrument was operated in both positive and negative ion electrospray mode. Needle voltage was optimized to +/- 3.5 kV, the drying and sheath gas temperatures were set to 300°C and the drying and sheath gas flow rates were set to 6 and 8 L/min, respectively. Data were collected in centroid mode from *m/z* 400–1700 in positive ion mode and *m/z* 200-1700 in negative ion mode at an acquisition rate of 1 spectrum/sec in the extended dynamic range mode (2 GHz), offering an in-spectrum dynamic range of 10^5 and a resolution of ± 10,000 FWHM in the lipid *m/z* range. To maintain mass accuracy during the analysis sequence, a reference mass solution was used containing reference ions (922.0097 for positive ESI mode, and 1033.9881 for negative ESI mode). Study samples were analyzed in randomized order in both ionization modes, with QC samples analyzed every 5 study samples. Samples were kept at 4°C in the autosampler tray while waiting for injection.

Tandem mass spectrometry (MS/MS) experiments were performed in the data dependent acquisition mode (DDA). A survey MS scan was alternated with three DDA MS/MS scans resulting in a cycle time of 4 seconds. Singly charged precursor ions were selected

based on abundance. After being fragmented twice, a particular *m/z* value was excluded for 30 s, allowing the MS/MS fragmentation of chromatographically resolved lipid isomers. Subsequent targeted MS/MS or further DDA experiments, thereby adding previously fragmented precursors in an exclusion list, increased the identification rate. The quadrupole was operated at narrow resolution and the collision energy was fixed at either 20 or 35 eV.

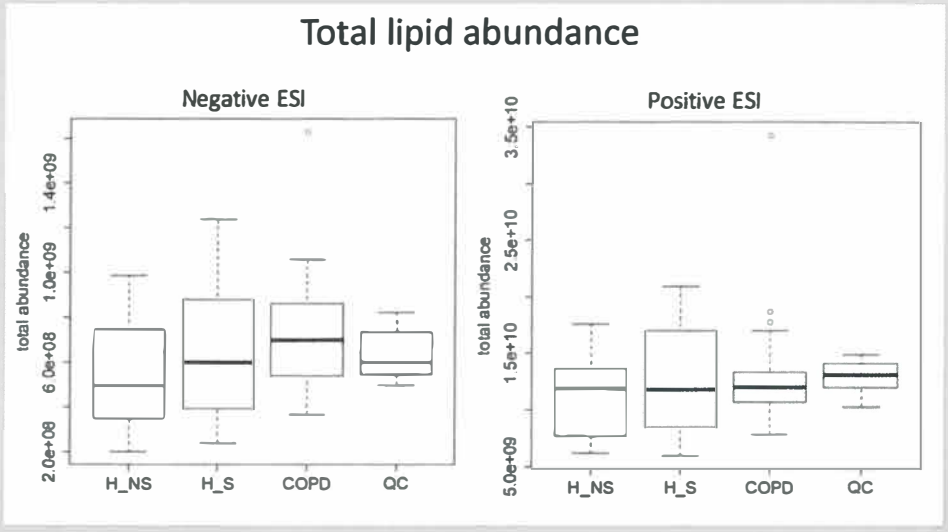
Lipid identification

Lipid identification results from an identification strategy that fully exploits the features of the Q-TOF-MS system. Molecular formulas, based on accurate mass, isotopic abundance, and spacing both in positive and negative ionization modes, are complemented with accurate mass database searching in an in-house build database (populated with LIPID MAPS and HMDB entries and theoretical lipid structures) and MS/MS measurement in both modes. Lipid fragmentation mechanisms/spectra in both positive and negative ionization modes have extensively been reported in literature.[3–12] Each lipid class displays characteristic fragmentation spectrum ions in positive and/or negative ESI mode, through neutral loss or the abundance of unique fragment ions. Other parameters such as lipid elution behavior and adduct formation interpretation further assisted in the identification. An in-house accurate mass retention time (AMRT) library was subsequently built with formula, exact mass and retention time of all identified lipids in comma-separated values (.csv) format (compatible with the MassHunter software), providing an automated and targeted data-processing of LC-MS lipid profiles.[3]

Data processing

Raw LC-MS data files were processed in an untargeted fashion using the Molecular Feature Extraction (MFE) algorithm incorporated in the MassHunter Qualitative Analysis 5.0 software package (Agilent Technologies). The resulting feature files were subsequently imported in MassProfiler Professional 12.0 (Agilent Technologies) which aligned, visualized and filtered the features. The filtered feature list was exported for recursive peak integration (i.e. targeted feature extraction) using the Find by Ion extraction algorithm in MassHunter Qualitative Analysis 5.0 using predefined mass (25 ppm) and retention time extraction windows (15 sec). The Agile integrator was used, with an absolute peak height cut-off of 1000 counts. After targeted extraction of features, samples were normalized using total lipid abundance, correcting for sputum dilution, and were imported in MassProfiler Professional for statistical analysis. No normalization step was required for cell extracts.

SUPPLEMENTAL RESULTS



Online supplemental Figure 1: Total lipid abundance in lung sputum did not significantly alter between smokers with COPD, smokers without COPD and never-smokers. . Box-and-whisker plots represent the minimum and maximum, interquartile range and median total lipid abundance for each sample group. H_NS = never-smokers, H_S = smokers without COPD, COPD = smokers with COPD, QC = quality control.

Supplementary table 1: 210 differentially regulated lipids in smokers with COPD and smokers without COPD

Sphingolipids						
Class	**Subclass**	**Lipid**	**Molecular structure**	**Fold change**	**p-value**	**RSD QC (%)**
ceramides	ceramides	Cer(d18:1/14:0)	C32H63NO3	1.84	0.001	9.86
		Cer(d18:1/15:0)	C33H65NO3	1.98	0.001	10.24
		Cer(d18:1/16:0)	C34H67NO3	1.83	0.001	10.24
		Cer(d18:1/16:0(2-OH))	C34H67NO4	1.58	0.005	8.49
		Cer(d18:1/16:1)	C34H65NO3	1.72	0.004	13.59
		Cer(d18:1/17:0)	C35H69NO3	1.93	0.001	8.27
		Cer(d18:1/17:1)	C35H67NO3	3.33	0.005	35.10
		Cer(d18:1/18:0)	C36H71NO3	1.67	0.001	8.73
		Cer(d18:1/18:1)	C36H69NO3	1.77	0.002	14.85
		Cer(d18:1/18:2)	C36H67NO3	1.67	0.001	8.68
		Cer(d18:1/19:0)	C37H73NO3	1.86	0.001	11.29
		Cer(d18:1/19:1)	C37H71NO3	4.04	0.001	37.32
		Cer(d18:1/20:0)	C38H75NO3	1.84	<0.001	8.79
		Cer(d18:1/20:1)	C38H73NO3	2.29	<0.001	8.59
		Cer(d18:1/20:2)	C38H71NO3	1.79	0.005	19.84
		Cer(d18:1/21:0)	C39H77NO3	1.79	<0.001	11.59
		Cer(d18:1/22:0)	C40H79NO3	1.81	<0.001	8.67
		Cer(d18:1/22:1)	C40H77NO3	1.66	0.001	7.88
		Cer(d18:1/22:2)	C40H75NO3	2.14	0.001	10.77
		Cer(d18:1/23:0)	C41H81NO3	1.69	0.001	8.20
		Cer(d18:1/23:1)	C41H79NO3	1.64	0.001	8.82
		Cer(d18:1/24:0)	C42H83NO3	1.51	0.004	9.28
		Cer(d18:1/24:1)	C42H81NO3	1.64	0.001	8.62
		Cer(d18:1/24:2)	C42H79NO3	2.03	0.001	10.63
		Cer(d18:1/25:1)	C43H83NO3	1.76	0.002	9.87
		Cer(d18:1/26:1)	C44H85NO3	1.75	0.003	11.72
		Cer(d18:1/26:2)	C44H83NO3	1.68	0.002	13.75
		Cer(d18:1/27:1)	C45H87NO3	1.92	0.003	12.25
	dihydroceramides	Cer(d18:0/14:0)	C32H65NO3	2.41	0.001	18.54
		Cer(d18:0/15:0)	C33H67NO3	2.49	<0.001	12.89
		Cer(d18:0/16:0)	C34H69NO3	2.51	<0.001	9.57
		Cer(d18:0/16:0(2-OH))	C34H69NO4	2.18	0.005	9.64
		Cer(d18:0/17:0)	C35H71NO3	2.65	0.001	9.71
		Cer(d18:0/18:0)	C36H73NO3	2.35	<0.001	7.75
		Cer(d18:0/19:0)	C37H75NO3	1.99	<0.001	10.27
		Cer(d18:0/20:0)	C38H77NO3	2.02	<0.001	8.89
		Cer(d18:0/21:0)	C39H79NO3	1.61	0.001	13.76
		Cer(d18:0/22:0)	C40H81NO3	1.79	0.001	12.54
		Cer(d18:0/23:0)	C41H83NO3	1.47	0.015	11.44
	phytoceramides	Cer(t18:0/14:0)	C32H65NO4	1.77	0.013	19.41
		Cer(t18:0/14:0(2-OH))	C32H65NO5	1.80	0.002	21.51
		Cer(t18:0/15:0)	C33H67NO4	1.69	0.005	14.03
		Cer(t18:0/16:0)	C34H69NO4	1.86	0.001	7.98
		Cer(t18:0/16:1)	C34H67NO4	2.35	0.001	11.46
		Cer(t18:0/16:2)	C34H65NO4	1.87	0.002	15.69
		Cer(t18:0/17:0)	C35H71NO4	1.61	0.017	13.25
		Cer(t18:0/18:0)	C36H73NO4	1.70	0.006	9.42
		Cer(t18:0/19:0)	C37H75NO4	1.43	0.031	8.13
		Cer(t18:0/20:0)	C38H77NO4	1.72	0.003	12.63
		Cer(t18:0/20:1)	C38H75NO4	1.74	0.006	17.58
		Cer(t18:0/22:0)	C40H81NO4	1.39	0.012	11.05
		Cer(t18:0/22:1)	C40H79NO4	1.75	0.004	11.28
		Cer(t18:0/22:2)	C40H77NO4	1.77	0.001	12.60
		Cer(t18:0/23:1)	C41H81NO4	1.46	0.016	8.14
		Cer(t18:0/24:1)	C42H83NO4	1.45	0.011	8.23
		Cer(t18:0/24:2)	C42H81NO4	1.87	0.001	8.83
		Cer(t18:0/25:1)	C43H85NO4	1.37	0.027	8.77
		Cer(t18:0/25:2)	C43H83NO4	1.68	0.003	11.32

Class	Subclass	Lipid	Molecular structure	Fold change	p-value	RSD QC (%)
sphingomyelins		SM(d18:0/14:0)	C37H77N2O6P	1.85	0.008	20.14
		SM(d18:0/15:0)	C38H79N2O6P	2.32	0.005	24.99
		SM(d18:0/16:0)	C39H81N2O6P	2.88	0.005	17.01
		SM(d18:0/18:0)	C41H85N2O6P	2.46	0.005	14.03
		SM(d18:0/20:0)	C43H89N2O6P	2.25	0.005	16.03
		SM(d18:0/22:0)	C45H93N2O6P	1.76	0.006	11.45
		SM(d18:0/24:0)	C47H97N2O6P	1.43	0.024	19.12
		SM(d18:0/24:1)	C47H95N2O6P	2.26	0.008	15.49
		SM(d18:1/14:0)	C37H75N2O6P	1.84	0.032	22.78
		SM(d18:1/15:0)	C38H77N2O6P	1.57	0.029	10.80
		SM(d18:1/16:0)	C39H79N2O6P	1.65	0.023	10.21
		SM(d18:1/17:0)	C40H81N2O6P	1.74	0.009	10.97
		SM(d18:1/18:0)	C41H83N2O6P	1.77	0.008	10.23
		SM(d18:1/19:0)	C42H85N2O6P	1.69	0.007	8.84
		SM(d18:1/20:0)	C43H87N2O6P	1.82	0.005	11.47
		SM(d18:1/21:0)	C44H89N2O6P	1.67	0.010	10.06
		SM(d18:1/22:0)	C45H91N2O6P	1.65	0.010	11.28
		SM(d18:1/22:1)	C45H89N2O6P	1.56	0.050	12.15
		SM(d18:1/23:0)	C46H93N2O6P	1.64	0.024	13.72
		SM(d18:1/24:0)	C47H95N2O6P	1.60	0.023	13.09
		SM(d18:1/24:1)	C47H93N2O6P	1.69	0.025	11.36
		SM(d18:1/24:2)	C47H91N2O6P	1.93	0.015	12.42
		SM(d18:1/26:1)	C49H97N2O6P	1.56	0.010	11.31
		SM(d18:1/26:2)	C49H95N2O6P	1.94	0.008	16.73
		SM(t18:0/14:0)	C37H77N2O7P	1.47	0.015	18.00
		SM(t18:0/16:0)	C39H81N2O7P	2.02	0.005	12.06
		SM(t18:0/16:1)	C39H79N2O7P	1.87	0.006	14.22
		SM(t18:0/18:1)	C41H83N2O7P	1.94	0.010	15.33
		SM(t18:0/22:0)	C45H93N2O7P	1.66	0.009	10.14
		SM(t18:0/23:0)	C46H95N2O7P	1.89	0.005	11.23
		SM(t18:0/24:0)	C47H97N2O7P	1.91	0.005	14.78
		SM(t18:0/24:1)	C47H95N2O7P	1.99	0.006	12.08
		SM(t18:0/24:2)	C47H93N2O7P	2.56	0.005	14.01
		SM(t18:0/25:1)	C48H97N2O7P	1.72	0.010	14.88
		SM(t18:0/26:1)	C49H99N2O7P	1.57	0.009	9.96
		SM(t18:0/26:2)	C49H97N2O7P	2.21	0.005	12.83
glycosphingolipids (1 sugar)	glucosyl/galactosyl-dihydroceramides	HexCer(d18:0/16:0)	C40H79NO8	2.69	0.002	10.40
	glucosyl/galactosyl-ceramides	HexCer(d18:1/16:0)	C40H77NO8	1.91	0.001	9.79
		HexCer(d18:1/16:0(2-OH))	C40H77NO9	1.96	0.001	9.49
		HexCer(d18:1/18:0)	C42H81NO8	1.57	0.035	9.89
		HexCer(d18:1/22:0)	C46H89NO8	1.35	0.012	7.61
		HexCer(d18:1/23:0)	C48H93NO8	1.26	0.005	9.12
		HexCer(d18:1/24:0(2-OH))	C48H93NO9	1.53	0.005	10.84
	glucosyl/galactosyl-phytoceramides	HexCer(t18:0/16:0)	C40H79NO9	2.00	0.001	7.79
		HexCer(t18:0/18:0)	C42H83NO9	1.55	0.043	9.13
		HexCer(t18:0/20:0)	C44H87NO9	1.70	0.019	10.85
		HexCer(t18:0/22:0)	C46H91NO9	1.57	0.003	7.27
		HexCer(t18:0/23:0)	C47H93NO9	1.56	0.005	9.80
		HexCer(t18:0/24:0)	C48H95NO9	1.44	0.007	6.64
		HexCer(t18:0/24:1)	C48H93NO9	1.47	0.010	7.85
		HexCer(t18:0/24:2)	C48H91NO9	2.46	0.001	13.84
glycosphingolipids (2 sugars)	lactosyl/digalactosyl-ceramides	Hex-HexCer(d18:1/16:0(2-OH))	C46H87NO14	2.02	0.001	10.02
		Hex-HexCer(d18:1/20:0(2-OH))	C50H95NO14	2.21	0.033	38.76
		Hex-HexCer(d18:1/22:0(2-OH))	C52H99NO14	2.20	0.002	20.83
		Hex-HexCer(d18:1/24:0(2-OH))	C54H103NO14	1.80	0.001	15.07
		Hex-HexCer(d18:1/26:0(2-OH))	C56H107NO14	1.64	0.012	16.04
	lactosyl/digalactosyl-phytoceramides	Hex-HexCer(t18:0/16:0)	C46H89NO14	2.17	0.001	9.98
		Hex-HexCer(t18:0/22:0)	C52H101NO14	1.99	0.001	19.03
		Hex-HexCer(t18:0/24:0)	C54H105NO14	1.78	0.001	15.22
		Hex-HexCer(t18:0/24:1)	C54H103NO14	1.53	0.011	10.19
		Hex-HexCer(t18:0/24:2)	C54H101NO14	2.86	0.009	11.87

Class	Subclass	Lipid	Molecular structure	Fold change	p-value	RSD QC (%)
glycosphingolipids (3 sugars)	trihexosylceramides	Hex-Hex-HexCer(d18:1/16:0)	C52H97NO18	2.57	0.001	13.30
		Hex-Hex-HexCer(d18:1/16:0(2-OH))	C52H97NO19	2.51	<0.001	18.24
		Hex-Hex-HexCer(d18:1/17:0)	C53H99NO18	3.50	0.001	16.48
		Hex-Hex-HexCer(d18:1/18:0)	C54H101NO18	2.34	0.011	26.88
		Hex-Hex-HexCer(d18:1/18:0(2-OH))	C54H101NO19	2.37	0.003	20.73
		Hex-Hex-HexCer(d18:1/18:2)	C54H97NO18	2.47	<0.001	18.53
		Hex-Hex-HexCer(d18:1/20:0)	C56H105NO18	1.96	0.016	17.61
		Hex-Hex-HexCer(d18:1/20:0(2-OH))	C56H105NO19	3.18	0.003	31.60
		Hex-Hex-HexCer(d18:1/20:1)	C56H103NO18	2.85	0.033	26.94
		Hex-Hex-HexCer(d18:1/21:0)	C57H107NO18	2.60	0.031	28.89
		Hex-Hex-HexCer(d18:1/22:0)	C58H109NO18	1.45	0.045	21.08
		Hex-Hex-HexCer(d18:1/22:0(2-OH))	C58H109NO19	2.48	0.003	27.34
		Hex-Hex-HexCer(d18:1/22:2)	C58H105NO18	2.88	0.009	33.49
		Hex-Hex-HexCer(d18:1/23:0)	C59H111NO18	1.48	0.029	18.28
		Hex-Hex-HexCer(d18:1/23:1)	C59H109NO18	1.97	0.040	18.30
		Hex-Hex-HexCer(d18:1/23:0(2-OH))	C59H111NO19	2.40	0.003	23.32
		Hex-Hex-HexCer(d18:1/24:0(2-OH))	C60H113NO19	1.87	0.006	22.17
		Hex-Hex-HexCer(d18:1/24:1)	C60H111NO18	1.55	0.012	12.74
		Hex-Hex-HexCer(d18:1/24:2)	C60H109NO18	1.74	0.016	20.72
		Hex-Hex-HexCer(d18:1/25:0(2-OH))	C61H115NO19	2.10	0.026	26.60
		Hex-Hex-HexCer(d18:1/26:1)	C62H115NO18	3.02	0.001	21.78
	trihexosyl-dihydroceramides	Hex-Hex-HexCer(d18:0/16:0)	C52H99NO18	3.96	0.001	13.19
		Hex-Hex-HexCer(d18:0/16:0(2-OH))	C52H99NO19	3.58	<0.001	19.50
		Hex-Hex-HexCer(d18:0/18:0)	C54H103NO18	4.47	0.002	15.86
		Hex-Hex-HexCer(d18:0/18:0(2-OH))	C54H103NO19	5.28	0.001	29.29
		Hex-Hex-HexCer(d18:0/20:0)	C56H107NO18	3.61	0.002	20.88
		Hex-Hex-HexCer(d18:0/22:0)	C58H111NO18	1.96	0.004	19.07
		Hex-Hex-HexCer(d18:0/22:0(2-OH))	C58H111NO19	3.11	0.002	21.21
		Hex-Hex-HexCer(d18:0/24:0(2-OH))	C60H115NO19	2.21	0.021	27.97
	trihexosyl-phytoceramides	Hex-Hex-HexCer(t18:0/16:0)	C52H99NO19	2.74	0.001	17.78
		Hex-Hex-HexCer(t18:0/16:0(2-OH))	C52H99NO20	2.92	0.003	15.28
		Hex-Hex-HexCer(t18:0/17:0)	C53H101NO19	3.26	0.002	14.44
		Hex-Hex-HexCer(t18:0/18:0)	C54H103NO19	2.41	0.007	26.33
		Hex-Hex-HexCer(t18:0/18:0(2-OH))	C54H103NO20	1.90	0.035	19.70
		Hex-Hex-HexCer(t18:0/20:0)	C56H107NO19	2.68	0.019	43.49
		Hex-Hex-HexCer(t18:0/20:0(2-OH))	C56H107NO20	2.31	0.033	25.83
		Hex-Hex-HexCer(t18:0/22:0)	C58H111NO19	1.74	0.024	21.84
		Hex-Hex-HexCer(t18:0/22:1)	C58H109NO19	2.17	0.048	47.33
		Hex-Hex-HexCer(t18:0/22:2)	C58H107NO19	5.15	0.002	16.38
		Hex-Hex-HexCer(t18:0/24:1)	C60H113NO19	1.64	0.026	18.31
		Hex-Hex-HexCer(t18:0/24:2)	C60H111NO19	4.50	0.003	24.85
glycosphingolipids (other)	neuraminic acid lactotrlaosylceramides	NeuAc-Hex-HexCer(d18:1/24:0)	C65H120N2O21	0.62	0.022	29.28
		HexNAc-Hex-Hex-HexCer(d18:1/16:0)	C60H110N2O23	1.91	0.013	18.98
		HexNAc-Hex-Hex-HexCer(d18:1/18:0)	C62H114N2O23	4.80	0.002	26.77
		HexNAc-Hex-Hex-HexCer(d18:1/20:0)	C64H118N2O23	5.09	0.002	37.29
		HexNAc-Hex-Hex-HexCer(d18:1/22:0)	C66H122N2O23	1.86	0.029	23.13
	lactotriaosyl-dihydroceramides	HexNAc-Hex-Hex-HexCer(d18:0/16:0(2-OH))	C60H112N2O24	6.23	0.001	41.90
	lactotrlaosyl-phytoceramides	HexNAc-Hex-Hex-HexCer(t18:0/16:1)	C60H110N2O24	3.09	0.001	22.65
		HexNAc-Hex-Hex-HexCer(t18:0/18:0)	C62H116N2O24	3.28	0.007	30.02
		HexNAc-Hex-Hex-HexCer(t18:0/24:0)	C68H128N2O24	3.19	0.008	53.71

Glycerophospolipids						
Class	Subclass	Lipid	Molecular structure	Fold change	p-value	RSD QC (%)
ethanolamines	phosphatidyl-ethanolamines	PE(18:0/20:4)	C43H78NO8P	1.59	0.014	7.91
		PE(38:6)	C43H74NO8P	1.55	0.013	15.17
	lysophosphatidyl-ethanolamines	PE(0:0/18:1)	C23H46NO7P	1.87	0.047	15.48
		PE(0:0/18:2)	C23H44NO7P	2.45	0.030	20.67
		PE(0:0/20:2)	C25H48NO7P	2.38	0.023	19.43
		PE(20:2/0:0)	C25H48NO7P	2.23	0.019	21.81
		PE(20:3/0:0)	C25H46NO7P	2.29	0.009	16.17
		PE(0:0/20:4)	C25H44NO7P	4.92	0.011	24.95
		PE(20:4/0:0)	C25H44NO7P	2.56	0.023	19.23
		PE(0:0/20:5)	C25H42NO7P	6.30	0.016	22.66
		PE(22:4/0:0)	C27H48NO7P	2.38	0.025	18.79
		PE(0:0/22:4)	C27H48NO7P	4.00	0.012	23.24
		PE(0:0/22:5)	C27H46NO7P	5.19	0.010	22.55
		PE(22:5/0:0)	C27H46NO7P	2.74	0.010	13.16
		PE(0:0/22:6)	C27H44NO7P	4.83	0.024	25.27
		PE(22:6/0:0)	C27H44NO7P	2.52	0.030	13.45
	plasmalogen phospatidyl-ethanolamines	PE(p16:0/18:1)	C39H76NO7P	1.64	0.003	7.07
		PE(p16:0/20:4)	C41H74NO7P	1.89	0.001	8.40
		PE(p16:0/22:4)	C43H78NO7P	2.08	0.001	6.62
		PE(p16:0/22:6)	C43H74NO7P	2.44	0.001	11.20
		PE(p17:0/22:6)	C44H76NO7P	2.02	0.005	10.00
		PE(p18:0/18:1)	C41H80NO7P	1.54	0.048	7.39
		PE(p18:0/18:2)	C41H78NO7P	1.64	0.012	9.19
		PE(p18:0/20:4)	C43H78NO7P	1.73	0.002	8.65
		PE(p18:0/20:5)	C43H76NO7P	1.47	0.005	19.19
		PE(p18:0/22:4)	C45H82NO7P	1.71	0.005	6.40
		PE(p18:0/22:6)	C45H78NO7P	2.18	0.001	6.68
		PE(p18:1/18:1)	C41H78NO7P	1.70	0.045	6.74
		PE(p20:0/18:1)	C43H84NO7P	1.91	0.018	8.14
		PEp(34:2)	C39H74NO7P	1.70	0.001	7.49
		PEp(38:5)	C43H76NO7P	1.56	0.011	9.40
		PEp(40:1)	C45H88NO7P	2.38	0.014	9.80
		PEp(42:3)	C47H88NO7P	1.91	0.027	16.69
		PEp(42:6)	C47H82NO7P	3.69	0.001	7.26
		PEp(44:6)	C49H86NO7P	3.93	0.001	12.21
		PEp(44:7)	C49H84NO7P	3.92	0.002	17.45

Prenol lipids						
Class	Subclass	Lipid	Molecular structure	Fold change	p-value	RSD QC (%)
tobacco compounds		solanesol	C45H74O	3.70	0.029	13.60
		solanesyl palmitate	C61H104O2	4.61	0.050	14.04
		solanesyl stearate	C63H108O2	8.19	0.025	18.60
		bomblprenone	C43H70O	3.10	0.025	12.54
		farnesylfarnesylacetone	C33H54O	2.53	0.041	10.71

Fold change = expression in smokers with COPD / expression in smokers without COPD. A fold change >1 is up-regulation in smokers with COPD. A fold change between 0 and 1 is down-regulation in smokers with COPD. The LipidMaps database was taken as the reference for the lipid nomenclature. LC-MS cannot discriminate between galactose and glucose, or N-acetylglucosamine and N-acetylgalactosamine. Therefore, lipid nomenclature contains hexose (Hex) instead of glucose or galactose. Relative standard deviation (RSD%) has been calculated from the QC samples (n=34).

Supplementary table 2: Differentially expressed lipids in smokers without COPD and never-smokers

Class	Subclass	Lipid	Molecular structure	Fold change	*p*-value	RSD QC (%)
ceramides	dihydroceramides	Cer(d18:0/16:0)	$C_{34}H_{69}NO_3$	1.71	0.021	9.57
glycosphingolipids	lactosyl/digalactosyl-ceramides	Hex-HexCer(d18:1/24:0(2-OH))	$C_{54}H_{103}NO_{14}$	1.67	0.003	15.07
	trihexosylceramides	Hex-Hex-HexCer(d18:1/16:0(2-OH))	$C_{52}H_{97}NO_{19}$	1.93	0.037	18.24
		Hex-Hex-HexCer(d18:1/18:2)	$C_{54}H_{97}NO_{18}$	1.85	0.047	18.53
		Hex-Hex-HexCer(d18:1/20:0(2-OH))	$C_{56}H_{105}NO_{19}$	2.03	0.021	31.60
		Hex-Hex-HexCer(d18:1/22:0(2-OH))	$C_{58}H_{109}NO_{19}$	3.24	0.001	27.34
		Hex-Hex-HexCer(d18:1/22:1)	$C_{58}H_{107}NO_{18}$	7.87	0.001	32.69
		Hex-Hex-HexCer(d18:1/23:0(2-OH))	$C_{59}H_{111}NO_{19}$	3.54	0.001	23.32
		Hex-Hex-HexCer(d18:1/23:0)	$C_{59}H_{111}NO_{18}$	1.89	0.034	18.28
		Hex-Hex-HexCer(d18:1/24:0(2-OH))	$C_{60}H_{113}NO_{19}$	3.58	0.001	22.17
		Hex-Hex-HexCer(d18:1/25:0(2-OH))	$C_{61}H_{115}NO_{19}$	4.47	0.001	26.60
		Hex-Hex-HexCer(d18:1/26:0(2-OH))	$C_{62}H_{117}NO_{19}$	5.35	0.001	33.31
		Hex-Hex-HexCer(d18:1/26:0)	$C_{62}H_{117}NO_{18}$	3.51	0.001	23.51
		Hex-Hex-HexCer(d18:1/26:1)	$C_{62}H_{115}NO_{18}$	2.95	0.002	21.78
	trihexosyl-dihydroceramides	Hex-Hex-HexCer(d18:0/16:0(2-OH))	$C_{52}H_{99}NO_{19}$	2.86	0.008	19.50
		Hex-Hex-HexCer(d18:0/22:0(2-OH))	$C_{58}H_{111}NO_{19}$	3.28	0.001	21.21
		Hex-Hex-HexCer(d18:0/24:0(2-OH))	$C_{60}H_{115}NO_{19}$	4.78	0.001	27.97
		Hex-Hex-HexCer(d18:0/26:0(2-OH))	$C_{62}H_{119}NO_{19}$	3.25	0.029	33.78
		Hex-Hex-HexCer(t18:0/24:0(2-OH))	$C_{60}H_{115}NO_{20}$	2.40	0.022	24.58
glycosphingolipids (other)	lactotriaosylceramides	HexNAc-Hex-Hex-HexCer(t18:0/18:0)	$C_{64}H_{120}N_2O_{24}$	3.25	0.001	33.55
	neuraminic acid	NeuAc-Hex-HexCer(d18:1/20:0)	$C_{61}H_{112}N_2O_{21}$	0.15	0.001	30.85
		NeuAc-Hex-HexCer(d18:1/24:1)	$C_{65}H_{118}N_2O_{21}$	0.36	0.001	26.14
tobacco compounds		solanesol	$C_{45}H_{74}O$	*	<0.001	13.60
		solanesyl palmitate	$C_{61}H_{104}O_2$	*	<0.001	14.04
		solanesyl stearate	$C_{63}H_{108}O_2$	*	<0.001	18.60
		bombiprenone	$C_{41}H_{70}O$	481.85	<0.001	12.54
		farnesylfarnesylacetone	$C_{33}H_{54}O$	*	<0.001	10.71
		geranylfarnesylacetone	$C_{28}H_{46}O$	*	<0.001	13.19

Fold change = expression in smokers without COPD / expression in never smokers. A fold change >1 is up-regulation in smokers without COPD. A fold change between 0 and 1 is down-regulation in smokers without COPD. * lipid not present in never-smokers. The LipidMaps database was taken as the reference for the lipid nomenclature. LC-MS cannot discriminate between galactose and glucose, or N-acetylglucosamine and N-acetylgalactosamine. Therefore, lipid nomenclature contains hexose (Hex) instead of glucose or galactose. Relative standard deviation (RSD%) has been calculated from the QC samples (n=34).

Supplementary table 3: Lipids that are differently regulated after 2-month smoking cessation than before smoking cessation in individuals with and without COPD

Class	Subclass	Lipid	Molecular structure	Fold change	*p*-value	RSD QC (%)
ceramides	ceramides	Cer(d18:1/25:1)	$C_{43}H_{83}NO_3$	0.69	0.021	6.53
		Cer(d18:1/25:2)	$C_{43}H_{81}NO_3$	0.75	0.047	8.79
		Cer(d18:1/26:2)	$C_{44}H_{83}NO_3$	0.76	0.038	6.80
	dihydroceramides	Cer(d18:0/15:0)	$C_{33}H_{67}NO_3$	0.70	0.048	7.71
		Cer(d18:0/16:0)	$C_{34}H_{69}NO_3$	0.57	0.018	7.27
		Cer(d18:0/17:0)	$C_{35}H_{71}NO_3$	0.56	0.029	9.02
		Cer(d18:0/18:0)	$C_{36}H_{73}NO_3$	0.62	0.021	5.80
		Cer(d18:0/20:0)	$C_{38}H_{77}NO_3$	0.70	0.021	5.79
		Cer(d18:0/22:0)	$C_{40}H_{81}NO_3$	0.76	0.029	8.41
glycosphingolipids	lactosyl/digalactosyl-ceramides	Hex-HexCer(d18:1/24:0(2-OH))	$C_{54}H_{103}NO_{14}$	0.81	0.047	9.36
	trihexosylceramides	Hex-Hex-HexCer(d18:1/22:0(2-OH))	$C_{58}H_{109}NO_{19}$	0.49	0.018	23.25
		Hex-Hex-HexCer(d18:1/23:0(2-OH))	$C_{59}H_{111}NO_{19}$	0.44	0.029	30.39
		Hex-Hex-HexCer(d18:1/24:0(2-OH))	$C_{60}H_{113}NO_{19}$	0.46	0.018	20.19
		Hex-Hex-HexCer(d18:1/26:0(2-OH))	$C_{62}H_{117}NO_{19}$	0.49	0.025	24.61
	trihexosyl-dihydroceramides	Hex-Hex-HexCer(d18:0/16:0(2-OH))	$C_{52}H_{99}NO_{19}$	0.52	0.018	16.46
		Hex-Hex-HexCer(d18:0/18:0(2-OH))	$C_{54}H_{103}NO_{19}$	0.38	0.024	44.83
		Hex-Hex-HexCer(d18:0/20:0(2-OH))	$C_{56}H_{107}NO_{19}$	0.32	0.048	25.30
		Hex-Hex-HexCer(d18:0/22:0(2-OH))	$C_{58}H_{111}NO_{19}$	0.37	0.018	45.45
		Hex-Hex-HexCer(d18:0/24:0(2-OH))	$C_{60}H_{115}NO_{19}$	0.40	0.018	22.59
		Hex-Hex-HexCer(d18:0/26:0(2-OH))	$C_{62}H_{119}NO_{19}$	0.37	0.038	33.77
	trihexosyl-phytoceramides	Hex-Hex-HexCer(t18:0/24:1)	$C_{60}H_{115}NO_{19}$	0.27	0.031	30.67
		Hex-Hex-HexCer(t18:0/24:2)	$C_{60}H_{111}NO_{19}$	0.56	0.018	19.96
glycosphingolipids (other)	lactotriaosylceramides	HexNAc-Hex-HexHexCer(d18:1/16:0(2-OH))	$C_{68}H_{126}N_2O_{24}$	61	0.048	26.60
		HexNAc-Hex-Hex-HexCer(d18:1/24:0(2-OH))	$C_{68}H_{126}N_2O_{24}$	0.27	0.018	36.60
		HexNAc-Hex-Hex-HexCer(d18:1/26:1)	$C_{70}H_{128}N_2O_{23}$	0.27	0.018	50.09
	lactotriaosyl-dihydroceramides	HexNAc-Hex-Hex-HexCer(d18:0/16:0)	$C_{60}H_{112}N_2O_{24}$	0.26	0.025	16.58
tobacco compounds		solanesol	$C_{45}H_{74}O$	*	0.020	10.01
		solanesyl palmitate	$C_{61}H_{104}O_2$	*	0.020	17.63

Fold change = expression after smoking cessation / expression before smoking cessation. A fold change >1 is up-regulation after smoking cessation. A fold change between 0 and 1 is down-regulation after smoking cessation. * lipid not present after smoking cessation. The LipidMaps database was taken as the reference for the lipid nomenclature. LC-MS cannot discriminate between galactose and glucose, or N-acetylglucosamine and N-acetylgalactosamine. Therefore, lipid nomenclature contains hexose (Hex) instead of glucose or galactose. Relative standard deviation (RSD%) has been calculated from the QC samples (n=13).

Supplementary table 4: Differentially regulated lipids after long-term exposure to low CSE concentration (2.5%)

Class	Subclass	Lipid	Molecular structure	Fold change	*p*-value	RSD QC (%)
ceramides	ceramides	Cer(d18:1/14:1)	$C_{32}H_{61}NO_3$	0.19	0.067	8.22
		Cer(d18:1/22:1)	$C_{40}H_{77}NO_3$	0.35	0.067	5.36
	dihydroceramides	Cer(d18:0/14:0)	$C_{32}H_{65}NO_3$	0.55	0.067	11.93
	phytoceramides	Cer(t18:0/17:0)	$C_{35}H_{71}NO_4$	9.07	0.067	7.89
		Cer(t18:0/23:0)	$C_{41}H_{83}NO_4$	7.40	0.067	6.52
		Cer(t18:0/25:0)	$C_{43}H_{87}NO_4$	4.36	0.067	5.59
sphingomyelins		SM(d18:1/14:0)	$C_{37}H_{75}N_2O_6P$	0.75	0.098	9.25
		SM(d18:1/14:1)	$C_{37}H_{73}N_2O_6P$	0.57	0.067	7.96
		SM(d18:1/24:2)	$C_{47}H_{91}N_2O_6P$	0.54	0.067	9.17
glycosphingolipids (2 sugars)	lactosyl/digalactosyl-ceramides	Hex-HexCer(d18:1/18:0)	$C_{48}H_{91}NO_{13}$	2.13	0.067	4.09
		Hex-HexCer(d18:1/24:0)	$C_{54}H_{103}NO_{13}$	1.80	0.067	6.49
glycosphingolipids (other)	lactotriaosylceramides	HexNAc-Hex-Hex-HexCer(d18:1/24:0)	$C_{68}H_{126}N_2O_{23}$	7.32	0.098	8.36
	neuraminic acid	NeuAc-Gal-GlcCer(d18:1/16:0)	$C_{57}H_{104}N_2O_{21}$	0.76	0.299	5.56
		NeuAc-Gal-GlcCer(d18:1/24:0)	$C_{65}H_{120}N_2O_{21}$	0.50	0.098	4.08
		NeuAc-Gal-GlcCer(d18:1/24:1)	$C_{65}H_{118}N_2O_{21}$	0.74	0.156	9.12

Fold change = expression in exposed cells / in non-exposed cells. A fold change >1 is up-regulation in exposed cells. A fold change between 0 and 1 is down-regulation in exposed cells. The LipidMaps database was taken as the reference for the lipid nomenclature. LC-MS cannot discriminate between galactose and glucose, or N-acetylglucosamine and N-acetylgalactosamine. E.g. LacCer can also be Gal-GalCer. Therefore, lipid nomenclature contains hexose (Hex) instead of glucose or galactose. Relative standard deviation (RSD%) has been calculated from the QC samples (n=6).

Supplementary table 5: Differentially regulated lipids after long-term exposure to high CSE concentration (10%)

Class	Subclass	Lipid	Molecular structure	Fold change	*p*-value	RSD QC (%)
ceramides	ceramides	Cer(d18:1/16:0)	$C_{34}H_{67}NO_3$	2.42	0.028	10.19
		Cer(d18:1/17:0)	$C_{35}H_{69}NO_3$	3.52	0.028	14.18
		Cer(d18:1/14:1)	$C_{32}H_{61}NO_3$	0.08	0.028	8.22
		Cer(d18:1/22:1)	$C_{40}H_{77}NO_3$	0.22	0.028	5.36
	dihydroceramides	Cer(d18:0/14:0)	$C_{32}H_{65}NO_3$	0.47	0.028	11.93
	phytoceramides	Cer(t18:0/17:0)	$C_{35}H_{71}NO_4$	8.59	0.028	7.89
		Cer(t18:0/23:0)	$C_{41}H_{83}NO_4$	8.21	0.028	6.52
		Cer(t18:0/25:0)	$C_{43}H_{87}NO_4$	5.00	0.028	5.59
sphingomyelins		SM(d18:0/14:0)	$C_{37}H_{77}N_2O_6P$	0.43	0.028	7.93
		SM(d18:1/12:0)	$C_{35}H_{71}N_2O_6P$	0.33	0.028	10.94
		SM(d18:1/14:0)	$C_{37}H_{75}N_2O_6P$	0.66	0.028	9.25
		SM(d18:1/16:0)	$C_{39}H_{79}N_2O_6P$	1.37	0.028	5.86
		SM(d18:1/17:0)	$C_{40}H_{81}N_2O_6P$	2.32	0.028	4.84
		SM(d18:1/14:1)	$C_{37}H_{73}N_2O_6P$	0.37	0.028	7.96
		SM(d18:1/16:1)	$C_{39}H_{77}N_2O_6P$	0.50	0.028	11.08
		SM(d18:1/18:1)	$C_{41}H_{81}N_2O_6P$	0.43	0.041	4.81
		SM(d18:1/24:2)	$C_{47}H_{91}N_2O_6P$	0.36	0.028	9.17
glycosphingolipids (2 sugars)	lactosyl/digalactosyl-ceramides	Hex-HexCer(d18:1/16:0)	$C_{46}H_{87}NO_{13}$	2.48	0.028	6.81
		Hex-HexCer(d18:1/24:0)	$C_{54}H_{101}NO_{13}$	2.58	0.041	6.49
glycosphingolipids (3 sugars)	trihexosylceramides	Hex-Hex-HexCer(d18:1/16:0)	$C_{52}H_{97}NO_{18}$	2.67	0.028	6.69
		Hex-Hex-HexCer(d18:1/20:0)	$C_{56}H_{105}NO_{18}$	2.67	0.041	9.57
		Hex-Hex-HexCer(d18:1/24:0)	$C_{60}H_{113}NO_{18}$	2.09	0.041	4.40
glycosphingolipids (other)	lactotriaosylceramides	HexNAc-Hex-Hex-HexCer(d18:1/24:1)	$C_{68}H_{128}N_2O_{23}$	9.07	0.028	11.17
		HexNAc-Hex-Hex-HexCer(d18:1/16:0)	$C_{60}H_{110}N_2O_{23}$	6.63	0.028	10.41
		HexNAc-Hex-Hex-HexCer(d18:1/22:0)	$C_{66}H_{122}N_2O_{23}$	3.45	0.028	15.65
		HexNAc-Hex-Hex-HexCer(d18:1/24:0)	$C_{68}H_{126}N_2O_{23}$	8.89	0.028	8.36
	neuraminic acid	NeuAc-Hex-HexCer(d18:1/16:0)	$C_{57}H_{104}N_2O_{21}$	0.54	0.041	5.56
		NeuAc-Hex-HexCer(d18:1/24:0)	$C_{65}H_{120}N_2O_{21}$	0.48	0.028	4.08
		NeuAc-Hex-HexCer(d18:1/24:1)	$C_{65}H_{118}N_2O_{21}$	0.56	0.028	9.12

Fold change = expression in exposed cells / in non-exposed cells. A fold change >1 is up-regulation in exposed cells. A fold change between 0 and 1 is down-regulation in exposed cells. The LipidMaps database was taken as the reference for the lipid nomenclature. LC-MS cannot discriminate between galactose and glucose, or N-acetylglucosamine and N-acetylgalactosamine. E.g. LacCer can also be Gal-GalCer. Therefore, lipid nomenclature contains hexose (Hex) instead of glucose or galactose. Relative standard deviation (RSD%) has been calculated from the QC samples (n=6).

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CHAPTER 10

Summary, Discussion and Future Perspectives

SUMMARY

Asthma and COPD are heterogeneous diseases composed of different phenotypes, and some phenotypes have been investigated in this thesis. The first part focuses on the effects of corticosteroid treatment and the second part of this thesis focuses on novel methods to investigate obstructive airway diseases, which may help to identify new phenotypes.

Corticosteroid sensitivity

Chapter 2 reviews the literature on the effects of corticosteroids in COPD. It shows that short-term treatment (<1 year) with ICS, both with and without long-acting β2-agonists, improves lung function and quality of life. In addition, several studies with longer follow-up demonstrated less decline in quality of life over time and fewer exacerbations. In contrast, long-term studies (≥1 year) were unable to show a substantial reduction in the decline of lung function in COPD. Based on these findings, it was concluded that the use of ICS does not influence the natural course of COPD. However, this conclusion has been challenged by two subsequent studies. The TORCH and GLUCOLD studies both showed a reduction in lung function decline over time with the use of ICS, demonstrating that at least a subgroup of COPD patients is sensitive to corticosteroid treatment. Taken together, the current evidence suggests that ICS can influence the natural course of COPD in a subgroup of patients. Although, to date it remains unclear which differential phenotypes determine a good corticosteroid response

Chapter 3 investigates the effect of smoking on corticosteroid response in asthma patients. In this chapter, we demonstrate that ex- and current-smokers have a different inflammatory profile than never-smokers with asthma. They have less eosinophils and more neutrophils in sputum and blood. In addition, we show that ex- and current-smokers have a reduced short-term corticosteroid response compared to never-smokers, although their long-term response appears to be comparable to never-smokers in this relatively small, but nevertheless considerable number of patients (n=64).

Chapter 4 investigates the corticosteroid sensitivity in obese asthma patients. This chapter shows that the level of lung function and bronchial hyperresponsiveness is comparable between obese (BMI ≥30 kg/m²) and lean (BMI <25 kg/m²) asthma patients. Interestingly, we found that obese asthma patients have more neutrophilic inflammation in sputum and blood. The latter may also explain the finding that obese asthmatics have a worse corticosteroid treatment response, as reflected by less improvement in lung function and eosinophilic inflammation, than lean patients after 2-week corticosteroid treatment.

In **Chapter 5** the corticosteroid skin-blanching test is used as a test for corticosteroid sensitivity. This study shows that the skin-blanching score is similar in asthma patients

and in healthy controls. However, asthma patients with airway obstruction (FEV_1 <80% of predicted) have a lower skin-blanching score than patients without, suggesting a reduced corticosteroid sensitivity. In addition, a lower skin-blanching score is associated with more severe airflow obstruction, higher age, female gender and absence of allergy, but not with use of inhaled or oral glucocorticosteroids or packyears smoked. Finally, we found that the skin-blanching score is lower during the summer season. These findings suggest that a reduced corticosteroid sensitivity is associated with a lower level of lung function both in asthma patients and in healthy controls.

Techniques to investigate obstructive airway diseases

Chapter 6 reports the association between small airways obstruction and the severity of bronchial hyperresponsiveness (BHR) in asthma. Asthma patients with small airways obstruction, defined as a mean expiratory flow at 50% of FVC (MEF_{50}) below the lower limit of normal, have more severe BHR than asthma patients without small airways obstruction. In addition, more severe small airways obstruction, as reflected by a lower MEF_{50}, is associated with more severe BHR, independently of large airways obstruction (FEV_1). These findings suggest that the small airways contribute significantly to the severity of BHR, which may have implications for therapy.

Chapter 7 investigates small airways dysfunction at baseline and during methacholine provocation in subjects with asymptomatic BHR, asthma patients and healthy controls. We demonstrate that subjects with asymptomatic BHR have a similar degree of small airways dysfunction as healthy controls, whereas asthma patients have more small airways dysfunction. During methacholine provocation tests, the degree of small airways dysfunction increases to a higher extent in subjects with asymptomatic BHR than in healthy controls, but this increase is less than in patients with asthma. Importantly, an increase in small airways dysfunction, but not large airways dysfunction, during a methacholine provocation test is associated with more dyspnea in subjects with asymptomatic BHR. These results suggest that subjects with asymptomatic BHR experience less symptoms because they have less small airways dysfunction.

Chapter 8 describes the range of airway wall thickness measured by high resolution CT-scans in a group of well characterized healthy subjects. This study shows that airway wall thickness decreases at a higher age, likely reflecting structural changes of the airways during aging. In addition, we show that airway wall thickness is higher in current-smokers than in never-smokers and that thicker airway walls are associated with lower levels of lung function, independent from age, gender, height and smoking status.

Chapter 9 investigates the role of differentially expressed lipids in COPD. This study shows that 55 lipids are upregulated in induced sputum of smokers with COPD compared to

smokers without COPD. 41 of these lipids are from the sphingolipid pathway, a pathway known to be involved in apoptosis and has previously been implicated in the development of emphysema. In contrast, only 12 lipids are upregulated in smokers without COPD compared to never-smokers without COPD and 7 of these are known tobacco components. Furthermore we performed analyses before and after smoking cessation, smoking being the only factor that is currently available to modify the course of the disease, i.e. reducing the relentlessly progressive lung function decline in COPD. We show that 2-month smoking cessation reduces expression of 17 lipids in sputum of smokers with and without COPD. This corroborates their role in COPD development. Additionally, long-term (6 months) exposure to cigarette smoke extract resulted in a significant change in the lipidome of the human bronchial epithelial cell line, BEAS-2B, comparable to differential findings in induced sputum of smokers with and without COPD. These results suggest that lipids may play an important role in the pathogenesis of COPD.

DISCUSSION AND FUTURE PERSPECTIVE

It is now widely accepted that corticosteroids are less effective in COPD than in asthma, as they appear not to influence the decline in FEV_1 in COPD. However, the GLUCOLD and TORCH studies challenge this belief, as they shows that ICS can reduce the decline in FEV_1, at least in a subgroup of patients.[1,2] Several factors may influence the corticosteroid sensitivity of COPD patients. First, the part of the lung that is most affected by the disease could be relevant. Some patients have predominantly parenchymal destruction, i.e. emphysema, whereas other patients have predominantly inflammation of the airways. ICS will likely be less effective in COPD patients where the inflammation has led to emphysema, since the destruction of parenchyma cannot be redressed by anti-inflammatory treatment. However, in patients where there is predominantly inflammation of the airways the anti-inflammatory effects of ICS are more likely to be effective. Studies supporting this hypothesis show that patients with more reversibility to β_2-agonists and more severe BHR, which can be considered as markers of ongoing inflammation of the airways, respond better to ICS treatment.[3–5] Additionally, the type of airway inflammation may be important. The most commonly observed type of inflammation in COPD is neutrophilic inflammation, which is associated with reduced corticosteroid sensitivity.[6] However, there are also COPD patients with eosinophilic airway inflammation and these patients show increased corticosteroid sensitivity.[7,8] Although studies have reported that higher corticosteroid sensitivity exists in patients with reversibility to β_2-agonists, more severe BHR or eosinophilic airway inflammation, the predictive value of these measurements is too low to make clinical decisions as to the treatment of individual patients, at least during stable disease. In contrast, during a COPD exacerbation, the blood eosinophil levels can be used to direct corticosteroid treatment.[9] Markers from new techniques, like gene expression, proteomics or lipidomics, and combininations of different measurements,

including genetics,[10] may result in better prediction models for corticosteroids sensitivity in COPD in the future.

Another possible explanation why even large studies did not find an effect of ICS on FEV_1 decline in COPD may be that these studies used large-particle ICS. Large-particle ICS will deposit preferentially in the large airways and less in the small airways.[11] However, there is strong evidence that the small airways are important in COPD. Hogg *et al.* showed that the small airways are narrowed and inflamed in COPD patients.[12] Furthermore, ventilation heterogeneity of the acinar airways measured with multiple breath nitrogen washout, a marker of small airways obstruction, is grossly abnormal in COPD patients.[13] There is also suggestive evidence that the small airways contribute to the severity of BHR in COPD patients.[14] Taken together, these results suggest that targeting the small airways with anti-inflammatory treatment may have beneficial effects in COPD. As small-particle ICS deposit in the small airways, it could be that they will have better results than large-particle ICS in COPD patients. So far, only one double-blind study with small-particle beclomethasone in 16 COPD patients has been performed.[15] This study showed no effect of 3-week small-particle treatment on small or large airway parameters compared to placebo. However, because of the small study size and short treatment duration, this study certainly does not exclude an effect of small particle treatment in COPD. Larger and longer studies are needed to investigate the effects of small-particle ICS in COPD. These studies should also focus on which patients would benefit from small-particle ICS treatment, for instance patients with baseline small airways obstruction or BHR. In asthma, small airways obstruction, i.e. acinar ventilation heterogeneity with multiple breath nitrogen washout, improved consistently after treatment with small-particle ICS only in those who had small airways obstruction at baseline.[16] Perhaps abnormal acinar ventilation heterogeneity can also identify COPD patients with small airways obstruction who would benefit from small-particle ICS or bronchodilator treatment. It should also be investigated at which time point small-particle treatment would be most beneficial. During the progression of COPD there is destruction of small airways, which is then followed by parenchymal destruction, leading to emphysema.[17] It is possible that emphysema can be prevented by early treatment of the small airways with small-particle ICS in COPD patients who do have small airways inflammation and obstruction, but have not yet developed emphysema.

Besides COPD, there are also phenotypes of asthma that are less responsive to corticosteroid treatment. This thesis provides evidence that both obese and smoking asthmatics show less improvement in FEV_1 after short-term corticosteroid treatment. The precise mechanisms behind this reduced corticosteroid responsiveness are unclear. The type of inflammation that these patients demonstrate appears to be important, as both obese and smoking asthma patients have more neutrophilic airway inflammation, which has been associated with a reduced corticosteroid treatment response in asthma.[18,19] It is also unclear if obesity changes the phenotype in established asthma to one that is less responsive to corticosteroids or that obesity induces new asthma with a phenotype that is less responsive

to corticosteroids. On one hand, pro-inflammatory cytokines produced by adipose tissue may influence the already present inflammation in the airways of asthma patients and thereby change the inflammatory pattern to one that is less responsive to corticosteroids. On the other hand, it has also been shown that obesity is a risk factor to develop asthma and in a study by Haldar *et al.* there was a cluster of obese asthma patients with late-onset asthma.[20,21] Perhaps the pro-inflammatory cytokines from adipose tissue promote airway inflammation in certain susceptible obese subjects, thereby causing a late-onset phenotype of asthma with a type of inflammation that is less responsive to corticosteroids. Longitudinal cohort studies may help to disentangle the interactions between the effects of obesity on existing asthma, on the development of asthma and corticosteroid responsiveness.

In smoking asthmatics, the responsiveness to corticosteroids is reduced after short-term ICS treatment. However, in a study by O'Byrne *et al.* responsiveness to ICS after 1 and 3 years of treatment was similar to the responsiveness in never-smokers, which is consistent with our findings of chapter 3.[22] In addition, a study in smoking asthmatics showed that the reduced corticosteroid responsiveness could be overcome by increasing the dose of ICS. These results suggest that the reduced corticosteroid responsiveness in smoking asthmatics can be attenuated by prolonged treatment and higher doses of ICS. It would be interesting to see if similar effects can also be observed in obese asthmatics.

It would be very useful to have a simple, non-invasive test to determine how a patient will respond to treatment with corticosteroids. In chapter 5 we describe that asthma patients and healthy controls with a lower skin-blanching score on the corticosteroid skin-blanching test also show lower levels of lung function. It is tempting to speculate that the corticosteroid skin-blanching test reflects the corticosteroid sensitivity of the airways in asthma and COPD and can thus be used to predict corticosteroid sensitivity in these diseases. So far, this has been investigated in only one study in a group of 22 asthma patients.[23] In this study, the skin-blanching score was not associated with changes in serum cortisol levels or severity of bronchial hyperresponsiveness after 3-week treatment with ICS. However, this does not per se mean that the skin-blanching test cannot be used to predict corticosteroid sensitivity. In chapter 5 we show that the skin-blanching score is influenced by a number of variables, like age, gender, absence or presence of allergy and even the season in which the test was performed. However, there is still a large part of the variability in the skin-blanching score that cannot be explained. Further research into the variability of the skin-blanching score, for instance by investigating the effect of genetic variation on the skin-blanching score, may reveal new insights in the mechanisms behind corticosteroid sensitivity. It is also possible that after correcting for variables influencing the skin-blanching score the corticosteroid skin-blanching test is able to predict the corticosteroid sensitivity or that applying the corticosteroid skin-blanching test in selected phenotypes will give more information. For instance, it could be that the corticosteroid skin-blanching test is not able to identify corticosteroid sensitivity in all asthma patients, but is able to identify corticosteroid sensitive patients in groups of patients that are generally considered to be corticosteroid insensitive, like smoking or obese asthma.

The second part of this thesis is on techniques that can identify new phenotypes of obstructive airway diseases, such as impulse oscillometry (IOS) to identify small airways obstruction, measuring airway wall thickness on high resolution CT-scans and identification of differentially regulated lipids in induced sputum. The phenotypes that can be identified with these techniques are of great interest for research, as they can increase our knowledge about the mechanisms underlying the development and progression of obstructive airway diseases, which can help in the development of new treatments. On the other hand, the implications of using the phenotypes thus found in clinical care or screening are less clear at the moment. There are several requirements that need to be fulfilled before a phenotype can be used in daily clinical practice or screening. First, the phenotype needs to be relevant. This means that in daily clinical practice that the phenotype should direct treatment, i.e. a different treatment or more / less intensive treatment. For screening purposes, the technique should be able to accurately identify patients at risk to develop the disease and for whom an intervention is available. For instance, asymptomatic smokers with a greatly increased risk to develop COPD could be actively encouraged to stop smoking. A phenotype that identifies subjects at risk to develop a disease which cannot be prevented by any intervention is of limited use. Second, a clinically useful phenotype needs to be identified by a test that can be incorporated in the daily clinical workflow, i.e. the test should be easy to perform at acceptable costs. Already in 2002, Green *et al.* demonstrated that guiding asthma treatment based on the level of sputum eosinophils significantly reduces asthma exacerbations and hospital admissions, without the need for additional anti-inflammatory treatment.[24] However, this method for guiding treatment has not been widely introduced in clinical care, mostly because the induced sputum process is labor intensive, time consuming and costly. Finally, new phenotypes and their treatment need to have been prospectively validated in randomized clinical trials before being introduced in guidelines and standard clinical care.

An important advantage of IOS to determine small airways obstruction is the ease of the measurement; the IOS machine can be attached to a normal spirometer, the measurement takes less than a minute to perform and is performed during tidal breathing, hence easy to perform by patients. Since the IOS measurement can be performed quickly, it is possible to investigate small airways obstruction during provocation tests, as we showed in chapter 7. This chapter demonstrated that subjects with asymptomatic BHR do not have small airways obstruction at baseline but do have small airways obstruction during provocation. BHR is a risk factor to develop both asthma and COPD.[25–28] Therefore, it is possible that BHR in these subjects is the first expression of small airway obstruction and inflammation, which in the future could lead to asthma or COPD. Studying the small airways in asymptomatic subjects with and without BHR, or alternatively studying very young children, and following these subjects longitudinally, might even show that asthma and COPD start in the small airways and from there spread to the large airways or parenchyma.

Imaging of the airways has great potential for phenotyping in obstructive airway diseases, most notably in COPD. Many new imaging techniques have been developed over

the past years in order to better investigate the lungs. In this thesis we describe the results of a method to measure airway wall thickness on high resolution CT-scans. We show that healthy current-smokers have thicker airway walls than healthy never-smokers, possibly indicating remodeling of the airways before symptoms and airflow obstruction become noticeable. This technique may be able to identify patients at risk to develop COPD. In COPD patients, thickening of the airway walls could be an indication of airway inflammation. Therefore it is possible that this technique can identify patients with airway inflammation, who may benefit from treatment with ICS. However, at the present time there is no evidence about the clinical relevance of the phenotypes that can be identified with this new imaging technique. There are several problems that need to be addressed before this technique can be used in clinical care. First, to investigate if phenotypes found with new imaging techniques really identify subjects at risk to develop COPD or COPD patients that respond differently to treatment, large prospective studies are needed. Second, the resolution of current CT-scanning devices is too low to measure airway walls of the small airways.[29] As the small airways are the location where narrowing and destruction of airways are most likely starting,[12] it is very likely that measurements of remodeling of the small airways will give more information than the current measurements of the large airways. Other imaging techniques to measure small airways obstruction have been developed. However, these techniques use indirect measurements of small airways obstruction, such as air-trapping on exhalation scans, and it remains questionable if these techniques are sensitive enough to detect subtle changes in small airways obstruction.[30–33] Future developments in image processing, such as the parametric response map (PRM) method, a voxel-wise image analysis technique, and ultra-high resolution image acquisition, like cone beam CT imaging, may improve measurements of the small airways in the future.[34–36] It is also possible that imaging techniques are not the best way to investigate the small airways and that non-imaging techniques like IOS and multiple breath NO washout are more suitable to investigate the small airways. Finally, there are problems with implementing these new imaging techniques in clinical care. The relatively high radiation exposure of high resolution CT-scans and the high costs of CT-scans limit their usefulness in screening healthy subjects for an increased risk of COPD. The radiation exposure of high resolution CT-scans also limits the use of repeated measurements for follow-up in patients with established disease. Magnetic Resonance Imaging (MRI) with hyperpolarized helium or xenon are imaging techniques without radiation exposure, however the availability to of hyperpolarized helium and xenon is very limited and MRI imaging is even more expensive than CT imaging.[35,37,38] In conclusion, new imaging techniques are currently providing new insights in the pathophysiology of obstructive airway diseases, but many hurdles need to be taken before these techniques can be implemented in clinical care.

New tools available to help investigate lung disease are 'high throughput' screening methods. These methods can quickly conduct a large number of genetic, chemical or pharmacological tests, making it possible to investigate many markers in a short time.

Examples are genotyping, gene expression and protein expression.[39] A relatively new group of molecules of interest are lipids. Until recently lipids were considered to be nothing more than building blocks of cell membranes and energy storage molecules. However, there is now increasing evidence that lipids also have immunomodulatory properties and are involved in various diseases.[40,41] A class of lipids that is implicated in inflammatory diseases, including COPD, are the sphingolipids.[42] In this thesis, liquid chromatography – mass spectrometry (LC-MS) was used to measure the expression of thousands of lipids in induced sputum of COPD patients and controls and in cell homogenates exposed to cigarette smoke extract. The results of these experiments strongly indicate a role for the upregulation of sphingolipids in the pathogenesis of COPD. However, we performed the first study in this area and our results need to be confirmed in further studies. Nevertheless our findings suggest that lipidomics may be a new and powerful tool in phenotyping airway diseases. Differences in the lipidome of different phenotypes of COPD, like patients with emphysema versus patients with small airway disease, may reveal new information about the pathophysiologic background of these phenotypes. Lipidomics may even be able to identify new phenotypes, as well as revealing new therapeutic targets for different phenotypes. Additionally, it is possible that the lipidomic profile changes early in the onset of COPD and that therefore lipidomics may be used as a biomarker in asymptomatic smokers.

Taken together, advances in research, through many modalities, will help to clarify the heterogeneity in obstructive lung diseases. This will help to identify different phenotypes of these diseases, each with their own specific pathophysiologic backgrounds, enabling targeted treatment. Eventually, this will lead to a shift from the classical one-size-fits-all treatment to treatment specifically tailored for individual patients, also called 'personalized medicine'.

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CHAPTER

Samenvatting in het Nederlands

OBSTRUCTIEVE LONGZIEKTEN

Astma en chronisch obstructief longlijden (COPD) vormen samen de obstructieve longziekten. Bij deze aandoeningen zorgt vernauwing van de luchtwegen voor moeite met in- en uitademen.

Astma

Astma is een ziekte die gekenmerkt wordt door ontsteking van de luchtwegen. Patiënten hebben periodieke aanvallen van benauwdheid, vaak met hoestklachten. Astmaklachten kunnen uitgelokt worden door allergenen zoals bijvoorbeeld huisstofmijt of pollen, of door aspecifieke prikkels zoals sigarettenrook, parfum of koude lucht, en ook door inspanning. Vaak hebben astmapatiënten 's nachts meer klachten. Er is een duidelijk verband tussen astma en allergie: veel astmapatiënten hebben een voorgeschiedenis van atopische klachten zoals eczeem en hooikoorts. De typische presentatie van allergisch astma wordt daarom ook wel de atopische mars genoemd. Dit beschrijft de progressie van eczeem bij baby's en peuters, naar hooikoorts en uiteindelijk astma bij kinderen. Maar niet-allergisch astma en astma op volwassen leeftijd komen ook veel voor. Astma is meestal een chronische ziekte en is tot nu toe niet te genezen.

Ontsteking van de luchtwegen is een essentieel onderdeel van astma, hoewel het type en de ernst van de ontsteking sterkt wisselt tussen patiënten. Bovendien correleert het type en de ernst van de ontsteking niet altijd met de mate van klachten die patiënten ervaren. Onderzoek heeft laten zien dat verschillende soorten ontstekingscellen in verhoogde mate voorkomen in de luchtwegen van astmapatiënten. Bij de meeste astmapatiënten zijn eosinofielen de meest voorkomende cellen, een ontsteking die meestal voorkomt bij mensen met allergisch astma. Andere types van ontsteking zijn: voornamelijk neutrofiel, gemengd eosinofiel en neutrofiel en paucigranulocytair (geen merkbare verhoging van ontstekingscellen). Het type van ontsteking bij astmapatiënten is relevant, omdat verschillende types ontsteking een andere respons op behandeling hebben.

COPD

COPD wordt gekenmerkt door luchtwegobstructie die niet volledig is op te heffen met luchtwegverwijders. Sigarettenrook is de belangrijkste oorzaak van COPD in de westerse wereld. Sigarettenrook veroorzaakt een abnormale ontstekingsreactie bij rokers die hiervoor gevoelig zijn. Deze ontstekingsreactie zorgt er voor dat de longfunctie bij deze mensen versneld achteruit gaat. Als COPD eenmaal ontstaan is kan stoppen met roken de versnelde achteruitgang in longfunctie afremmen. COPD is een belangrijke aandoening die zorgt voor veel ziektelast en mortaliteit wereldwijd; op het ogenblik is COPD de vierde meest voorkomende doodsoorzaak in de wereld.

COPD leidt tot veranderingen in verschillende delen van de long. Per patiënt zijn er grote verschillen in welke mate bepaalde delen van de long zijn aangedaan. In het parenchym

(de longblaasjes) zorgt de ontstekingsreactie door sigarettenrook tot vernietiging van het parenchym, ook wel bekend als emfyseem. In de luchtwegen zorgt de ontstekingsreactie voor verdikking van de luchtwegwand al dan niet in combinatie met toegenomen productie van slijm.

FENOTYPES VAN OBSTRUCTIEVE LONGZIEKTEN

Astma en COPD zijn complexe ziekte die beïnvloed worden door genetische en omgevingsfactoren. Hierdoor zijn er verschillende klinische presentaties, ook wel fenotypes genoemd. Het bestuderen van deze fenotypes is van belang, omdat de prognose en respons op behandelingen verschillen tussen fenotypes. In dit proefschrift onderzoeken we verschillende fenotypes van obstructieve longziekten. Het proefschrift bestaat uit twee delen. Het eerste deel beschrijft de respons op behandeling met corticosteroïden bij verschillende fenotypes van astma en COPD. Het tweede deel beschrijft verschillende onderzoeksmethodes waarmee obstructieve longziekten kunnen worden onderzocht en waarmee in de toekomst mogelijk nieuwe fenotypes geïdentificeerd kunnen worden.

CORTICOSTEROÏD GEVOELIGHEID

Corticosteroïden zijn ontstekingsremmende medicijnen die gebruikt worden bij de behandeling van zowel astma als COPD. Bij astma zijn geïnhaleerde corticosteroïden (ICS) de hoeksteen van de behandeling. Ze verbeteren luchtwegklachten, kwaliteit van leven en longfunctie, verminderen bronchiale hyperreactiviteit, ontsteking in de luchtwegen, de frequentie en ernst van exacerbaties en het risico op overlijden door astma. Bij COPD lijkt de effectiviteit van ICS minder uitgesproken te zijn. Echter, ook bij astma reageren niet alle patiënten even goed op behandeling met ICS en er zijn zelfs astmapatiënten die helemaal niet reageren op behandeling met ICS.

In **Hoofdstuk 2** wordt een overzicht gegeven van de literatuur over het gebruik van corticosteroïden bij COPD. Dit hoofdstuk laat zien dat kortdurende behandeling met ICS de longfunctie en kwaliteit van leven verbeteren. Verder verminderen ICS bij langdurig gebruik de achteruitgang in kwaliteit van leven en het aantal exacerbaties. Echter, een aantal langdurige (>1 jaar) onderzoeken lieten geen verbetering in achteruitgang longfunctie zien. Hierop werd geconcludeerd dat ICS het natuurlijk beloop van COPD niet beïnvloeden. Deze conclusie wordt in twijfel getrokken door twee meer recente onderzoeken, de 'Toward a Revolution in COPD Health' (TORCH) en de 'Groningen Leiden Universities Corticosteroids in Obstructive Lung Disease' (GLUCOLD) studies. Deze twee onderzoeken laten beide wel een verbetering in de achteruitgang in longfunctie zien en geven daarmee aan dat ICS het natuurlijk beloop van COPD wel kunnen beïnvloeden, al was het alleen maar bij een

subgroep van de patiënten. Mogelijk zijn dit patiënten waarbij er voornamelijk ontsteking in de luchtwegen is en minder in het parenchym. Op het ogenblik is het nog niet mogelijk om die patiënten te identificeren die het meeste baat zullen hebben bij behandeling met ICS..

Aangezien COPD patiënten minder goed reageren op behandeling met ICS en roken de belangrijkste risicofactor is voor het ontwikkelen van COPD, wordt in **hoofdstuk 3** onderzocht wat het effect van roken is binnen astma. In dit hoofdstuk wordt het type ontsteking en de behandelrespons op ICS van voormalig rokende, huidig rokende en nooit rokende astmapatiënten besproken. We laten zien dat zowel voormalig rokende als huidig rokende astmapatiënten minder eosinofielen en meer neutrofielen hebben in hun sputum en bloed dan nooit rokers. Ook laten we zien dat voormalig en huidige rokers minder verbeteren na korte termijn behandeling met ICS, echter de lange termijn respons lijkt wel vergelijkbaar te zijn met die van nooit rokers.

Vervolgens onderzochten we of andere astmafenotypes ook een lagere behandelrespons op corticosteroïden hebben, zoals astmapatiënten met obesitas. **Hoofdstuk 4** behandelt het type ontsteking en de behandelrespons op corticosteroïden bij astmapatiënten met obesitas en astmapatiënten met normaal gewicht. Dit hoofdstuk toont aan dat er bij de start van het onderzoek geen verschil is in longfunctie of mate van bronchiale hyperreactiviteit tussen astmapatiënten met obesitas (body mass index [BMI] ≥ 30 kg/m^2) en astmapatiënten met normaal gewicht (BMI <25 kg/m^2). Obese patiënten hebben wel meer neutrofiele inflammatie. Na een behandeling van 2 weken met corticosteroïden hebben obese patiënten een significant mindere verbetering van longfunctie en percentage sputum eosinofielen dan patiënten met normaal gewicht. Deze verminderde verbetering suggereert dat astmapatiënten met obesitas een lagere behandelrespons op corticosteroïden hebben dan patiënten met een normaal gewicht, mogelijk door de meer neutrofiele ontsteking van de luchtwegen.

In **hoofdstuk 5** wordt een test beschreven waarmee de gevoeligheid voor corticosteroïden gemeten kan worden: de corticosteroïd huidtest. Bij deze test worden verschillende concentraties van het corticosteroïd budesonide op de huid aangebracht. Hoe gevoeliger een persoon is voor corticosteroïden, hoe meer de huid verbleekt. Dit onderzoek toont aan dat er geen verschil is in gevoeligheid voor corticosteroïden tussen astmapatiënten en gezonde controles. Echter, astmapatiënten met luchtwegobstructie (FEV_1 <80%) hebben een lagere corticosteroïd gevoeligheid dan astmapatiënten zonder luchtwegobstructie. Verder is een lagere corticosteroïd gevoeligheid geassocieerd met slechtere longfunctie (FEV_1 % van voorspeld), hogere leeftijd, vrouwelijk geslacht en afwezigheid van allergie, maar niet met gebruik van corticosteroïden of het aantal gerookte pakjaren. Bovendien is de uitslag van de corticosteroïd huidtest lager bij een test afgenomen in de zomer. Deze resultaten laten zien dat de aangeboren gevoeligheid voor corticosteroïden invloed heeft op longfunctie, zowel bij astmapatiënten als bij gezonde mensen.

METHODES VOOR ONDERZOEK IN LONGZIEKTEN

Het tweede gedeelte van dit proefschrift behandeld onderzoeksmethodes die nieuwe inzichten kunnen geven bij obstructieve longziekten en die in de toekomst kunnen helpen met het identificeren van nieuwe fenotypes.

Impuls oscillometrie om kleine luchtwegobstructie te meten bij bronchiale hyperreactiviteit

De afgelopen jaren is er toenemende interesse in de rol van kleine luchtwegen bij astma. De kleine luchtwegen zijn luchtwegen met een externe diameter <2 mm. Vroeger werden de kleine luchtwegen de 'stille zone' genoemd, aangezien ze onder normale omstandigheden maar ongeveer 10% bijdragen aan de totale luchtwegweerstand. Recente onderzoeken laten echter zien dat de kleine luchtwegen de voornaamste locatie van luchtwegobstructie zijn bij astmapatiënten.

Een ander belangrijk kenmerk van astma is bronchiale hyperreactiviteit (BHR), vernauwing van de luchtwegen als reactie op een prikkel die onschuldig is voor een gezond persoon, zoals sigarettenrook, parfum, koude lucht en droge of juist vochtige lucht. De aanwezigheid van BHR kan gemeten worden tijdens een provocatietest. Hierbij ademt een patiënt toenemende concentraties van een prikkelende stof in, bijvoorbeeld metacholine, histamine of adenosine 5'-monophosphate. De ernst van de hyperreactiviteit wordt uitgedrukt als de concentratie van een prikkelende stof waarop de FEV_1 met 20% afneemt. Het is algemeen geaccepteerd dat vernauwing in de grote luchtwegen bijdraagt aan de ernst van BHR, echter er zijn ook aanwijzingen dat astmapatiënten met BHR toegenomen vernauwing van de kleine luchtwegen hebben.

Hoofdstuk 6 laat zien dat patenten met vernauwing van de kleine luchtwegen ernstiger BHR hebben dan astmapatiënten zonder vernauwing van de kleine luchtwegen. Bovendien is ernstigere vernauwing van de kleine luchtwegen geassocieerd met ernstiger BHR, onafhankelijk van de mate van vernauwing in de grote luchtwegen. Deze bevindingen suggereren dat de kleine luchtwegen bijdragen aan de ernst van BHR.

Een methode om obstructie van de kleine luchtwegen te meten is met 'impulse oscillometry' (IOS). Tijdens een IOS meting wordt tijdens normale ademhaling door een luidspreker een drukgolf van verschillende frequenties door de luchtwegen gestuurd. De resulterende veranderingen in luchtstroom en druk kunnen voor elke frequentie gemeten worden bij de mond. Vervolgens kan de weerstand in de kleine luchtwegen berekend worden, gebaseerd op het principe de lagere frequenties door de hele long voorgeleidt worden terwijl de hoge frequenties alleen de grote luchtwegen bereiken. Een IOS meting is gemakkelijk uit te voeren, aangezien de meting maar enkele seconden duurt en geen inspanning vereist van de patiënt. Hierdoor is het mogelijk een meting te verrichten na elke stap van provocatietest en kan dus vernauwing van de kleine luchtwegen tijdens een provocatietest gemeten worden.

Hoofdstuk 7 beschrijft de mate van vernauwing van de kleine luchtwegen bij mensen met asymptomatische BHR (wel BHR, maar geen astma of luchtwegklachten), astmapatiënten en gezonde controles (geen BHR, geen luchtwegklachten) zowel op baseline als tijdens een provocatietest. Dit hoofdstuk laat zien dat op baseline mensen met asymptomatische BHR even veel vernauwing van de kleine luchtwegen hebben als gezonde controles, terwijl astmapatiënten juist meer vernauwing van de kleine luchtwegen hebben. Echter tijdens een provocatietest hebben mensen met asymptomatische BHR juist meer vernauwing van de kleine luchtwegen dan gezonde controles, maar nog wel minder dan astmapatiënten. Bovendien is meer vernauwing van de kleine luchtwegen tijdens een provocatietest geassocieerd met meer benauwdheid tijdens de provocatietest bij de mensen met asymptomatische BHR. Deze resultaten suggereren dat mensen met asymptomatische BHR minder klachten ervaren dan astmapatiënten, omdat ze minder obstructie van de kleine luchtwegen hebben. n mensen met asymptomatische BHR (wel BHR, maar geen astma of klachten)en.

Luchtwegwanddikte op CT-scans

Een belangrijk kenmerk van obstructievelongziekten is verandering van luchtwegwanden. De chronische ontstekingen van de luchtwegen bij astma en COPD veroorzaakt veranderingen in het bekledend epitheel, de basaalmembraan, de extra-cellulaire matrix en in het gladde spierweefsel rondom de luchtweg. Deze veranderingen leiden uiteindelijk tot verdikking van de luchtwegwand. Een nieuwe, niet-invasieve methode om de verandering van de luchtwegwand te onderzoeken is door de luchtwegwanddikte te meten op 'computed tomography' (CT) scans. **Hoofdstuk 8** beschrijft de luchtwegwanddikte bij een groep gezonde vrijwilligers. We laten zien dat de luchtwegwanddikte afneemt bij hogere leeftijd en dat de luchtwegwand dikker is bij rokers dan bij niet-rokers. Bovendien laten we zien dat een dikkere luchtwegwand geassocieerd is met lagere longfunctie, onafhankelijk van leeftijd, lengte, geslacht, en rookstatus. Mogelijk is de dikkere luchtwegwand bij rokers een teken van verandering van de luchtwegen nog voordat klachten of luchtwegobstructie ontstaan. In de toekomst kan het meten van de luchtwegwanddikte op CT-scans mogelijk gebruikt worden voor het opsporen van rokers met een verhoogd risico om COPD te ontwikkelen.

Lipidomics in COPD

Lipidomics is het onderzoeken van de expressievan lipiden in weefsels oflichaamsvloeistoffen. Lang is gedacht dat lipiden alleen bouwstoffen van celwanden of moleculen voor energieopslag waren. Er is echter een toenemende interesse in de invloed van lipiden op inflammatie. Een groep lipiden, de sphingolipiden, zijn betrokken bij zowel apoptose en cel overleving. **Hoofdstuk 9** onderzoekt de expressie van lipiden zowel in geïnduceerd sputum van COPD patiënten en gezonde controles, maar ook in cellijnen die langere tijd (6 maanden) aan sigarettenrookextract (CSE) zijn blootgesteld. We laten zien dat 55 lipiden (waarvan 41 sphingolipiden) een hogere expressie hebben in sputum van COPD patiënten dan in sputum

van gezonde rokers. Als we gezonde rokers vergelijken met niet-rokende gezonde deelnemers hebben maar 12 lipiden een hogere expressie bij de rokers dan bij de niet-rokers en 8 hiervan zijn bestanddelen van sigarettenrook. We laten ook zien dat 2 maanden na stoppen met roken de expressie van 16 lipiden bij gezonde rokers en COPD patiënten een lagere expressie hebben dan voor het stoppen met roken. In epitheliale cellijnen zorgt de blootstelling aan CSE voor een verandering in de expressie van 25 lipiden. Deze veranderingen komen grotendeels overeen met de veranderingen in het patiëntenmateriaal. Deze resultaten suggereren een belangrijke rol voor veranderingen in lipiden expressie, voornamelijk van de sphingolipiden, bij het ontstaan van COPD. Dit nieuwe onderzoeksveld kan de kennis over het ontstaan van COPD vergroten en mogelijk leiden tot de ontdekking van nieuwe aangrijpingspunten voor medicatie en biomarkers voor COPD.

CONCLUSIE

Vooruitgangen in het onderzoek naar de heterogeniteit van obstructieve longziekten, met behulp van verschillende onderzoeksmethoden, kan inzicht verschaffen in de verschillende fenotypes van deze ziektes. Hierdoor zullen in de toekomst beter gerichte behandelingen beschikbaar komen. Dit zal uiteindelijk leiden dat niet elke astma of COPD patiënt op dezelfde manier behandeld zal worden, maar op een manier die afgesteld is op het specifieke fenotype van de patiënt, ook wel 'personalized medicine' genoemd.

DANKWOORD

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