Novel views on endotyping asthma, its remission, and COPD

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Chapter 1

General introduction

1.1 Asthma

Asthma is the most common inflammatory disease of the lung, affecting around 300 million individuals worldwide, with still increasing prevalence [1]. Due to the high incidence, the chronicity of the condition, and morbidity of the patients, the disease imposes a significant burden on the health care system [1]. Asthma causes symptoms such as wheezing, shortness of breath, chest tightness and cough, that fluctuate in severity over time [1]. These symptoms are associated with the level of airflow obstruction, which in turn is caused by bronchoconstriction (i.e. contraction of smooth muscle bundles around the airways), increased mucus production, and airway wall thickening (due to e.g. influx of inflammatory cells and airway wall remodeling) [1].

The word asthma comes from the Greek ασθμα, literally meaning "hard breathing" or "death rattle" [2]. Homer used this term to describe the extreme breathlessness of Hector after being nearly defeated by Ajax, during the battle of Troy [3]. The various connotations of asthma and its symptoms highlight the fact that it is not one, homogenous condition [4]. Indeed, the previously described causes of airflow obstruction also suggest multiple underlying pathophysiological processes [5]. Although widely recognized as a heterogeneous disease, the diagnosis and assessment of asthma are predominantly focussed on the clinical expression of the disease, such as symptoms and loss of pulmonary function. The severity of airflow limitation, as measured by the reduction in forced expiratory volume in one second (FEV_1) and its variability over time, after salbutamol, or after a bronchoconstrictive agent, provides the most useful information to the physician with respect to diagnosis and treatment options [1,6].

Based on demographics and clinical features, asthmatics can be clustered into "phenotypes". Phenotypes are defined as 'observable properties of an organism produced by the interaction of its genes and the environment' [7–9]. Examples of asthma phenotypes are shown in table 1 [9].

Table 1: asthma phenotypes in relation to clinical and pathological characteristics

Phenotype	Natural history	Clinical and physiological features	Pathobiology and biomarkers
Early-onset allergic	Early onset; mild to severe	Allergic symptoms and other diseases	Eosinophils; specific IgE; TH2 cytokines; thick subepithelial basement membrane
Late-onset	Adult onset; often severe	Sinusitis; less allergic	Corticosteroid-refractory eosinophilia; IL-5
Exercise-induced	-	Mild; intermittent with exercise	Mast-cell activation; TH2 cytokines; cysteinyl leukotrienes
Obesity-related	Adult onset	Women are primarily affected; very symptomatic; airway hyperresponsiveness less clear	Lack of TH2 biomarkers; oxidative stress
Smoking-related	-	Low FEV_1; more air trapping	Sputum neutrophilia; TH17 pathways; IL-8

Adapted with (Wenzel SE, et al. Nat Med. 2012 May 4;18(5):716-25)

Decades ago, these phenotypes, which were the only way to describe any disease, largely lacked clinical consequences [4,10]. The introduction of 'endotypes' provides caregivers with more insight into who will and will not respond to therapy [8]. Endotypes are subtypes of a phenotype, defined by a distinct pathogenic mechanism [5,8,10,11]. Approaches to define endotypes in asthma can, among others, be based on features of airway wall remodeling [11–13], eosinophilic inflammation in different lung compartments [11,13], changes in the microbiome [14], volatile compartment composition in exhaled breath [15], gene-expression signatures (e.g. single-cell RNA-sequencing) [16,17], proteomics (i.e. set of proteins expressed in a sample), and other –omic modalities [8] (figure 1). The description of asthma endotypes enables us to integrate clinical features, established laboratory parameters, and novel biomarkers, in order to elucidate the multi-layered heterogeneity of asthma. Endotyping started around 1958, when Brown *et al.* showed that sputum eosinophils predict corticosteroid responsiveness in asthma [18]. Thereafter, different endotypes were determined, such as eosinophilic and non-eosinophilic asthma [5,9,19,20]. Nowadays, distinct pathogenic pathways (examples listed in table 1) are recognised, which were mainly identified in experimental animal models. The attribution of discrete pathogenic mechanisms to specific endotypes of asthma allows tailored interventions, such as anti-IgE [21], anti-

IL-4/-IL-13 [22], anti-IL-13 [23], and anti-IL5 [24]. However, some endotypes, like non-eosinophilic asthma, lack a clear pathogenic mechanism and/or viable therapeutic options. A better understanding of the underlying biological mechanisms driving these endotypes should unveil new treatment targets.

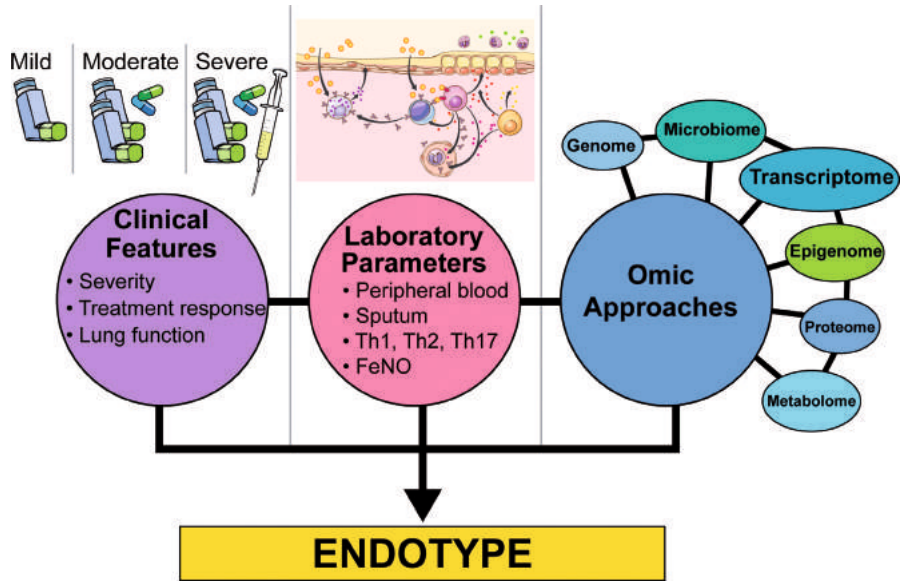

Figure 1: Approaches to endotype asthma.
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Airway inflammation is a prominent feature of asthma. Evidence of elevated type 2-helper T cell (TH2)-associated responses like increased eosinophil numbers in blood, sputum or biopsies or elevated exhaled NO levels, can be found in more than 80% of children and 35 – 50% of adults with asthma [16,25–28]. TH2-driven inflammation is seen in the majority of early-onset allergic, eosinophilic late-onset, and exercise-induced asthmatics [9]. TH2 pathways can be addressed by targeted therapy (e.g. inhaled corticosteroid, anti-IL-5, and anti-IL-13 treatment) and offer opportunities to be linked as a biomarker for therapy response [23,29–31]. One proposed biomarker for TH2 inflammation is periostin, an IL-5 and IL-13 inducible extracellular matrix protein secreted by structural cells of the airways such as basal epithelial cells [17,32]. The role of periostin in asthma and TH2-driven inflammatory responses is an area of active research [33]. The gene encoding periostin is part of a transcriptional TH2 signature in sputum,

that has been successfully used as a biomarker for the response to corticosteroids [16], and has been put forward as a biomarker for anti-IL13 responsiveness in asthmatics [23]. Notwithstanding, currently available literature does not sufficiently support the use of serum periostin levels as a biomarker in clinical practice.

A relatively unexplored, difficult to treat phenotype, is asthma with obesity [34]. Both asthma and obesity are based on observable properties (i.e. phenotypes) and asthma with obesity can thus be defined as a mix of phenotypes, yet the underlying interaction between these diseases is likely to induce a discrete asthma endotype [35,36]. For instance, a previous study found no difference in asthma severity between obese and non-obese asthma patients, but the asthma patients with obesity were characterized by an increased number of neutrophils in the blood and sputum [37]. This is of interest since neutrophilic inflammation has been described as a clinical feature associated with specific asthma endotypes and might reflect a specific pathogenic mechanism (e.g. TH17 inflammation) amendable to tailored therapy [38]. Moreover, while it is known that patients with obesity have a higher risk for developing asthma compared to the general population [36], the risk factors causing this relation might be related to insulin resistance or other components of the metabolic syndrome, rather than to the higher body mass index per se [35,39]. Understanding the unique mechanisms of the asthma-obesity syndrome could potentially reveal new therapeutic options for this phenotype-combination.

1.2 Asthma remission

Even though asthma is a chronic respiratory disease for which a curative intervention is not available, it has been reported that asthma patients can go into spontaneous remission [40]. Patients in asthma remission are no longer burdened by symptoms, and no longer require any asthma medication. Notwithstanding the lack of symptoms and medication use, patients in asthma remission might still have (asymptomatic) bronchial hyperresponsiveness, low lung function, and ongoing airway inflammation [13]. This situation is referred to as clinical remission. Interestingly, a subset of asthma remission patients has normal lung function and absence of bronchial hyperresponsiveness. This situation is referred to as complete asthma remission [40]. In other words, individuals in complete asthma remission, have been diagnosed with asthma in the past, but

would now be categorized as respiratory healthy by clinicians. Figure 2 shows that individuals with clinical asthma remission have similar blood eosinophil levels as patients with asthma without ICS treatment, and comparable eosinophilic peroxidase immunopositivity in bronchial biopsies compared to ICS-using and –naïve asthmatics. Individuals with complete asthma remission had the lowest degree of eosinophilic inflammation. In agreement with this, complete asthma remission might be the disease state closest to cured asthma.

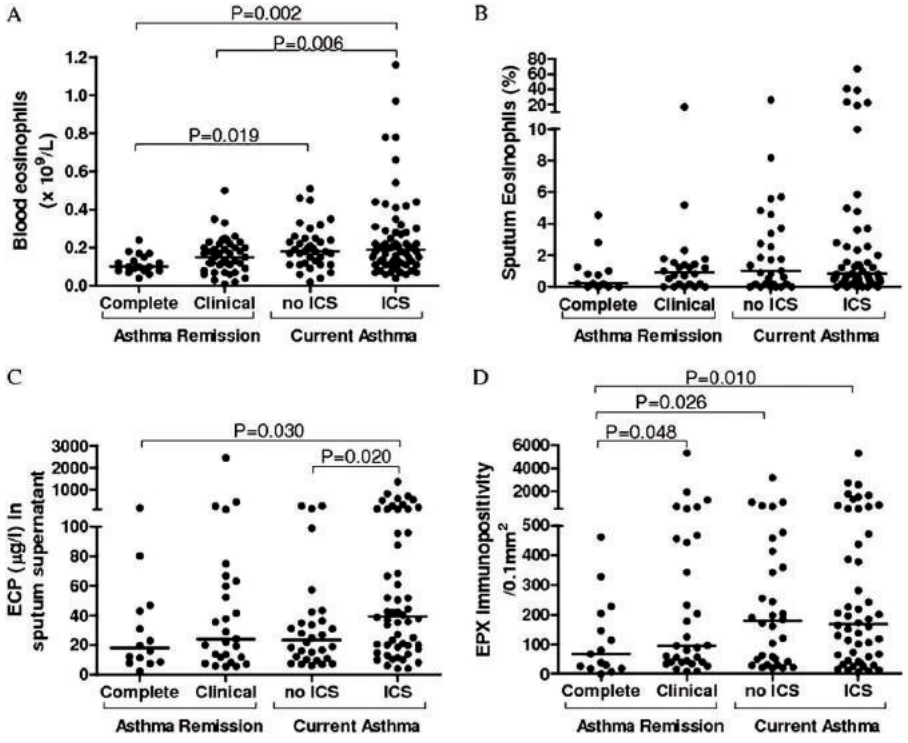

Figure 2: eosinophilic inflammation in inhaled corticosteroid (ICS) naïve and ICS-using asthmatics, clinical- and complete asthma remission subjects. **A:** absolute blood eosinophils, **B:** percentage of sputum eosinophils, **C:** eosinophil cationic protein (ECP) in sputum supernatant, **D:** eosinophilic peroxidase (EPX) immunopositivity (pixels) in bronchial biopsies P values of EPX immunopositivity are after correction for age, sex, and smoking using multiple regression analysis. Horizontal bars represent median values. Reprinted with permission (Broekema M et al. Am J Respir Crit Care Med. 2011 Feb 1;183(3):310-6).

To date, the molecular and cellular mechanisms of asthma remission are not known. Understanding its biology is an important research goal as it could potentially reveal

novel biological targets that can be addressed therapeutically to induce complete asthma remission and cure the disease. As such, eliciting complete asthma remission in patients with persistent disease would be, together with asthma prevention, the ultimate therapeutic goal. Nevertheless, the vast majority of studies focus on clinical asthma remission. But as shown in figure 2, clinical remission still demonstrates features that are similar to persistent asthma. Therefore, it can be hypothesized that the study of complete asthma remission has a higher chance to reveal biological pathways with the capacity to treat asthma or even induce asthma remission.

1.3 Small airways dysfunction

Asthma affects the entire bronchial tree, including the small airways [41,42]. Small airways disease is important since these airways are estimated to comprise 80 - 90% of the total airway surface area [43,44]. The small airways, defined as those with an internal diameter ≤2mm [45], significantly contribute to the airway resistance in patients with obstructive pulmonary disease [41]. Figure 3 illustrates the hypothesis of small airways physiology; in the healthy population, airway resistance gradually decreases from large to small airways due to an exponential increase of the surface area. In conditions with small airways disease such as asthma this is not the case, as inflammatory infiltrates partially block the most distal airways [46,47], causing less peripheral airflow, consequently decreasing gas diffusion in the alveoli [42]. And while the surface of the lumen and airway walls has shrunk, it is postulated that small airways disease reduces both particle deposition and exhalation [42]. Moreover, airway wall remodeling can increase its stiffness, thereby affecting the elasticity and resistance of the small airways [48].

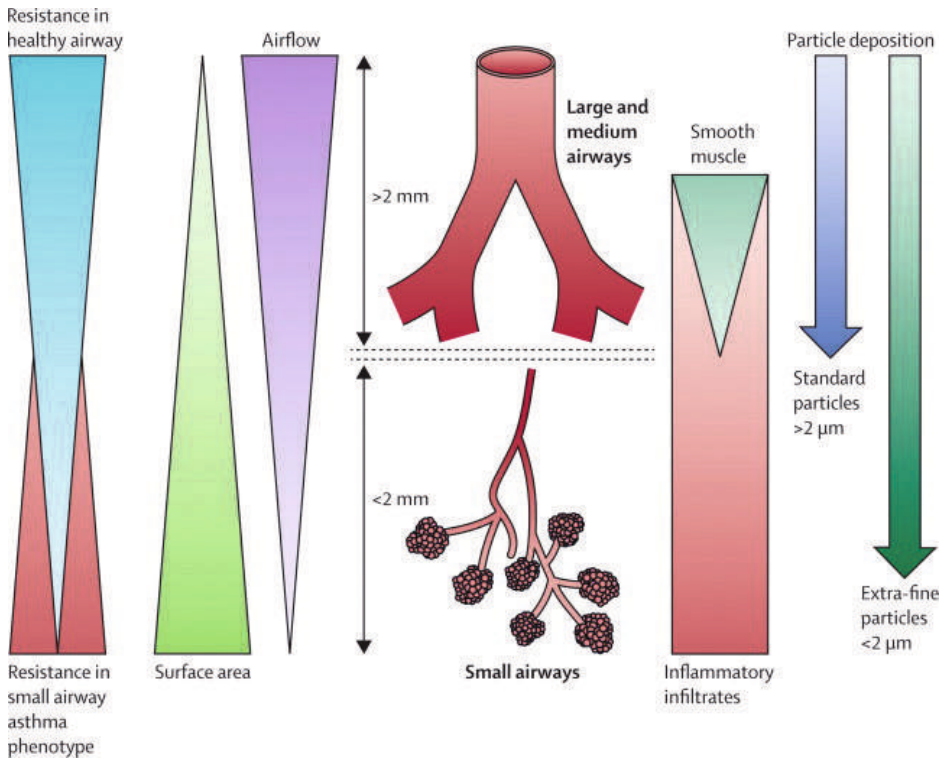

Figure 3: small airway asthma phenotype.
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There are various tools to assess small airways dysfunction [42,49]; these are presented in table 2. The different physiological tests can identify different aspects of the small airways [41], which are increasingly altered per GINA severity stage [41]. However, there is no gold standard for many of these tests, and some are difficult to perform for the severely obstructed patient. For clinical practice, it is important that these measurements delineate different types of small airways disease, which differ in treatment options (e.g. use of ultra-fine inhaled corticosteroids [50]) or prognosis [51].

Table 2: the assessment of small airways

Tool	Outcome	Measures
Spirometry	Dynamic volumes and flow	$FEF_{25-75\%}$
Whole-body plethysmography	Lung volumes and hyperinflation/ air trapping and resistance	Raw, functional residual capacity, ratio of residual volume to total lung capacity
Multiple Breath Nitrogen Washout (MBNW)	Airtrapping and ventilation heterogeneity	Functional residual capacity, ratio of closing volume to vital capacity, ratio of residual volume to total lung capacity, S_{acin}, S_{cond}
Impulse oscillometry (IOS)	Airway resistance and reactance	R_5-R_{20}, reactance area under curve, reactance at 5 Hz, resonant frequency
Exhaled fractional nitric oxide (FeNO)	Airway inflammation	Alveolar and bronchial nitric oxide fractions
Imaging	Air trapping and regional distribution	High-resolution CT parametric response mapping, gamma scintigraphy, PET, MRI, OCT
Bronchoscopy	Airway resistance and inflammation	Wedged airway resistance, transbronchial biopsy, bronchoalveolar lavage
Induced sputum sample investigation	Airway inflammation	Cell and cytokine profile

***$FEF_{25-75\%}$**: forced mid-expiratory flow between 25% and 75% of forced vital capacity. **S_{acin}**: acinar (diffusion) dependent ventilation heterogeneity. **S_{cond}**: conductive (convection) dependent ventilation heterogeneity. **R_5-R_{20}**: peripheral airways resistance as difference between measurements at 5 Hz and 20 Hz. **Raw**: total airway resistance, **OCT**: optical coherence tomography.*
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1.4 COPD

Chronic Obstructive Pulmonary Disease (COPD) is the third leading cause of death worldwide, accounting for approximately three million deaths annually (i.e. 6% of all deaths globally), and its prevalence is still rising [52]. COPD is characterized by respiratory symptoms and persistent airflow obstruction (i.e. FEV_1/FVC ratio <70%) due to airway and alveolar abnormalities (e.g. obstructive bronchiolitis, emphysema, chronic mucus hypersecretion) caused by significant exposure to noxious particles or gases [53]. It is widely accepted that smoking is the main risk factor for COPD, yet only 20 – 30% of smokers will ultimately develop the disease [54]. The classification of disease severity is usually based on exacerbation frequency and the severity of airflow obstruction, but it does not accurately capture the heterogeneity of COPD.

Further clinical characterization, such as measuring blood and sputum inflammatory cell counts, alpha-1-antitrypsine levels (i.e. genetic predisposition), residual volume, diffusion capacity of carbon monoxide, and bronchial hyperresponsiveness, as well as imaging of the lung using inspiration-expiration CT-scans, is required to define COPD endotypes. The classification of COPD patients into specific endotypes enables clinicians to improve treatment of COPD patients by prescribing a more personalized therapy [53,55]. For instance, COPD patients with elevated sputum eosinophils need to be treated with inhaled corticosteroids [55]. Other treatment options for COPD endotypes – so called 'treatable traits' – are: lung volume reduction therapy for COPD patients with hyperinflation and heterogeneous emphysema [56], phosphodiesterase-4 inhibitors for patients with severe airflow limitation and symptoms of chronic bronchitis [57], and long-term low-dose macrolides for patients with airway bacterial colonisation quantified by sputum culture [55], or in case of frequent exacerbations.

COPD is a chronic inflammatory disease of the respiratory tract and lung parenchyma in response to chronic irritants such as cigarette smoke [53], and is associated with increased numbers of macrophages in the peripheral airways, lung parenchyma and pulmonary vessels, and with increased numbers of activated neutrophils and lymphocytes [53]. In a sub-population of COPD patients, there may also be increased levels of eosinophils [58–60], a TH2 driven inflammatory cell type. The latter inflammatory cell type is of clinical interest, for two reasons. First, eosinophilia in patients with COPD is associated with a higher frequency of exacerbations [61]. Second, numerous studies have shown that blood eosinophil counts predict the magnitude of the effect of inhaled corticosteroids [62–64]. Consequently, thresholds for blood eosinophils (i.e. <100 or >300 cells/µl) have been implemented in the GOLD guidelines for treatment of COPD [53]. Although Casanova *et al.* found that 43.8% of COPD patients have eosinophilia (i.e. >300 cells/µl) [60], individual predictions of response are still far from ideal, especially for patients with blood eosinophils between 100 and 300 cells/µl. As also mentioned for asthma, periostin has potential as a biomarker for TH2-driven inflammation and ICS responsiveness in asthma. Yet, its applicability as a biomarker for ICS responsiveness in COPD has been investigated to a limited extent only.

1.5 Airway wall remodeling

Asthma is characterised by structural changes of the airways, so-called airway wall remodeling [65]. Eventually, this results in airway wall thickening in proportion to disease severity and duration [66,67]. Factors involved in remodeling are: epithelial fragility, subepithelial collagen deposition including basement membrane thickening, smooth muscle hypertrophy, mucus metaplasia, angiogenesis and increased extracellular matrix (e.g. glyco-) proteins [68,69]. Figure 3 shows a cross-section of a normal compared to a remodeled airway wall, the latter from severe asthma. Airway remodeling is linked to clinical features such as lung function impairment, hyperresponsiveness, mucus hypersecretion, and air trapping [70–74].

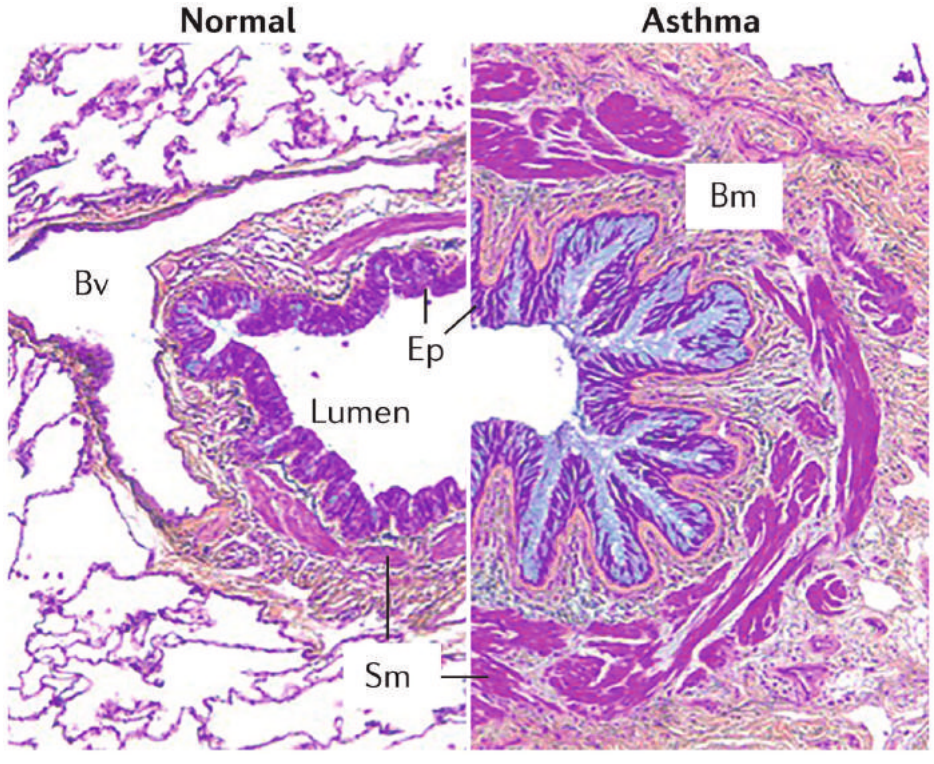

Figure 3: cross section of a severe asthmatic airway (right) compared with a normal airway (left). Asthma involves mucosal inflammation that most frequently consists of activated eosinophils, mast cells and T lymphocytes within the context of a remodelled airway with mucous metaplasia, an increase in smooth muscle **(Sm)**, fibrosis and angiogenesis. **Bm:** basement membrane, **Bv:** blood vessel, **Ep:** epithelium. Reprinted with permission (Holgate ST, et al. Nat Rev Dis Primers. 2015 Sep 10;1:15025).

Quantifying parameters associated with airway wall remodeling is of interest for two reasons: first of all, early stages of pulmonary obstructive conditions could be detected by measuring (biomarkers of) sub-symptomatic airway wall remodeling; various studies have found that remodeling of the airways already occurs in early and mild stages of asthma [75,76], also preceding the development of asthma in at-risk children, younger than six years [77]. Second, the parameters of airway wall remodeling can then be correlated with types of inflammatory processes and with inhaled, systemic, or intervention therapy response [78,79]. This would ultimately allow clinicians to use these remodeling features as a biomarker for a certain endotype, providing stratification of treatment options for the patients.

Currently, two diagnostic tools are available: high-resolution CT and assessment of the airway wall by bronchial biopsy [80,81]. Unfortunately, measuring bronchial wall and lumen area using a CT-scan does not provide information on the cause of airway wall thickening. Inspection of the bronchial biopsy is the gold standard to determine airway remodeling, but is a burden to patients and is time-consuming due to processing and staining of the biopsy sections. Additionally, a biopsy is only a small segment, which is routinely generalized to reflect the status of the airway wall remodelling along the small airways. Thus, current tools to detect and quantify airway remodeling are limited, especially *in vivo*. Optical Coherence Tomography (OCT) is a novel imaging technique that produces infrared-refraction images, both cross-sectional and sagittal (up to 5cm). A few studies already investigated OCT intensity areas in the airway walls between patients with obstructive pulmonary diseases and healthy participants; Ding *et al.* saw smaller luminal areas and thicker airway wall areas in higher COPD GOLD stages, compared to their never-smoking or smoking control peers [82]. Another study successfully used OCT imaging to determine *in vivo* elastic airway wall properties in individuals with and without obstructive pulmonary disease [83]. In asthma, the mucosal- and epithelial thickness measured by OCT was higher than in allergic- and non-allergic non-asthmatic controls [84]. However, no literature is available with respect to what component in the airway wall causes the OCT to refract and reflect infrared light, or what asthma phenotypes have other OCT signals. Most importantly, a relationship between OCT imaging and biomarkers for airway wall remodelling has not been described to date. Although OCT tissue intensity has been linked to collagen

deposition in the ovaria and skin [85,86], this has not yet been investigated in the airways.

1.6 Outline of the thesis

The overall aim of this thesis was to provide an overview of what is known about the asthma-obesity and asthma remission phenotypes, add knowledge to these topics, and to introduce new methods to:

- Endotype asthma, by single-cell RNA-sequencing.
- Endotype COPD, by measuring serum periostin levels and performing transcriptomic clustering.
- Analyze airway wall remodeling, which occurs in both obstructive pulmonary diseases.

In **Chapter 2**, we provide an overview of the current literature about clinical and complete asthma remission. We discuss the definition, prevalence, clinical characteristics, inflammatory markers, histological signs and genotypes linked to these phenomena. Next to current knowledge, we highlight future studies that will enable further exploration of the pathophysiology of asthma remission.

In 1972-1976, children diagnosed with clinical asthma were extensively characterized and re-examined in young adulthood and late adulthood. In these latter visits, the subjects were divided in three groups: persistent asthma, clinical- and complete asthma remission. The aim of the study in **Chapter 3** was to determine whether asthma remission persisted during this long-term follow-up, and which childhood factors are associated with clinical and/or complete asthma remission during adulthood.

Chapter 4 focuses on the number of exhaled particles in asthma patients, asthma remission subjects, and healthy individuals, enrolled in the *exploring Asthma ReMission by Single-cell TRansciptiONal sequencinG* (ARMSTRONG) study. Small airway disease is thought to close distal airways, consequently blocking non-volatile particles to be exhaled. Analysis of Particles of Exhaled Air (PExA) is a novel tool that enables measurement of the exhaled particle mass, which are produced by opening- and closing

of the lining tract fluid in the small airways. Our hypothesis is that the PExA mass is reduced in asthmatics and individuals with clinical asthma remission, while subject with complete asthma remission and healthy controls exhale more PExA mass. In line with this, we hypothesize that PExA correlates well with already established small airways parameters.

Chapter 5 describes the differences of small airways disease and inflammation in asthmatics, in subjects in clinical or complete asthma remission, and in healthy individuals, who participated in the ARMSTRONG cohort. Although it is known that subjects with clinical remission still express a degree of lung function impairment, bronchial hyperresponsiveness and comparable levels of airway inflammation [13], no data is published addressing their small airways function and inflammatory levels, compared to both asthmatics and healthy controls. We hypothesize that the parameters linked to small airways dysfunction and airway inflammation are similar in asthmatics and subjects with clinical asthma remission. Second, we anticipate that complete asthma remission subjects regain their pulmonary function, showing comparable features like healthy controls.

In **Chapter 6**, we apply a recently published model that predicts asthma remission on our own Dutch asthma remission cohorts. Authors of the *Childhood Asthma Management Program* (CAMP) trial showed that a combination of clinical features yields more that 80% probability to achieve asthma remission in young adulthood [87].

In **Chapter 7**, we review the asthma-obesity relationship. We focus on the increased risk to develop asthma in overweight and obese individuals, the effect of physical inactivity on asthma, the link between obesity and airway inflammation, the mechanical effects of obesity on asthma, the anti-inflammatory therapy response in obese asthmatics, the improvements of asthma symptoms due to weight loss, and the overweight comorbidities affecting asthma.

In **Chapter 8,** we apply single-cell transcriptomics – a novel technique for asthma endotyping - on bronchial biopsies, bronchial brushes and nasal brushes extracted from asthmatics and healthy controls in the ARMSTRONG study, as well as lung parenchyma tissue from donor lungs. Two methods of single-cell RNA-sequencing are

used: SmartSeq2 analysis of FACS-sorted epithelial and CD4 T cells from airway wall and the analysis of total cell suspensions using the 10XGenomics Chromium platform for single cell RNA sequencing [88,89]. Based on the transcriptomic profile of each cell, a cellular landscape of upper- and lower airways, and lung parenchyma is charted, which enables us to identify proportions and transcriptional cell-typing of structural and inflammatory cells between locations and asthma versus healthy.

In **Chapter 9**, we assess whether airway remodeling can be assessed by optical coherence tomography (OCT). In this chapter, we align histological stainings of airway wall sections with paired ex-vivo OCT-images, both derived from five lobectomy specimens. The airway wall sections are stained for various extracellular matrix components, i.e. total collagen, collagen A1, Masson's Trichrome, elastin, and fibronectin. We correlate the area and intensity of both the stained airway wall and the OCT images.

In **Chapter 10**, we investigate whether serum periostin – a marker linked to TH2-driven inflammation and thereby a potential marker for ICS responsiveness in asthma – is elevated in COPD patients compared to healthy controls. Second, we assess if periostin levels are associated with cross-sectional and longitudinal characteristics, including levels of inflammatory cells in three compartments (i.e. blood, sputum, and bronchial biopsies) and amount of bronchial extracellular matrix components in COPD patients.

In **Chapter 11**, the potential to use gene-expression to further endotype conditions such as asthma and COPD is explored. In 2014, Baines *et al.*, demonstrated that unbiased clustering based on gene-expression was able to distinguish three different phenotypes in asthma [90]. In this chapter, we use a COPD-associated gene signature from another cohort to cluster COPD patients based on their RNA sequencing data obtained from bronchial biopsies during the *Groningen and Leiden Universities Corticosteroids in Obstructive Lung Disease* (GLUCOLD) study [91,92]. Subsequently, we assess whether these clusters were clinically different cross-sectionally and longitudinally.

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Chapter 2

The pathophysiology of asthma remission

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Abstract

Asthma is a chronic respiratory condition, which is highly prevalent worldwide. Although no cure is currently available, it is well recognized that some asthma patients can spontaneously enter remission of the disease later in life. Asthma remission is characterized by absence of symptoms and lack of asthma-medication use. Subjects in asthma remission can be divided into two groups: those in clinical remission and those in complete remission. In clinical asthma remission, subjects still have a degree of lung functional impairment or bronchial hyperresponsiveness, while in complete asthma remission, these features are no longer present. Over longer periods, the latter group is less likely to relapse. This remission group is of great scientific interest due to the higher potential to find biomarkers or biological pathways that elicit or are associated with asthma remission.

Despite the fact that the definition of asthma remission varies between studies, some factors are reproducibly observed to be associated with remitted asthma. Among these are lower levels of inflammatory markers, which are lowest in complete remission. Additionally, in both groups some degree of airway remodeling is present. Still, the pathological disease state of asthma remission has been poorly investigated. Future research should focus on at least two aspects: further characterisation of the small airways and airway walls in order to determine histologically true remission, and more thorough biological pathway analyses to explore triggers that elicit this phenomenon. Ultimately, this will result in pharmacological targets that provide the potential to steer the course of asthma towards remission.

Introduction

Asthma is a usually chronic respiratory disease with an estimated 300 million individuals affected worldwide. It is characterized by variable airflow obstruction associated with symptoms of dyspnea, cough and bronchial hyperresponsiveness as outlined in the GINA guidelines [1]. Current treatments fail to cure the disease. Nevertheless, it has been reported that asthma can go into spontaneous remission [2,3], meaning that asthmatics at some point are no longer burdened by the disease, and do no longer require any asthma medication. These “ex-asthmatics” are labelled to be in clinical asthma remission, but might still have (asymptomatic) bronchial hyperresponsiveness or a low lung function [4]. In fewer cases, subjects go into complete asthma remission, additionally having no pulmonary function impairment or bronchial hyperresponsiveness [2,3]. To date, most discussion of asthma treatment goals revolves around disease control [1,5,6], whereas exploring the induction of asthma remission as a therapeutic goal has so far attracted little interest. In this review, we highlight the definition, prevalence and characteristics of asthma remission. Next, we describe factors associated with the induction of asthma remission, inflammatory markers, histological signs and genotypes linked to this phenomenon. Finally, we discuss current research on identifying biological pathways that could trigger asthma remission, which may be used for therapeutics in the future.

Definition of asthma remission

Defining asthma remission seems straightforward, but is not an easy task. Asthma is a usually chronic disease characterized by variable airflow obstruction, bronchial hyperresponsiveness and inflammation, and disease severity fluctuates over time. As such, episodes with little or no disease activity can alternate with periods of more disease symptoms and renewed dependence on medication use. Consequently, patients in remission of the disease have a certain risk of relapse [2,3,7,8]. This resembles “remission” of cancer [9], in which the disappearance of signs and symptoms does not ensure that the disease is cured. Yet, it is thought that remission of cancer is the closest to cure and has smaller chance of relapse, especially in “complete remission” of cancer, certainly for many non-operable lung cancers (National Cancer Institute, 2018). The risk of relapse also pertains to other inflammatory diseases, such as rheumatoid arthritis [6,10], inflammatory bowel disease [11], and multiple sclerosis [12]. In principle,

the relapse risk depends on how strict remission is defined and what features must be absent. Thus to apply similar reasoning to asthma where the label of remission needs to be associated with minimal occurrence of relapse, the definition of asthma remission should be strict including an absence of symptoms, its period, no medication use, as well as absence of lung function impairment and bronchial hyperresponsiveness. The complexities of and guidelines for defining asthma remission are discussed below.

Symptom perception

The asthmatic's perception of the severity of symptoms has a dominant important role in the effective management of asthma [13]. In line with this, individuals with asthma remission might also have poor perception of symptoms and feel no need for treatment. Yet, the mechanisms underlying poor perception are not well understood [14]. One concept explaining poor perception is that of temporal adaptation; the diminished perception of symptoms is caused by psychological modification due to chronic obstruction and dyspnea [14]. In other words, individuals with remitted asthma could experience symptoms differently than family and associates would judge them.

Symptom-free period

According to most definitions, individuals in asthma remission should not have experienced any degree of asthma-related symptoms during a reasonable period of time. In the many studies carried out so far, a broad range of symptom-free periods is used to define asthma remission [6]. Table 1 shows that the duration of absence of symptoms was on average one year, with a range of 6 months to 5 years. Studies are limited in their accuracy to determine the symptom-free period for several reasons. First, in retrospective cohorts, asthma remission should not be defined as having "no medical records for current asthma" since these individuals could have been treated somewhere else. Second, questions such as "did you experience asthma symptoms in the previous year?" are dichotomous and do not allow for reporting subtle symptoms, resulting in an overestimation of remission prevalence in large survey studies. We think it is highly probable that standardized questioning with several lines (e.g. wheeze, dyspnea on exertion, several triggers) will identify more subjects who still have some symptoms. And last, determining the symptom-free period is affected by selection bias; asthma symptoms might be underestimated by the clinical researcher and the participant who wants to enroll, when investigating the relatively rare occurrence of

asthma remission [6]. Since individuals with remitted asthma are difficult to find, researchers might underrate wheezing in order to fill the cohort. In addition, it can be debated whether patients who re-experience symptoms during methacholine and adeno-'5-monophosphate provocation tests should be labelled as symptom free [15], while healthy non-asthmatic individuals would not experience dyspnea [16]. In principle, true asthma remission should be defined as having no asthma symptoms (i.e. wheezing, asthma attacks, including dyspnea during provocation) for at least one year.

Medication use

A key factor that should be assessed in order to ascertain asthma remission is absence of medication use. In contrast to fully controlled asthma, patients are usually considered to be in asthma remission when they did not take any asthma-related medication for at least one year. Thus in order to define true asthma remission, individuals should not take any symptom-relievers and anti-inflammatory agents, including immunosuppressant medication used for other diseases.

Lung function and bronchial hyperresponsiveness

In order to make the definition of asthma remission less dependent on symptom perception, Vonk *et al.* suggested dividing remission into clinical and complete asthma remission. Both definitions share the absence of wheeze, asthma attacks and use of asthma medication for more than one year. Yet in clinical asthma remission, individuals still have a positive bronchial hyperresponsiveness (BHR) test and/or lung function impairment, while in subjects with complete asthma remission, these features are absent [2]. Defining complete asthma remission results in fewer subjects who meet these criteria [2,3,17–19]. Despite the scarcity of subjects, studying complete asthma remission has two advantages. First, it is of scientific interest; this strict phenotype has higher potential to elucidate biological biomarkers and pathways that are associated with asthma remission [4,20]. Second, the risk of asthma relapse is lower in complete remission subjects: one quarter compared to two-third in clinical asthma remission subjects [3].

Figure 1: Pubmed search

Last searched on 6-1-2019

(asthma[ti] **AND** (remission[tiab] OR outgrow[tiab] OR "free from symptoms"[tiab] OR remitted[tiab] OR "no symptoms"[tiab] OR "asymptomatic"[tiab] OR "absence of symptoms"[tiab] OR "natural history"[tiab] OR "course of asthma"[tiab])) **AND** (prevalence[tiab] OR rate*[tiab] OR outcome[tiab] OR predictors[tiab] OR prognos*[tiab] OR factors[tiab] OR characteriz*[tiab] OR determinants[tiab])

Results: 704 studies

Excluded
Non-English: 103 studies

Title of interest

Included
Relevant studies: **41**
Found in relevant studies: 3
(- 8 overlapping cohorts)
Relevant cohorts: 36

Excluded
Reviews: **21**
Only abstract: **3**
Wrong topic: **42**
Asthma diagnosis <6 years: **2**

Table 1: various definitions, prevalence rates and factors associated with asthma remission

Study *36 cohorts*	**Cohort design** *Asthma diagnosis and quality of the study*	**N**	***Age*** *Enrollment*	**Definition of asthma remission**	**Classification of remission**	**Proportion**	**Associated with remission**
The Childhood Asthma Management Program (CAMP), USA [17,21]	**Prospective cohort** with **15**-year clinical FU. **Asthma**: enrolled asthmatic children in CAMP trial, mild-to-moderate persistent asthma with positive methacholine test. **High quality:** large cohort, well-defined and clinically assessed asthma and asthma remission diagnosis.	909	5-12Y	**No signs of:** asthma reported symptoms **Medication use:** no asthma-medication **Period:** 1 year	**Remission**	6% (18Y)	- No positive SPT's - Fewer positive SPT's - Less sensitive to allergens - Mild symptoms in childhood - Higher baseline FEV_1
					Remission	-	- Not defined
					Clinical remission, i.e. FEV_1/FVC ratio >80%	26% (23Y)	- Female sex - Less BHR at baseline - Higher baseline FEV_1 - Higher baseline FEV_1/FVC - Lower blood IgE - Lower blood eosinophils - Wheezes during colds
					Complete remission, i.e. FEV_1/FVC ratio >80%, PC_{20} methacholine >25mg/ml	15% (23Y)	- Female sex - Less BHR at baseline - Higher baseline FEV_1 - Higher baseline FEV_1/FVC - Lower blood IgE - Lower blood eosinophils - Wheezes during colds
Outpatient cohort of Pediatric clinic of Golestan University Hospital In Ahvaz, Iran [22]	**Prospective cohort** with **5**-year clinical FU. **Asthma**: registered in outpatient pediatric clinic with ≥2 asthma attacks in past. **High quality:** well-defined and clinically assessed asthma and asthma remission diagnosis.	197	6-10Y	**No signs of:** asthma symptoms **Medication use:** no ICS or SABA **Period:** 1 year	**Remission** FEV_1/FVC >80%, exercise challenge test <15% decline after 6-8 minutes of running	**33**% (15Y)	- No family history of asthma - No passive smoking - No eczema

Table 1: (continued)

Study *36 cohorts*	**Cohort design** *Asthma diagnosis and quality of the study*	**N**	***Age*** *Enrollment*	**Definition of asthma remission**	**Classification of remission**	**Proportion**	**Associated with remission**
Outpatient cohort of Hacettepe University Pediatric Allergy and Asthma Unit, Turkey [23]	**Prospective cohort** with **11**-year clinical FU. **Asthma**: registered at outpatient clinic with ≥1 visit every 2 year for 6 years, reversible airway obstruction with spirometry. **High quality:** well-defined and clinically assessed asthma and asthma remission diagnosis.	115	5Y	**No signs of:** asthma symptoms **Medication use:** no controller medication **Period:** 1 year	**Remission**	**53**% (17Y)	- Male sex - No blood eosinophilia
					Clinical remission, i.e. % pred. ≤80%, PC_{20} methacholine ≤8mg/ml	26% (17Y)	- Male sex
					Complete remission, i.e. FEV_1 % pred. >80%, PC_{20} methacholine >8mg/ml	27% (17Y)	- Not described
Isle of Wight Birth Cohort, United Kingdom [24-26]	**Prospective cohort** with **18**-year clinical FU. **Asthma**: physician diagnosed <10 year, asthma treatment in the last year. **High quality:** reasonably defined asthma diagnosis, well-defined and clinically assessed asthma remission diagnosis.	181	1Y	**No signs of:** asthma symptoms **Medication use:** no asthma-medication **Period:** 1 year	**Remission**	**31**% (18Y)	- Male sex - Less BHR at baseline - Less atopy
					Clinical remission, i.e. PC_{20} methacholine <8mg/ml	11% (18Y)	
					Complete remission, i.e. PC_{20} methacholine ≥8mg/ml	10% (18Y)	
Outpatient cohort of Marmara University Pediatric Allergy and Immunology Department, Turkey [27]	**Retrospective cohort** with **10**-year clinical FU. **Asthma**: diagnosis based on GINA and ARIA guidelines. **High quality:** reasonably defined asthma diagnosis, well-defined and clinically assessed asthma remission diagnosis.	62	2-8Y	**No signs of:** asthma symptoms **Medication use:** no asthma-medication **Period:** 1 year	**Remission**	**50**% (16Y)	- Negative family history - Less BHR at baseline - Absence of rhinitis - Higher childhood FEV_1 - Higher childhood $FEF_{25-75\%}$
					Clinical remission, i.e. PC_{20} methacholine <8mg/ml, negative SPT	16% (16Y)	- Not described
					Complete remission, i.e. PC_{20} methacholine ≥8mg/ml, negative SPT	34% (16Y)	- Not described

Table 1: (continued)

Study *36 cohorts*	**Cohort design** *Asthma diagnosis and quality of the study*	**N**	***Age*** *Enrollment*	**Definition of asthma remission**	**Classification of remission**	**Proportion**	**Associated with remission**
Obstructive Lung Disease in Northern Sweden Studies (OLIN), Sweden [28-29]	**Prospective cohort** with **12**- year clinical FU. **Asthma**: physician diagnosed by pediatricians. **High quality:** reasonably defined asthma diagnosis, well-defined and clinically assessed asthma remission diagnosis.	248	7-8Y	**No signs of:** wheeze **Medication use:** no asthma-medication **Period:** 3 years	**Remission**, mean FEV_1 % pred. 90%, median PC_{20} methacholine: 3.4mg/ml	**21**% (19Y)	- Male sex - No SPT positivity to animals
Outpatient cohort of Pediatric Allergy Outpatient Unit at the Central Hospital of Skövde, Sweden [30]	**Prospective cohort** with **21**-year clinical FU. **Asthma**: registered at outpatient clinic, ≥3 episodes of wheezing. **High quality:** reasonably defined asthma diagnosis, well-defined and clinically assessed asthma remission diagnosis.	55	5-14Y	**No signs of:** asthma symptoms **Medication use:** no asthma-medication **Period:** 1 year	**Remission**	**16**% (30Y)	- Male sex
Outpatient cohort of Pediatric pulmonology department of the University Medical Center Groningen, The Netherlands [2]	**Prospective cohort** with **30**-year clinical FU. **Asthma**: registered at outpatient clinic, physician diagnosed, positive histamine test. **High quality:** well-defined and clinically assessed asthma and asthma remission diagnosis.	119	5-14Y	**No signs of:** wheeze or asthma attacks **Medication use:** no ICS **Period:** 1 year	**Remission**	**52**% (32-42Y)	- Higher baseline FEV_1 - Higher increase in FEV_1 - Less pack years in adulthood
					Clinical remission, i.e. PC_{20} methacholine ≤16mg/ml or FEV_1 % pred. ≤90%	30% (32-42Y)	- Not described
					Complete remission, i.e. PC_{20} methacholine and >16mg/ml, FEV_1 % pred. >90%	22% (32-42Y)	- Not described

Table 1: (continued)

Study *36 cohorts*	**Cohort design** *Asthma diagnosis and quality of the study*	**N**	***Age*** *Enrollment*	**Definition of asthma remission**	**Classification of remission**	**Proportion**	**Associated with remission**
Outpatient cohort of Pediatric pulmonology department of the University Medical Center Groningen, The Netherlands [31]	**Prospective cohort** with **39**-year clinical FU. **Asthma**: registered at outpatient clinic, physician diagnosed, positive histamine test. **High quality:** well-defined and clinically assessed asthma and asthma remission diagnosis.	63	7-12Y	**No signs of:** asthma symptoms **Medication use:** no asthma-medication **Period:** 1 year	**Remission**	**18**% (25Y)	- Not wheezing during cold - No pneumonia in childhood - Dusty house in childhood - Leukemia in family history - No FEV_1 <80% in childhood
						40% (49Y)	- Having pets in childhood
					Clinical remission, i.e. FEV_1 % pred. ≤90% or PC_{20} methacholine ≤9.8mg/ml	11% (25Y)	- Not described
						30% (49Y)	- Not described
					Complete remission, i.e. FEV_1 % pred. >90% and PC_{20} methacholine >9.8mg/ml	7% (25Y)	- Not wheezing during cold - No maternal atopy - Leukemia in family history - Having a higher FEV_1/FVC - SPT positivity to mould
						10% (49Y)	- Not found
Adult-Onset Asthma and Inflammatory Subphenotypes (ADONIS), The Netherlands [32]	**Prospective cohort** with **5**-year clinical FU. **Asthma**: physician diagnosed, reversibility ≥12% or positive methacholine test, excluded if asthma in childhood. **High quality:** well-defined and clinically assessed asthma and asthma remission diagnosis, yet wide age-range of enrollment.	194	18-75Y	**No signs of:** asthma symptoms **Medication use:** no asthma-medication **Period:** 1 year	**Remission**	**16**% (+5Y)	- Lower ICS dosage at onset - Less BHR at baseline - No nasal polyps - Less blood neutrophils
					Clinical remission, i.e. PC_{20} methacholine <4mg/ml	6% (+5Y)	- Not described
					Complete remission, i.e. FEV_1 % pred. >80% and PC_{20} methacholine >4mg/ml	10% (+5Y)	- Not described

Table 1: (continued)

Study *36 cohorts*	**Cohort design** *Asthma diagnosis and quality of the study*	**N**	***Age*** *Enrollment*	**Definition of asthma remission**	**Classification of remission**	**Proportion**	**Associated with remission**
Outpatient cohort of *Department of Chest Diseases, Ankara University School of Medicine, Turkey* [33]	**Retrospective cohort** with **7**-year clinical FU. **Asthma**: registered at outpatient clinic, diagnosed according to the GINA guidelines. **High quality:** reasonably defined asthma diagnosis, well-defined and clinically assessed asthma and asthma remission diagnosis.	200	<47Y	**No signs of:** asthma symptoms **Medication use:** no asthma-medication **Period:** 2 years	**Remission**	**11**% (53Y)	- Younger age of onset
					Clinical remission, i.e. positive methacholine test	4% (53Y)	- Not described
					Complete remission, i.e. negative methacholine test	2% (53Y)	- Not described
Dunedin Multi-disciplinary Health and Development Study (DMHDS), New Zealand [19]	**Prospective cohort** with **26**-year clinical FU. **Asthma**: questionnaire-based, wheezing reported. **Moderate quality:** debatable definition of asthma diagnosis, clinically assessed asthma remission diagnosis, yet no medication use described.	613	3Y	**No signs of:** wheeze **Medication use:** not defined **Period:** 2 years	**Remission**	**15**% (26Y)	- Not described
					Clinical remission, i.e. PC_{20} methacholine ≤8mg/ml or reversibility ≥10% at any assessment from 9-21 Y	10% (26Y)	- Not described
					Complete remission, i.e. PC_{20} methacholine >8mg/ml and reversibility <10% at any assessment from 9-21 Y	5% (26Y)	- Not described
Childhood Asthma Study (CAS), USA [34]	**Prospective cohort** with **11**-year clinical FU. **Asthma**: physician diagnosed and treated for ≥1 year. **Moderate quality:** reasonably defined asthma diagnosis, well-defined and clinically assessed asthma remission diagnosis, immunotherapy use in asthma remission group.	85	5-12Y	**No signs of:** asthma symptoms **Medication use:** no asthma-medication **Period:** 1 year	**Remission*** FEV_1 % pred. >80%, FEV_1/FVC ratio >80%, mean PC_{20} methacholine: 0.2mg/ml **46*% *used active immunotherapy*	**15**% (23Y)	- Lower blood IgE - Fewer positive SPT's

Table 1: (continued)

Study *36 cohorts*	**Cohort design** *Asthma diagnosis and quality of the study*	**N**	***Age*** *Enrollment*	**Definition of asthma remission**	**Classification of remission**	**Proportion**	**Associated with remission**
Melbourne Asthma Study (MAS), Australia [35-38]	**Prospective cohort** with **42**-year clinical FU. **Asthma**: reports of wheezing by self-report by the parent at recruitment. **Severe asthma:** ≥10 attacks in 2 years before age or persistent symptoms at age 10, according to GINA guidelines. **Moderate quality:** debateable asthma definition, well-defined and clinically assessed asthma remission diagnosis, multiple follow-up visits.	269	7-10Y	**No signs of:** wheeze **Medication use:** no asthma-medication **Period:** 3 years	**Remission**	20% (14 Y) 33% (21 Y) 40% (42 Y) 52% (50 Y)	- Male sex - Mild symptoms in childhood - No childhood hayfever - No childhood eczema - No SPT positivity
Outpatient cohort Asthma clinic at Beatrix-oord hospital Haren, The Netherlands [18]	**Prospective cohort** with **25**-year clinical FU. **Asthma**: registered at outpatient clinic, physician diagnosed, positive histamine test. **Moderate quality:** well-defined asthma diagnosis, clinically assessed asthma remission, yet pulmonary medication was used within the remission-group.	181	13-44Y	**No signs of:** cough, sputum, dyspnea, wheeze and asthma attacks **Medication use:** not defined **Period:** 3 years	**Remission***	**40**% (48Y)	- Male sex - Younger age of onset - Higher baseline FEV_1 - Less BHR at baseline
					Clinical remission, i.e. PC_{20} histamine ≤4mg/ml or FEV_1 % pred. ≤90%	25% (48Y)	- Not described
					Complete remission, i.e. PC_{20} histamine >4mg/ml and FEV_1 % pred. >90% **5% used pulmonary medication*	11% (48Y)	- Not described
Military service men in 1987–1990 referred to the Central Military Hospital, Finland [39]	**Prospective cohort** with **20**-year clinical FU. **Asthma**: based on medical records; asthma symptoms, medication use, lung function and allergy tests. **Moderate quality:** reasonably defined asthma diagnosis, well-defined and clinically assessed asthma remission diagnosis, yet predominantly men.	119	19-21Y	**No signs of:** asthma symptoms **Medication use:** no asthma-medication **Period:** 3 years	**Remission**	**12**% (41Y)	- Not described

Table 1: (continued)

Study *36 cohorts*	**Cohort design** *Asthma diagnosis and quality of the study*	**N**	***Age*** *Enrollment*	**Definition of asthma remission**	**Classification of remission**	**Proportion**	**Associated with remission**
European Community Respiratory Health Survey II (ECRHSII), Europe, North America, Oceania [40]	**Prospective cohort** with **9**-year of clinical FU. **Asthma**: physician diagnosed, asthma-like symptoms and/or medication in the last year. **Moderate quality:** debatable definition of asthma, clinically assessed asthma remission diagnosis, yet ICS use in subset of asthma remission subjects.	856	20-44Y	**No signs of:** asthma-like symptoms or asthma attacks **Medication use:** no asthma-medication **Period:** 1 year	**Remission*** **16% used ICS in the last 12 months*	**12**% (35Y)	- Higher baseline FEV_1 - Lowest increase of BMI
Risk Factors for Asthma in Adults Study (RAV), Denmark [41]	**Prospective cohort** with **9**-year clinical FU. **Asthma**: questionnaire based, 'have you ever had asthma?', combined with asthma-like symptoms, use of medication in the last year or airflow obstruction. **Moderate quality:** questionnaire based asthma diagnosis, well-defined and clinically assessed asthma remission diagnosis.	239	20-44Y	**No signs of:** asthma symptoms **Medication use:** no asthma-medication **Period:** 1 year	**Remission**	**28**% (+9Y)	Not described
Seinäjoki Adult-onset Asthma Study (SAAS), Finland [42]	**Prospective cohort** with **12**-year clinical FU. **Asthma**: physician diagnosed, objective lung function measurements showing reversible obstruction, symptoms of asthma. **High quality:** reasonably defined asthma diagnosis, asthma remission definition 6 months and still could have a degree of symptoms.	203	46Y	**No signs of:** asthma symptoms, Asthma Control Test score of 25 **Medication use:** no asthma-medication **Period:** 6 months	**Remission**	**6**% (58Y)	- Higher baseline FEV_1/FVC - Lower blood IgE
					Clinically assessed, i.e. FEV_1 % pred. >80%, FEV_1/FVC >70%, reversibility <12%, FeNO ≤20ppb	3% (58Y)	- Not described

Table 1: (continued)

Study *36 cohorts*	**Cohort design** *Asthma diagnosis and quality of the study*	**N**	***Age*** *Enrollment*	**Definition of asthma remission**	**Classification of remission**	**Proportion**	**Associated with remission**
Lung Disease in Northern Sweden study (OLIN), Sweden [43]	**Prospective cohort** with **10**-year clinical FU. **Asthma:** ≥2 asthma attacks during last year, reversibility >15% or PC_{20} methacholine <4mg/ml, including ≥3 following: 1. Recurrent wheeze, 2. Attacks of shortness of breath, 3. ≥2 asthma provoking factors, 4. Normal breathing between asthma attacks or periods of asthma. **Moderate quality:** debatable definition of asthma, well-defined asthma remission, yet including subjects with persistent wheeze and medicine use.	267	35-66Y	**No signs of:** recurrent wheeze or attacks of shortness of breath **Medication use:** no asthma-medication **Period:** 1 year	**Remission***	**6**% (+10Y)	- Younger age of onset - Mild asthma in adulthood - Cessation of smoking
					Clinically assessed, i.e. FEV_1 % pred. ≥80%* **Including few subjects with persistent wheeze and medicine use*	4% (+10Y)	
Environment and Childhood Asthma (ECA) Study in Oslo, Norway [44]	**Prospective cohort** with **10**-year questionnaire FU. **Asthma**: 2 of 3 criteria: 1. Symptoms 0-10 years, 2. Doctor's diagnosis, 3. Use of asthma medication 0-10 years. **Lower quality:** debatable definition of asthma, asthma remission defined by no current record of asthma, including children with doctor's diagnosis <6 years.	616	0Y	**No signs of:** dyspnea, chest tightness and/or wheezing (no record of current asthma) **Medication use:** no asthma-medication **Period:** 1 year	**Remission*** **including children with doctor's diagnosis <6 years.*	**55**% (10Y)	- Female sex
Tucson Children's Respiratory Study, USA [45]	**Prospective cohort** with **16**-year clinical FU. **Asthma**: reporting presence of wheezing >3 episodes in previous year in at least on survey or physician diagnosed. **Lower quality:** debatable definition of asthma, undefined medication use in asthma remission.	166	2Y	**No signs of:** wheeze **Medication use:** not defined **Period:** 1 year	**Remission**	**42**% (13-16Y)	- No obesity - No early onset of puberty - No childhood sinusitis - No positive SPT

Table 1: (continued)

Study *36 cohorts*	**Cohort design** *Asthma diagnosis and quality of the study*	**N**	***Age*** *Enrollment*	**Definition of asthma remission**	**Classification of remission**	**Proportion**	**Associated with remission**
Comprehensive medical record database of the Olmsted Medical Center, USA [46]	**Retrospective cohort** with **10**-year of database FU. **Asthma**: extensive criteria list in publication. **Lower quality:** reasonably defined asthma diagnosis using a database, debatable definition of asthma remission.	117	8Y	**No signs of:** no medical records indicating asthma symptoms, visits or admissions **Medication use:** no asthma-medication **Period:** 3 years	**Remission**	**24**% (18Y)	- Caucasian ethnicity
Population-based sample of 11.048 neonates, Greece [47]	**Prospective cohort** with **18**-year questionnaire FU. **Asthma**: physician diagnosed and treated at some point in life. **Lower quality:** debatable definition of asthma, asthma remission defined by no current record of asthma, including children with doctor's diagnosis <6 years.	562	0Y	**No signs of:** asthma symptoms (no record of current asthma **Medication use:** not defined **Period:** 1 year	**Remission*** **including children with doctor's diagnosis <6 years.*	**69**% (18Y)	- Female sex - No family history of atopy - Smoking cessation - No maternal smoking during pregnancy.
Dunedin Multi-disciplinary Health and Development Study (DMHDS), New Zealand [5]	**Prospective cohort** with **23**- year clinical FU. **Asthma**: questionnaire-based: 'do you have asthma?' **Lower quality:** questionnaire-based definition of asthma diagnosis, undefined medication use in asthma remission .	176	3Y	**No signs of:** wheeze **Medication use:** not defined **Period:** 1 year	**Remission**	**39**% (18Y)	- Older age of onset - Higher baseline FEV_1 - Higher baseline FEV_1/FVC - Less BHR at baseline - Less reversibility
						25% (26Y)	- Not described

Table 1: (continued)

Study *36 cohorts*	**Cohort design** *Asthma diagnosis and quality of the study*	**N**	***Age*** *Enrollment*	**Definition of asthma remission**	**Classification of remission**	**Proportion**	**Associated with remission**
Comprehensive medical evaluation for eligibility for national service between 1999 and 2008, Israel [48]	**Cross-sectional cohort** with **3**-year of database FU. **Asthma**: medical records indicating asthma symptoms, requiring medication, FEV_1 <80% and/or positive exercise challenge test. **Lower quality:** cross-sectional design, database record defined asthma and asthma remission diagnosis.	26.400	17Y	**No signs of:** asthma symptoms **Medication use:** no asthma-medication **Period:** 3 years	**Complete remission,** i.e. FEV_1 and FEV_1/FVC >80%, no decline of <10% in FEV_1 exercise challenge test, methacholine challenge PC_{20} >8mg/ml	22% (17Y)	- Not described
Random stratified cluster sample of non-Mexican white American households in Tucson, USA [7]	**Prospective cohort** with **9**-year of clinical FU. **Asthma**: questionnaire-based 'have you ever had asthma?', 'how many asthma attacks have you had in the past year?', 'how often are you bothered by attacks of shortness of breath and wheezing?' **Lower quality:** questionnaire based asthma diagnosis, wide age-range at enrollment, debatable asthma remission definition, limited assessment of asthma remission at follow-up.	136	6-80Y	**No signs of:** asthma attacks, <2 attacks of shortness of breath with wheezing **Medication use:** no asthma-medication **Period:** 1 year	**Remission**	**22**% (overall) 35% (<10Y) 65% (10-19Y) 28% (20-29Y) 15% (30-39Y) 6% (40-49Y) 18% (50-59Y) 27% (60-69Y) 25% (70-79Y)	- Mild asthma in adulthood - Younger age of onset - Higher baseline FEV_1 - Co-existing emphysema
Outpatient cohort of Prince of Wales's General Hospital Department of Pediatrics and Allergy, London [49]	**Retrospective cohort** with **20**-year questionnaire FU. **Asthma**: recurrent ≥3 attacks of paroxysmal dyspnea with wheezing. **Lower quality:** debateable definition of asthma, undefined medication use in asthma remission.	267	<12Y	**No signs of:** asthma symptoms **Medication use:** not defined **Period:** 2 years	**Remission**	**28**% (<32Y)	- Not described

Table 1: (continued)

Study *36 cohorts*	**Cohort design** *Asthma diagnosis and quality of the study*	**N**	***Age*** *Enrollment*	**Definition of asthma remission**	**Classification of remission**	**Proportion**	**Associated with remission**
European Community Respiratory Health Survey I (ECRHSI), Europe, North America, Oceania [50]	**Cross-sectional cohort**, survey-based. **Asthma:** questionnaire-based : 'have you ever had asthma ?' and 'how old were you when you had your first asthma attack?' **Lower quality:** cross-sectional design, questionnaire based asthma diagnosis, asthma remission defined by no current record of asthma.	1.558	0-44Y	**No signs of:** asthma attacks (no record of current asthma) **Medication use:** no asthma-medication **Period:** 2 years	**Remission**	**43**% (<10Y) 34% (10-20Y) 16% (20-44Y)	- Younger age of onset - Negative family history - Less acute resp. infections - Contact with older children - Pets in childhood household
Tasmanian Longitudinal Health Study (TAHS, Australia [51]	**Prospective cohort** with **36**-year questionnaire FU. **Asthma:** questionnaire-based, 'have you ever had asthma? **Lower quality:** questionnaire based asthma- and asthma remission diagnosis.	1.620	7-13Y	**No signs of:** asthma attacks (no record of current asthma) **Medication use:** no asthma-medication **Period:** 2 years	**Remission**	**65**% (46Y)	- Male sex - Younger age of onset - No maternal asthma - No pneumonia in childhood
Alumnae address database of the Brown University School of Medicine, USA [52]	**Prospective cohort** with **23**-year questionnaire FU. **Asthma**: physician diagnosed, history of ≥3 clinically recurrent, reversible episodes of wheezing and dyspnea. **Lower quality:** debatable definition of asthma, undefined medication use in asthma remission.	84	16-20Y	**No signs of:** asthma symptoms **Medication use:** not defined **Period:** 5 years	**Remission**	**40**% (40Y)	- Younger age of onset
Three population-based multicentre studies: ECRHS-Italy, ISAYA and GEIRD performed in Italy [53]	**Cross-sectional cohort**, survey-based. **Asthma:** questionnaire based, 'have you ever had asthma?', 'how old were you when you have your first attack of asthma?' **Lower quality:** cross-sectional design, questionnaire based asthma diagnosis, asthma remission defined by no current record of asthma, wide age-range of enrollment.	3.087	20-84Y	**No signs of:** asthma attacks (no record of current asthma) **Medication use:** inhalers, aerosols or tablets **Period:** 2 years	**Remission**	**65**% (0-14Y) 36% (15-29Y) 21% (>30Y)	- Male sex - Younger age of onset

Table 1: (continued)

Study *36 cohorts*	**Cohort design** *Asthma diagnosis and quality of the study*	**N**	***Age*** *Enrollment*	**Definition of asthma remission**	**Classification of remission**	**Proportion**	**Associated with remission**
Italian Study on Asthma in Young Adults (ISAYA), Italy [54]	**Prospective cohort** with 9-year questionnaire FU. **Asthma:** self-reported physician's diagnosis of asthma and ≥1 asthma attack in last year and/or current use of medication. **Lower quality:** questionnaire based asthma- and asthma remission diagnosis.	214	21-47Y	**No signs of:** wheeze, tightness of the chest, shortness of breath, asthma attacks **Medication use:** no asthma-medication **Period:** 1 year	**Remission**	**30**% (+9Y)	- Older age of onset
Respiratory Health in Northern Europe (RHINE), Iceland, Norway, Sweden, Denmark and Estonia [55]	**Prospective cohort** with **12**-year questionnaire FU. **Asthma:** questionnaire based, 'have you ever had asthma?', 'how old were you when you have your first attack of asthma?' **Lower quality:** questionnaire based asthma- and asthma remission diagnosis.	1.153	28-56Y	**No signs of:** asthma symptoms, i.e. "which was the latest year you experienced asthma symptoms?" **Medication use:** no asthma-medication **Period:** 2 years	**Remission**	**19**% (+12Y)	- Cessation of smoking
Hiroshima COPD Cohort Study, Japan [56]	**Cross-sectional cohort**, survey-based. **Asthma:** questionnaire based, 'Were you ever diagnosed with asthma by a physician?', 'Have you been awakened in the last 12 months by an attack of shortness of breath or wheezing when you did not have a cold?' **Lower quality:** cross-sectional design, questionnaire based asthma diagnosis, asthma remission defined by no current record of asthma.	388	35-60Y	**No signs of:** asthma symptoms (no record of current asthma) **Medication use:** not defined **Period:** 1 year	**Remission*** **Remitted childhood asthma divided by total childhood asthma.*	**74**%	- Not described

BHR: bronchial hyperresponsiveness, BMI: body mass index, FU: follow-up, ICS: inhaled corticosteroids, SABA: short-acting beta-agonist, SPT: skin prick test.

Prevalence of asthma remission

The prevalence of the asthma remission has a very broad range in studies so far, for three main reasons. First, the age at baseline varies with the type of cohort (e.g. birth cohorts, outpatient clinic cohorts, retrospective national service databases, cross-sectional international surveys, and follow-up on finished clinical trials). Second, some research groups clinically assessed subjects at baseline and at follow-up, while others defined asthma and its remission solely on a questionnaire-based answer. Questionnaire-based studies generally had higher prevalence rates and did not always specify medication use. And last, the years of follow-up ranged from five years to four decades, increasing the difficulty in comparing the results per study. For these reasons and for estimation of prevalence rates, in this manuscript we only included studies meeting the following criteria: clinically assessed asthma diagnosis or medical record diagnosis based on GINA guidelines [1], clinically assessed asthma remission status, and specifically defined asthma remission criteria (i.e. no asthma symptoms such as wheeze or asthma attacks, no asthma-related medication, for at least 1 year). Table 1 lists an overview of 36 cohorts assessing the prevalence of asthma remission ordered by age at baseline and years of follow-up (figure 1 for PubMed search term). Eleven of the cohorts (white colored rows) were used to estimate the prevalence. We excluded cohorts only enrolling children with a doctor's diagnosis of asthma before the age of six, due to the fact that the diagnosis in this age group is mixed with transient wheezers, who are not the same as asthmatics [57].

The proportions of asthma remission per age group at follow-up were 33 – 53% in adolescence (<18 years), 6 – 33% in young adulthood (≥18 and ≤30 years), 11 – 52% in adulthood (>30 years). The majority of the studies focused on asthmatic children and their chance of going into asthma remission. This focus is likely due to the following reasons: asthma is the most common non-communicable disease among children [58], the remission proportion is highest in this age sub-group [50,59,60], and elucidating this phenomenon within this population is of highest prognostic value [17]. Studies determining the adult-onset asthma remission proportion on the other hand, are limited. This is because cohorts usually include individuals with child- and adulthood-onset of asthma, consequently mixing both groups when determining asthma remission. A recent prospective study only assessed adult-onset asthma, and found a remission prevalence of 16% within five years [32]. Based on these data, it is

premature to state that childhood-onset phenotype of asthma has a higher chance of going into remission compared to the adult-onset phenotype of the disease.

A few studies included lung function and histamine or methacholine provocation tests, thus allowing estimations for complete versus clinical asthma remission rates. The majority of the studies found a higher proportion of clinical asthma remission (ranging 10-30%) than the complete asthma remission status (ranging 5-22%). Two studies however, found higher prevalence proportions of complete compared to clinical remission [27,32]. An explanation for this difference could be that Westerhof *et al.* used a >4mg/ml methacholine threshold cut-off, classifying clinical remission subjects with mild bronchial hyperresponsiveness in the complete remission group.

Predictors of asthma remission

Despite the previously described differences between studies, there appears to be a degree of consistency in the factors that were associated with asthma remission later in life. The following baseline characteristics were positively associated with a higher asthma remission prevalence (last column in table 1) younger age of onset, mild asthma at onset, male sex, higher baseline lung function, less bronchial hyperresponsiveness at baseline, lower blood eosinophils and IgE at baseline, lower skin prick test scores (SPT), no comorbidities (i.e. nasal polyps, eczema, atopy or rhinitis), no pneumonia in the past, a negative family history of asthma and atopy, cessation of smoking and environmental factors (e.g. pets in household). The majority of the listed factors are well established due to the fact that these are also inversely related to uncontrolled asthma [1]. In the following section, some of these factors are further discussed.

Male sex

One of the acknowledged factors associated with asthma remission is male sex [18,24,28,30,35,51]. Before puberty, the prevalence of asthma is higher in boys than in girls. However, in adulthood, the prevalence of asthma reverses to be higher in females [61]. Female sex hormones have been linked to asthma and its morbidity; the risk of developing asthma is increased for those with a higher cumulative female sex hormone concentration seen in pregnancy [62] and early-onset menarche [61,63,64]. Additionally, 30 - 40% of female asthmatics experience perimenstrual asthma worsening [65,66]. The TRIALS study assessed associations of transition though puberty with asthma

remission in 2,230 male and female subjects [67]. The authors found a higher prevalence of asthma in girls aged 16 compared to boys, which was related to a higher incidence and lower remission rate of asthma in females compared to their male peers. From these studies, the hypothesis is that a lower level of female sex hormones might result in a higher chance of asthma remission.

There are three other potential mechanisms to explain why females have a less chance of asthma remission. First, there is a difference in physical growth of the lungs from birth into adulthood between boys and girls [65]; boys tend to have a later growth spurt than girls, which makes them more prone to wheezing due to smaller diameters of the airways. Second, the TRIALS study identified obesity as an additional/independent risk factor for asthma in female subjects both in cross-sectional and longitudinal analyses. Obesity is accompanied by an increased production of estrogens, with – next to the effect on puberty - potential effects on asthma as well [68]. And last, methacholine hyperresponsiveness is more severe in the post-pubertal female asthmatics compared to their male peers [69]. With these associations, targetting sex hormones - such as oral contraceptives - might work as therapy. Unfortunately, studies investigating the effect of oral contraceptive pills on asthma published contradicting results; one survey found a reduced prevalence of current wheeze in women with a history of asthma while on contraceptives [70], while another found no association [62]. In contrast, oral contraceptive pills have also been associated with an increased risk for asthma [64,71], and shown to have DNA methylating effects on polymorphisms of the GATA3 gene, a master regulator of TH2 cell differentiation, which is related to a higher risk of developing asthma [72].

Severity of asthma

The severity of asthma-onset is a recognized factor influencing the outcome of asthma remission [35,43,59]. In moderate and severe cases of asthma, the disease activity stays much the same over long periods of time [19,35,73], while subjects with mild and intermittent symptoms are likely to experience asthma remission. The Melbourne Asthma Study followed clinical and lung function features of childhood asthmatics aged 7 - 10 until the age of 50 [35]. Within this cohort, the children who solely wheezed during bronchitis and respiratory infections had the highest chance of remitted symptoms through all years ranging from 40 - 65%. Strikingly, severe asthmatic children had

the lowest remission rates; around 10%. Even though we have to keep in mind that the wheeze bronchitis- is not the same as the asthma-phenotype, figure 2 illustrates that an increase in asthma severity is negatively correlated with the remission rate.

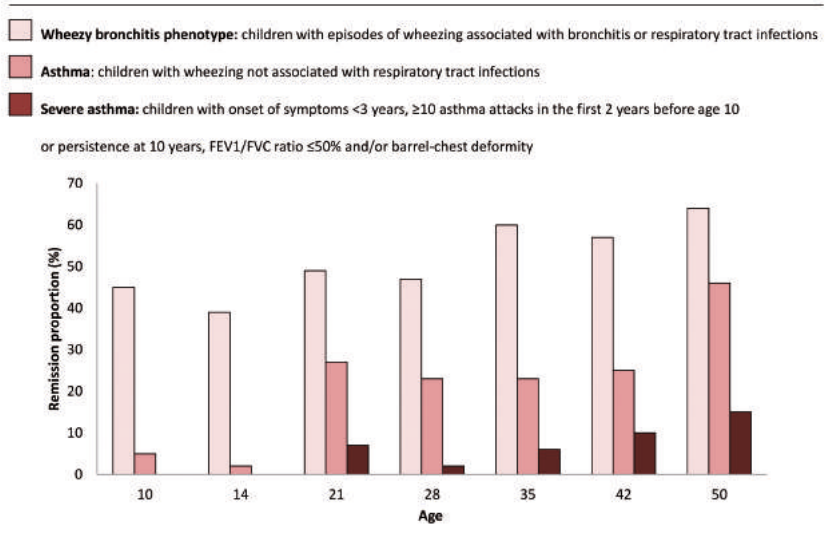

Figure 2: remission percentages at age 10 to 50 in children, enrolled at age 7 and divided in three asthma severity levels. This figure shows that less severe asthma is associated with a higher chance of remission later in life.
Reproduced, with permission (Tai A, et al. J Allergy Clin Immunol. 2014 Jun;133(6):1572-8.e3).

Very recently, authors of the CAMP-trial assessed whether clinical features in childhood asthmatics could predict asthma outcome later in life [17]. A model based on childhood clinical features was made to predict asthma remission in young adulthood. With a baseline FEV_1/FVC ratio ≥85%, a PC_{20} methacholine ≥1mg/ml and blood eosinophil count of <500 cells/µl, the probability of asthma remission at age 23 was 82.6%. In other words, this study demonstrated that subjects with milder asthma in childhood (i.e. no pulmonary obstruction, relatively mild bronchial hyperresponsiveness and low blood eosinophils) have a higher chance of going into remission. We applied this model on our own cohorts and found that children with these features had the highest chance of remission as well [74].

Pneumonia

Several studies found that the occurrence of pneumonia during childhood was associated with a reduced likelihood to go into asthma remission later in life [3,51,75]. This is in agreement with the hypothesis that childhood lower respiratory tract infections (LRTI), such as pneumonia, could influence asthma persistence [76,77] or trigger its inception [78]. The incidence of pneumonia is estimated to be 7.4% in the first three years of life with Respiratory Syncytial Virus (RSV) as the most common infectious agent in children [77]. The Tasmanian Longitudinal Health Study (TAHS) found that a higher frequency of infectious diseases in childhood protected against asthma later in life, but pneumonia was positively associated with self- or parent-reported asthma until adolescence [76]. These findings suggest a balance between infections and asthma persistence in children.

Other pediatric studies relied on radiologically diagnosed infiltrations [77,79,80], and saw an increase of asthma diagnoses after admissions due to childhood pneumonia and also bronchiolitis . The authors of the Tucson Children's Respiratory Study included children in the first years of life with radiologically confirmed pneumonia, and re-examined them up until eleven years of age [77]. Here, children with pneumonia had lower levels of FEV_1 and FEF25-75% compared to the unaffected children. More interesting, but unfortunately with insufficient subjects to draw a strong conclusion, a negative trend was seen in the maximal expiratory flow in neonates two months after birth, prior to these infections. This implies that these children might already have diminished lung function since birth, making them more susceptible for LRTIs later on. Thus, in accordance to the susceptibility-theory, individuals who go into asthma remission might be born with a better lung function than asthmatics with persistent disease activity.

Only one study was found that investigated pneumonia in adulthood and asthma remission. The ADONIS project enrolled 194 adult-onset asthmatics diagnosed in the previous year, assessed the patient reported trigger of asthma-onset and followed the five year course of asthma [81]. While the majority of patients could not recall any triggers (38%), 8% of the subjects thought that a pneumonia was the trigger. Adult-onset asthmatics that were thought to have been elicited by pneumonia, had a significantly higher chance of clinically verified asthma remission over the next five years.

Although the exact role of pneumonia on the course of asthma remains vague, it is clear that individuals affected by these infections are not burdened by asthma persistence per se. What triggers asthma persistence or remission may depend on peri- and postnatal factors, the severity of the infection, the ensuing local damage, microbe type and exposure duration [82,83]. Still, it is difficult to disentangle subjects developing asthma due to LRTIs from subjects that were already susceptible for asthma, subsequently having a higher risk of respiratory infections [80]. It is highly probable that LTRIs are merely a sign of underlying asthma susceptibility [79]. Abnormal lung function, altered airway structure and immune responses to viral infections all predispose infants to lower respiratory infections, which could further damage the airways leading to respiratory morbidity later in life [84,85]. Accordingly, individuals with asthma remission might be less susceptible (e.g. normal lung function, unaltered airway structure and immune response), enabling them to recover faster from respiratory infections.

Leukemia

We have previously shown that another factor associated with asthma remission is having a positive family history for leukemia [3]. In this study, children who had a first or second-degree family member were associated with both clinical and complete asthma remission at 25 years. Our results are in agreement with previously published findings as there have several links between leukemia and allergic diseases. In contrast, children with atopic first-degree relatives seemed to have a lower risk for acute lymphocytic leukemia (ALL) [86,87]. Various studies found a reduced chance to develop childhood asthma while affected by ALL [87–89]. Children born by caesarean delivery – causing a deviant immune-maturation - developed more asthma and ALL than children delivered via the vaginal route [90]. A meta-analysis found a significant pooled odds-ratio of ALL in atopy (0.69 ,95% CI 0.54 – 0.89) [91], while a more current meta-analysis updated a negative trend between ALL and asthma by Linabery *et al.* (OR 0.79, 95% CI 0.61-1.02) to a non-significant odds-ratio (P = 0.45) [92]. These findings suggest that a subset of allergic diseases and ALL have a protective effect on each other. Both conditions are linked to the hygiene hypothesis [93], proposed by Strachan to explain the rising prevalence of allergy in the western population (Strachan, 1989). However, this would imply similar incidence patterns. The asthma-leukemia relation may also be false due to several reasons. One, the mentioned pooled case-control studies that assess allergy do so by parental recall of allergies [93]. The parents of children affected by ALL may be more

likely to imagine factors that may have caused their child's condition, leading to false positive associations. A second reason is that treatment for leukemia might alleviate asthma symptoms, thereby mimicking remission while extreme immunosuppression was given. A case report described asthma remission after high-dose chemotherapy and autologous stem transplantation for breast cancer [94] , suggesting a beneficial effect of this therapy. On the other hand, chemotherapy for ALL in children was able to suppress asthma, but did not lead to long-term relief [95]. To date, no studies elucidated the biological connection to asthma and leukemia, leaving the inverse relationship of asthma remission and leukemia as a mere signal.

Intervention to induce asthma remission

The majority of factors that are associated with asthma remission cannot be altered or treated. Some treatments such as ICS and biological treatments can induce full control of symptoms but have not been associated with real remission. A few factors however, can be influenced and are thought to have an effect on asthma remission; weight loss and immunotherapy. It is noteworthy that bilateral lung transplantation has been linked to asthma remission as well [96,97], still this controversial intervention followed by immunosuppression is not discussed in this review.

Weight loss

The asthma-obesity relationship is well described, although the mechanisms underlying it are not well understood [98]. It is hypothesized that this relation is affected by a different type of inflammation [99] and by other comorbidities such as gastroesophageal reflux and diabetes mellitus type 2 [100,101]. Weight loss has shown positive effects on several measures of asthma-control [102–105], but it is unclear whether weight loss could lead to clinical asthma remission. De Marco *et al.* saw that asthma remission was negatively associated with an increase in BMI over 10 years follow-up [40]. Taking this into account, it is possible that non-surgical weight loss can induce asthma remission. Yet, due to the number of subjects needed to be followed up to assess asthma remission, non-surgical weight loss interventions are laborious to carry out and are at risk for confounding.

The other option is surgical weight loss, such as bariatric surgery, which has also been associated with improved asthma control [106–109], but again not necessarily to

remission. Macgregor and Greenberg studied 40 morbidly obese patients with severe (i.e. >10 asthma attacks) or moderately severe (i.e. 6 - 10 attacks) asthma, and saw that after 4 years 49% of the subjects reported asthma remission, while the rest all experienced less symptoms and medication usage [110]. The United Kingdom National Bariatric Surgery Registry analyzed the prevalence of comorbidities after this surgical intervention over five years [111]. Of the 50,782 entries, 19% had asthma and were all treated with either inhalers or additional medication. After one year, the prevalence of clinician verified asthma significantly decreased to 14%. Intriguingly, the prevalence of asthma remained somewhat the same after the additional four years of follow-up, indicating that the effect of bariatric surgery on asthma is predominantly within the first year after treatment. The pathophysiology for this might be that in some cases the sudden weight loss improves lung mechanics or alleviates the chronic inflammation due to obesity, decreasing the symptoms to an extent that subjects are not burdened anymore, while in others, the asthmatic inflammation remained the main component of the chronic inflammation.

Immunotherapy

Various studies found that negativity of skin prick tests (SPTs) was associated with asthma remission [21,28,34,35]. Allergen avoidance is also related to asthma remission [112], although it is debateable whether avoiding these triggers is similar to true remission.

Lee *et al.* performed a retrospective cohort study of 627 adults with allergic asthma who were sensitized to house dust mite and/or pollens and underwent subcutaneous immunotherapy [113]. All participants had documented symptoms, were either bronchial hyperresponsive to methacholine (PC_{20} ≤25mg/ml) or reversible to salbutamol (≥12% and ≥200ml), and had positivity to at least one inhalant allergen during a SPT. In this study, the cumulative incidence of asthma remission continuously increased up to 87% until the 12th year, with an average maintenance period of 5.1 years. Similar results were found in a smaller retrospective study, including 39 mild-moderate asthmatic children treated with a three-year sublingual immunotherapy with a mixture of Dermatophagoides [114]. Again, high remission rates of 95% were reported. However, both studies were flawed in design: individuals with asthma remission could still use bronchodilators in these cohorts, or could have symptoms if they did not respond to

methacholine. Second, both studies did not investigate if asthma relapsed after subjects were withdrawn from immunotherapy. Last and most important, no asthma-control groups were assessed to take into account the natural course of asthma.

Possibly due to these shortcomings, other studies did not see an effect of allergen immunotherapy on the remission rate [115]. A double-blind placebo controlled trial was conducted two decades ago, enrolling moderate-to-severe asthmatic children and administering subcutaneous injections of either seven aeroallergen extracts or placebo for ≥18 months [116]. Here, asthma remission (i.e. no medication after 30 months) was achieved in 8% of the immunotherapy- and 9% in the placebo-group, indicating that the injections did not seem to be beneficial for inducing the remission of asthma. Since the latter study has the most scientific credibility, it is not likely that immunotherapy induces asthma remission.

Airway inflammation in asthma remission

It has been previously described in other reviews that the level of airway inflammation has a relationship with the development of asthma remission over time [6,117]. Figure 3 illustrates a theory in which asthma severity is correlated to whether a subject experiences symptoms or experiences lung function impairment.

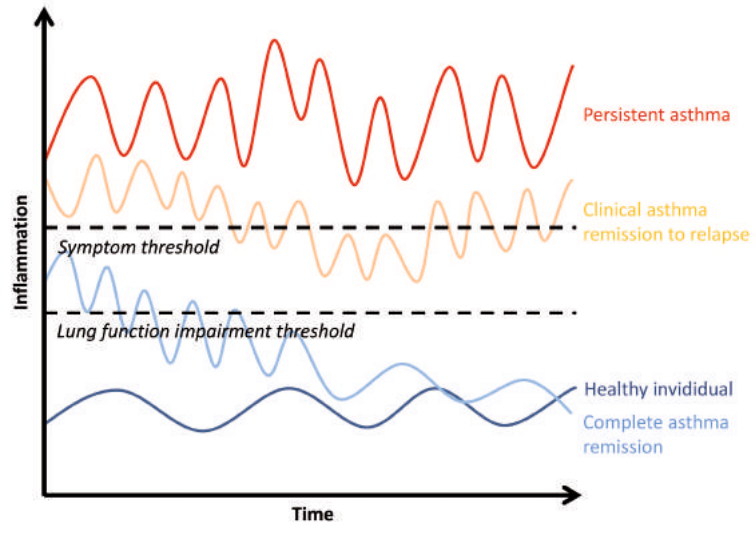

Figure 3: theoretical trajectory of persistent asthma, asthma remission and relapse over time. Adapted, with permission (Upham JW, et al. Pharmacol Ther. 2011 Apr;130(1):38-45).

In accordance with figure 3, individuals with remitted asthma might still have ongoing airway inflammation [4]. A variety of studies assessed the inflammatory markers in different compartments (e.g. blood, sputum, biopsy), subsequently comparing their presence in asthma remission subjects with either asthmatics, healthy controls, or both (see table 2). In general, the majority of findings were consistent (i.e. markers were higher in asthma remission compared to healthy control and lower compared to persistent asthma), although some studies found no significant differences between the groups. Eosinophils, either in blood, sputum, bronchial alveolar lavage (BAL), or bronchial biopsy, were the most studied. Of interest, eosinophil cationic protein and eosinophilic peroxidase levels were significantly lower in complete asthma remission subjects compared to persistent asthmatics, but no significant difference was found when comparing the latter with clinical asthma remission. This suggests some eosinophilic activity in the clinical asthma remission group [4], which might have clinical consequences; this same cohort was followed for five years to show that asthmatics with fast FEV_1 decline (i.e. >30ml/year) were linked to higher levels of eosinophils in sputum and biopsies, which was not seen in the complete asthma remission subjects and asthmatics with slow FEV_1 decline [118]. Other biomarkers that were significantly different between the groups were blood IgE, blood and subepithelial IL-5, exhaled fractional and (sub-)epithelial inducible nitric oxide, sputum Tumour Necrosis Factor α and (sub-)epithelial tryptase and chymase. The majority of these inflammatory markers are recognised for their link to the TH2 pathway [119–121].

Table 2: biomarkers associated with clinical and complete asthma remission in cross-sectional studies

Biomarkers	Significantly higher/lower than in healthy controls (P<0.05)			Significantly higher/lower than in persistent asthmatics (P<0.05)		
	Higher (+)	*Lower (-)*	*Non-significance*	*Higher (+)*	*Lower (-)*	*Non-significance*
Blood eosinophils	Kim *et al.* 2018 Boulet *et al.* 2012b [ComR]		Xu *et al.* 2000 Waserman *et al.* 2012 Volbeda *et al.* 2010		Broekema *et al.* 2011 Kim *et al.* 2018 Noma *et al.* 1999 Waserman *et al.* 2012	Boulet *et al.* 2012b
Blood IgE	Kim *et al.* 2018 Noma *et al.* 1999 van den Toorn *et al.* 2001		Waserman *et al.* 2012 Boulet *et al.* 2012[ComR] Volbeda *et al.* 2010		Xu *et al.* 2000 van den Toorn *et al.* 2001 Broekema *et al.* 2011	Kim *et al.* 2018 Waserman *et al.* 2012 Andersson *et al.* 2013 Panhuysen *et al.* 1997 Boulet *et al.* 2012 [ComR]
Blood IL-5 activity	Noma *et al.* 1999 Tomiita *et al.* 2015				Noma *et al.* 1999	Tomiita *et al.* 2015
Blood IL-10 activity			Tomiita *et al.* 2015			Tomiita *et al.* 2015
Blood regulating T-cells [B]			Boulet *et al.* 2012 [ComR]		Boulet *et al.* 2012b [ComR]	
Exhaled air FeNO	van den Toorn *et al.* 2000		-		Arshad *et al.* 2014	van den Toorn *et al.* 2000
Sputum eosinophils	Obase *et al.* 2001		Boulet *et al.* 2012 [ComR]		Waserman *et al.* 2012 Volbeda *et al.* 2010[A]	Boulet *et al.* 2012 [ComR] Broekema *et al.* 2011 Arshad *et al.* 2014 Obase *et al.* 2001
Sputum ECP	Obase *et al.* 2001		Waserman *et al.* 2012 Broekema *et al.* 2011		Waserman *et al.* 2012 Broekema *et al.* 2011[ComR]	Broekema *et al.* 2011 Arshad *et al.* 2014 Obase *et al.* 2011
Sputum neutrophils			Boulet *et al.* 2012 [ComR] Broekema *et al.* 2011 Volbeda *et al.* 2010			Boulet *et al.* 2012 [ComR] Broekema *et al.* 2011 Waserman *et al.* 2012 Volbeda *et al.* 2010 Arshad *et al.* 2014

Table 2: (continued)

Biomarkers	Significantly higher/lower than in healthy controls (P<0.05)			Significantly higher/lower than in persistent asthmatics (P<0.05)		
	Higher (+)	*Lower (-)*	*Non-significance*	*Higher (+)*	*Lower (-)*	*Non-significance*
Sputum histamine			-		Broekema *et al.* 2011	
Sputum macrophages			Waserman *et al.* 2012 Volbeda *et al.* 2010			Waserman *et al.* 2012 Volbeda *et al.* 2010
Sputum lymphocytes			Waserman *et al.* 2012 Volbeda *et al.* 2010	Broekema *et al.* 2011 ClinR		Waserman *et al.* 2012 Volbeda *et al.* 2010 Broekema *et al.* 2011 ComR
Sputum TNF-α	Obase *et al.* 2001		Waserman *et al.* 2012		Waserman *et al.* 2012	Obase *et al.* 2011
Sputum IL-5			Waserman *et al.* 2012			Waserman *et al.* 2012
Sputum IL-10			Waserman *et al.* 2012			Waserman *et al.* 2012
Sputum IL-12			Waserman *et al.* 2012		Waserman *et al.* 2012	
BAL eosinophils	Warke *et al.* 2002					
BAL neutrophils			Warke *et al.* 2002			
BAL mast cells			Warke *et al.* 2002			
BAL macrophages			Warke *et al.* 2002			
BAL lymphocytes			Warke *et al.* 2002			
Subepithelial eosinophils			van den Toorn *et al.* 2001			Broekema *et al.* 2011
Epithelial eosinophils			van den Toorn *et al.* 2001			van den Toorn *et al.* 2001
EPX immunopositivity			-		Broekema *et al.* 2011 ComR	Broekema *et al.* 2011 ClinR
Subepithelial neutrophils			-			Broekema *et al.* 2011
Subepithelial tryptase	van den Toorn *et al.* 2001			van den Toorn *et al.* 2001		
Epithelial tryptase			van den Toorn *et al.* 2001	van den Toorn *et al.* 2001		Broekema *et al.* 2011
Subepithelial chymase			van den Toorn *et al.* 2001	van den Toorn *et al.* 2001		
Epithelial chymase			van den Toorn *et al.* 2001	van den Toorn *et al.* 2001		

Table 2: (continued)

Biomarkers	Significantly higher/lower than in healthy controls (P<0.05)			Significantly higher/lower than in persistent asthmatics (P<0.05)		
	Higher (+)	*Lower (-)*	*Non-significance*	*Higher (+)*	*Lower (-)*	*Non-significance*
Subepithelial macrophages			van den Toorn *et al.* 2001			van den Toorn *et al.* 2001 Broekema *et al.* 2011
Epithelial macrophages			van den Toorn *et al.* 2001			van den Toorn *et al.* 2001
Subepithelial CD^{4+} T-cells			van den Toorn *et al.* 2001			Broekema *et al.* 2011 van den Toorn *et al.* 2001
Epithelial CD^{4+} T-cells			van den Toorn *et al.* 2001			van den Toorn *et al.* 2001
Subepithelial CD^{8+} T-cells			van den Toorn *et al.* 2001			Broekema *et al.* 2011 van den Toorn *et al.* 2001
Epithelial CD^{8+} T-cells			van den Toorn *et al.* 2001			van den Toorn *et al.* 2001
Subepithelial CD^{25+} T-cells			van den Toorn *et al.* 2001			van den Toorn *et al.* 2001
Epithelial CD^{25+} T-cells			van den Toorn *et al.* 2001			van den Toorn *et al.* 2001
Subepithelial CD^{69+} T-cells			van den Toorn *et al.* 2001			van den Toorn *et al.* 2001
Epithelial CD^{69+} T-cells			van den Toorn *et al.* 2001			van den Toorn *et al.* 2001
Subepithelial CD^{20+} B-cells			-			Broekema *et al.* 2011
Subepithelial IL-5	van den Toorn *et al.* 2001					van den Toorn *et al.* 2001
Epithelial IL-5			van den Toorn *et al.* 2001			van den Toorn *et al.* 2001
Subepithelial INOS			van den Toorn *et al.* 2001	van den Toorn *et al.* 2001		
Epithelial INOS	van den Toorn *et al.* 2001					van den Toorn *et al.* 2001

***Arshad** et al.* 2014 [24]: n = 108/45/0, *mean age* 18, ***Andersson** et al.* 2013 [28]: n = 84/43/0, *age range* 16-17, ***Boulet** et al.* 2012 [122]: n = 29/42/15 *mean age* 32, ***Broekema** et al.* 2011 [4]: n = 103/62/0, *mean age* 49, *range* 18-75, ***Kim** et al.* 2014 [123]: n = 31/30/31, *mean age* 8, ***Noma** et al.* 1999 [124]: n = 6/7/7, *age range* 6-35, ***Obase** et al.* 2001 [125]: n = 20/20/80, *mean age, range* 20-29, ***Panhuysen** et al.* 1997 [18]: n = 161/201/0 *mean age* 48, *range* 35-71, ***Tomiita** et al.* 2015 [119]: n = 18/15/14, *mean age* 21, ***van den Toorn** et al.* 2000 & 2011 [120,121]: n = 19/18/17, *mean age* 22, *range* 18-25, ***Broekema** et al.* 2010 [118]: n = 46/7/0 *mean age* 49, *range* 18-70, ***Warke** et al.* 2002 [126]: n = 0/25/35, *mean age* 7, ***Waserman** et al.* 2012 [127]: n = 15/15/15, *mean age* 14, *range* 12-18, ***Xu** et al.* 2000 [128]: n = 0/20/30, *age range* >18.

A: Adenosine-'5-monophosphate provocation test induced eosinophilia, B: capacity of T-regs to suppress proliferation of effector T-cells, BAL: bronchial alveolar lavage, ClinR: only in clinical asthma remission, ComR: only in complete asthma remission, ECP: eosinophil cationic protein, EPX: eosinophilic peroxidase, FeNO: fractional exhaled nitric oxide, INOS: inducible nitric oxide synthase

Airway remodeling in asthma remission

Chronic inflammation of the airways may lead to altered structure in the airway wall, referred to as remodeling [129]. Airway remodeling, such as increased basement membrane thickness, can occur early in childhood and is associated with an increased risk of developing clinical asthma [130]. Studies investigating airway remodeling in asthma remission are scarce [4,121]. A cross-sectional study assessing remodeling enrolled 54 never-smoking adolescents aged 18 - 25 in three groups; asthmatics, asthma remission subjects and healthy controls [121]. Bronchial biopsies were obtained from the segmental divisions of the main bronchi. While the reticular basement membrane thickness in asthma and asthma remission subjects was similar (11.5µm ±1.5 versus 10.9µm ±1.3 respectively), both were significantly thicker than those in the healthy controls (7.9µ ±1.0, both P<0.001). Additionally, the reticular basement membrane to total membrane ratio of the remission subjects differed significantly from the values obtained from the asthmatics and in controls, falling between these two ranges. The collagen III density in the biopsies – a component of airway remodeling – was not significantly different between the groups. Another group investigated the phenomenon of airway remodeling in a comparable design, including 129 adults of all ages and dividing remission subjects in either clinical- or complete remission [4]. The authors found that asthmatics and individuals with clinical remission had a higher degree of inflammatory markers in blood and biopsies than asthmatics had, but basement membrane thickness was not significantly different. Of interest, asthmatics who used ICS had a significantly lower basement membrane thickness compared to ICS-naïve asthmatics and clinical remission subjects (5.3µm [2.8-8.2] versus 5.7µm [2.8-12.6] and 6.5µm [3.8-11.7, P=0.04 and P<0.001 respectively). Again, no difference in collagen III stained submucosae was found between the groups.

These studies suggest that basement membrane thickening is still present in clinical and complete remission. The authors questioned if basement membrane thickening by itself could be a risk factor for asthma relapse, or if it is just an end-stage of disease with histological "scarring" [4]. To answer this question, asthma remission subjects need to be followed at different and longer time points, undergoing bronchoscopies for such histological assessments. Unfortunately, these longitudinal studies are hard to perform and as such the question still remains to be addressed.

Genotyping asthma remission

Only three genetic studies have been performed on asthma remission subjects. To explore if SERPINE1 polymorphism is linked to asthma remission, Dijkstra *et al.* re-evaluated a longitudinal cohort of 281 asthmatics and asthma remission subjects [18,131], with an independent population-based cohort as a control group. Asthmatics with the 5G allele had significantly higher serum total IgE levels, a lower FEV_1, and a faster annual FEV_1 decline compared to the control cohort. More interestingly, complete asthma remission was significantly more prevalent in subjects with the 4G/4G genotype (20%), compared to the 4G/5G (11%) or 5G/5G genotype (4%). The SERPINE1 -675 4G/5G promotor polymorphism regulates plasminogen activator inhibitor (PAI)-1 levels, a key regulator of the plasminogen activator system, and has been associated with elevated serum IgE levels [132,133], and both the development and the severity of asthma [133–135]. The authors concluded that these findings could reflect differences in chronic airway inflammation and remodeling between the genotypes.

Genome-wide association studies (GWAS) have provided insights into the origins of asthma and identified multiple genes associated with its development [136], but GWAS studies examining remission are more scarce. In a recent GWAS study, Vonk et al investigated 612 persistent asthmatics, 178 clinical remission subjects and 55 complete remission subjects as an identification cohort and replicated the results in two smaller independent cohorts [20]. Only one single nucleotide polymorphism (SNP) could be replicated in clinical remission, while in complete asthma remission, two SNPs were replicated: the top SNP, rs6581895, almost reached genome-wide significance and was an expression quantitative locus (eQTL) for fibroblast growth factor receptor substrate 2 (FRS2) and chaperonin containing TCP1 subunit 2 (CCT2). FRS2 is a critical regulator of VEGF receptor signalling in lung tissue, which may affect angiogenesis [137], potentially contributing to the resolution of inflammation. CCT2 has been associated with cell growth [138] and maintenance of cell proliferation [139]. The second SNP, rs1420101, is a cis-eQTL for IL1RL1 and IL18R1 a trans-eQTL for IL13 in lung tissue. Intriguingly, the expression of IL1RL1, IL18R and IL13 are associated with a risk for asthma [140,141]. This could imply that these variants play a role in regulating type 2 inflammation [20].

Finally, there is increasing interest in the role of the airway microbiome in respiratory diseases. The microbiome may play a significant role in airway remodeling through the

stimulation of various immune and inflammatory pathways, subsequently affecting the course of asthma [142]. A cross-sectional cohort of 30 children with asthma remission, 31 with persistent asthma and 30 controls were studied in the KOREA study [143]. DNA was extracted from nasopharyngeal swabs, to analyze the composition of microbiota among the groups and their clinical features. Genera that comprised more than 1% of the microbiota in over 50% of the samples were analyzed. The authors found a significantly higher abundance of Staphylococcus in the asthma-group (13%), compared to the asthma remission (8%) and controls (2%). The relative highest abundance of Haemophilus influenzae was seen in the healthy controls, while Fusobacterium was seen relatively highest in the remission-group. Additionally, there was a significant negative correlation between the separate abundance of Staphylococcus and Firmicutes with bronchial hyperresponsiveness, and a negative correlation with Streptococcus and lung function. This could imply that a different airway microbiome might contribute to the severity of bronchial hyperresponsiveness, resulting in a different course of asthma in children [143].

Taken all together, these three studies implicate pathophysiological pathways that have not yet been studied thoroughly in asthma remission. They found significant differences between asthma remission and both asthma and controls, strengthening the hypothesis that asthma remission is a valid pathological state, with the potential of identifying a causal pathway leading to remission. In this sense, complete asthma remission is most likely to be a more rewarding candidate state for research compared to clinical asthma remission.

Pharmacological potential of achieving asthma remission

As described previously, two interventions are thought to have the potential to induce long-term asthma remission: weight reduction and hormonal therapy. And by elucidating the pathways associated with the relatively new disease state called complete asthma remission (e.g. inflammatory- and gene-expression features) there is an opportunity for identifying new potential targets. As an analogy: in the last decades, understanding short-term variation as related to eosinophil numbers in asthmatics has greatly assisted the introduction of anti-IL5 therapy [144,145]. Similarly, but on a much longer time scale, understanding indicators or pathways associated with complete remission could also help introducing new therapeutic targets.

There are various novel therapies for asthma that are under development [146]: Dupilumab [147], a monoclonal antibody that blocks the common receptor for IL-4 and IL-13, Tezepelumab [148], a monoclonal antibody directed against thymic stromal lymphopoeitin which is produced by epithelial cells and affects multiple immune cells, and Fevipiprant [149], an agent that blocks the prostaglandin D2 receptor CRTH2. Unsurprisingly, these three options are predominantly tested on severe asthmatics, but in future might be used to achieve asthma remission in less severe cases.

Conclusions and future perspectives

Defining asthma remission is a complex issue; to date, it is poorly understood even though it has been the ultimate therapeutic goal for so long. When determining the prevalence, risk factors, and clinical correlates of remission based on ill-defined criteria, the relevance can be questioned. However, when defined properly, such as in complete asthma remission, we might find biological triggers that actually cause asthma to spontaneously remit. We believe that complete asthma remission is a more robust pathological disease state, which has more prognostic and scientific value than clinical asthma remission. Future research is needed to explore its phenotype and underlying mechanisms. To further look into the clinical features of asthma remission, it could be rewarding to assess small airways disease using novel diagnostic tools, such as measuring and analysing particles in exhaled air [150], functional CT imaging by parametric response mapping of the lungs [151], optical coherence tomography of the airway walls [152], and further characterization of the histology in bronchial biopsies. These new techniques might give us more insight in the detection and monitoring of small airways disease and remodeling, both being proposed contributors to persistence of asthma. To disentangle underlying mechanisms, single-cell RNA-sequencing of inflammatory- and epithelial cells has the potential to find targets which may induce asthma remission [153]

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Chapter 3

Childhood factors associated with complete and clinical asthma remission at 25 and 49 years

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Abstract

While asthma is a chronic respiratory disease without cure, asthma remission is the highest attainable goal for patients. The aim of this study is to assess asthma (remission) persistence till age of 49 and which factors in childhood are associated with clinical and/or complete asthma remission.

In 1972-1976, children diagnosed with clinical asthma were characterized (questionnaires, peripheral blood, bronchial hyperresponsiveness, lung function and skin prick tests (SPT)) and were re-examined in young adulthood and late adulthood. In these latter visits, the subjects were divided in three groups: persistent asthma (PersA), clinical- (ClinR) and complete asthma remission (ComR).

188 subjects were seen at age 25 (±21), while 102 were seen at age 48.9 (±2.1). 66 subjects were completely characterized in all three visits. At 49 years, 75.0% of the ComR subjects did not relapse in asthma. Childhood factors associated with ComR compared to PersA were having a leukaemia positive family history, a higher FEV_1/FVC ratio, a positive mould SPT, less childhood wheeze during a cold and having an atopic mother.

It is possible to have persistent remission after childhood asthma over 39 years of follow-up. Therefore is important to find out which genetic and/or environmental factors drive asthma remission.

Introduction

Asthma can go into remission later in life in approximately 35% of all patients [1]. Asthma remission is associated with childhood onset of asthma [2, 3], the male sex, smoking cessation, initially less severe airway obstruction and, notably, more severe bronchial hyperresponsiveness (BHR) [4, 5]. Unfortunately, patients in asthma remission may show relapse later in life. Data regarding childhood factors that predict clinical asthma remission in adulthood are sparse, and even fewer data on complete asthma remission [6] or the persistence of asthma remission throughout the lifespan are presently available. Therefore, the aim of the current study is to explore whether asthma remission persists in children, followed from childhood up to an average age of 49 years. Furthermore, we determined which factors in childhood are associated with clinical and/or complete asthma remission that persists during adulthood.

Methods

An asthma cohort was created from 1972–1976 (visit 1) and re-investigated from 1987–1989 (visit 2) [7] and 2013–2014 (visit 3). At visit 1, 406 children diagnosed with asthma were referred to the University Medical Center Groningen, and characterised by extensive standardised questionnaires, peripheral blood measurements, BHR, lung function and skin prick tests. At visit 2, 285 children (209 children with BHR at visit 1) were re-examined in young adulthood. At visit 3, 102 subjects with BHR at visit 1 were included and re-examined.

Predicted spirometry values at all visits were those of Quanjer [16]. At visit 1 and 2, the histamine concentration (range 0.5 to 32 mg/ml) with a decrease in FEV_1 >10% from pre-challenge FEV_1 was taken as the threshold for BHR. At visit 3, the BHR-test was discontinued if the FEV1 decreased ≥20% from pre-challenge FEV_1 or if the highest dose of methacholine bromide (range 0.038 – 39.3 mg/ml) had been given. The skin prick test (SPT) was regarded positive if the mean of the perpendicular diameters of the wheal was > 0.7 times the mean diameter of histamine as positive control.

The presence of persistent asthma (PersA), clinical asthma remission (ClinR) and complete asthma remission (ComR) was determined at visits 2 and 3. ClinR was defined

as the absence of symptoms (dyspnoea attacks and/or wheezing), without asthma medication over the last year, irrespective of the presence or absence of a positive BHR test (visit 2: PC_{10} (provocative concentration causing a 10% fall in FEV_1 (forced expiratory volume in 1 second) histamine ≤16 mg/ml; visit 3: PC_{20} methacholin ≤39.3 mg/ml) and/or normal FEV_1 (>80% predicted pre-bronchodilator). ComR was defined as ClinR, combined with a negative BHR test and a normal FEV_1 (>80% predicted pre-bronchodilator) (figure 1). Subjects with PersA still had symptoms and/or used asthma medication. Associations between childhood factors and asthma outcome (ComR versus PersA and ComR/ClinR versus PersA) at visits 2 and 3 were studied, by performing multivariate logistic regression with adjustments for sex and age at visit 1 using the SPSS 22 software (SPSS Inc, Chicago, IL, USA). A P-value <0.05 was considered statistically significant.

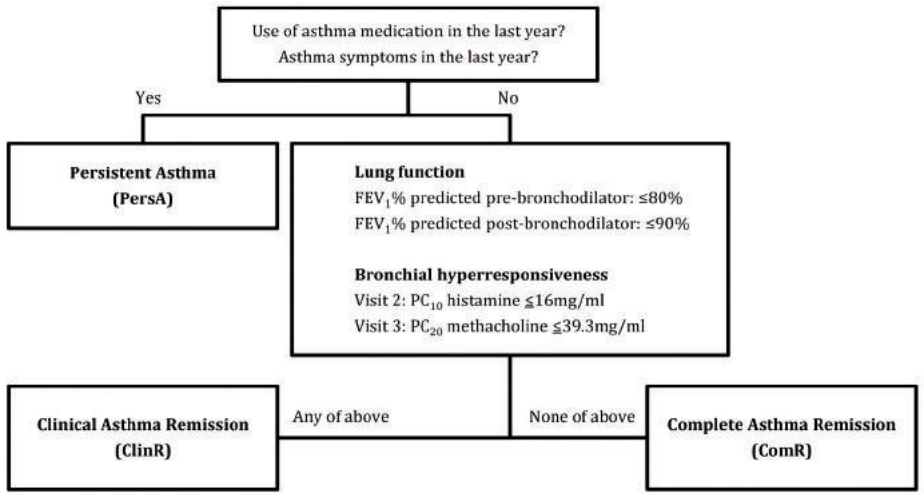

Figure 1: flowchart to define persistent asthma, clinical asthma remission and complete asthma remission.

Results and discussion

Of the initial cohort of 406 children, 209 had BHR at visit 1. At visit 2, 188 subjects were re-assessed at a mean age of 25 (±2) years, of whom 25 only completed questionnaires. At visit 3, 102 subjects with a mean age of 49 (±2) years were re-assessed, of whom 34 only completed questionnaires (figure 2).

	Visit 1 (1972–76) 406 subjects	Visit 2 (1987-89) 209 subjects	Visit 3 (2013-14) 209 subjects
Excluded	187 No BHR 10 Incomplete childhood data	21 Lost to follow-up	65 No response 31 Untraceable 6 Not cooperative 5 Deceased
Included	209 subjects	188 subjects	102 subjects
Asthma outcome	-	154 PersA (81.9%) 20 ClinR (10.6%) 14 ComR (7.5%)	61 PersA (59.8%) 31 ClinR (30.4%) 10 ComR (9.8%)

Figure 2: subject recruitment during al visits.

Subjects who completed questionnaires only at visits 2 or 3 could only be classified as ClinR or PersA, as indications of BHR and FEV_1 were missing. Subjects with questionnaire data at visit 2 only, had significantly lower FEV_1/IVC (inspiratory vital capacity) values at baseline, compared to subjects with both questionnaire and spirometry data, without any other statistically significant demographic differences (table 1).

The prevalence of complete remission was 7% at age 25 and 10% at age 49, indicating that ComR is a rare phenomenon. To our knowledge, only one other study using the same definition reported that 22% of children with asthma were in ComR after a follow-up of 30 years [6]. The prevalence of ComR in our study is probably an underestimation, because information on BHR and FEV_1 was missing in almost half of the ClinR subjects.

Sixty-three subjects were fully examined during both follow-up visits (figure 3). Of all subjects with PersA at visit 2, 15 developed ClinR (29%) and four developed ComR (8%) at visit 3. Seven out of the 63 subjects (11%) had ComR/ClinR between the ages 25 to 49. The prevalence of asthma-relapse at visit 3 was 36%, i.e. clinical asthma after ComR/ClinR at visit 2. Notably, using the strict definition of remission, 75% of the subjects with ComR at age 25 had no relapse of asthma by the age of 49.

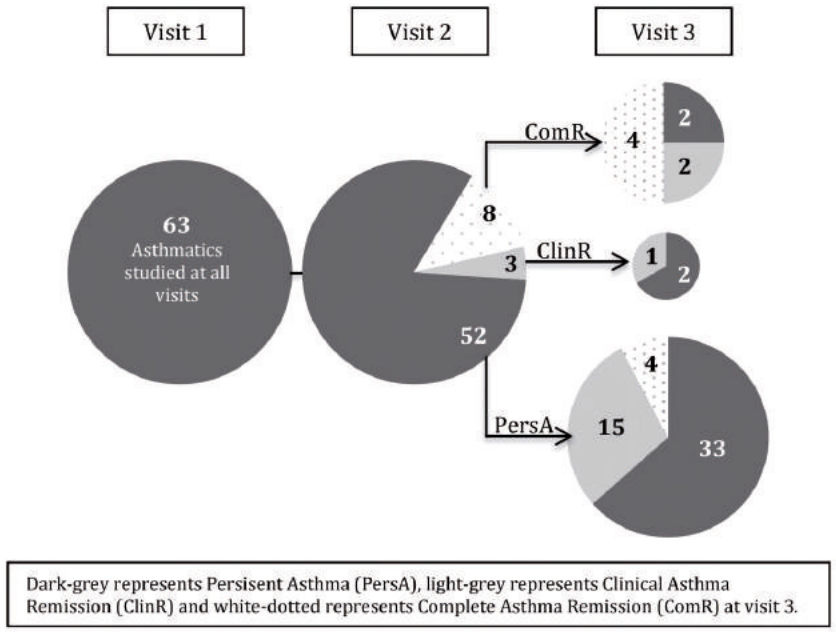

Figure 3: timeline of asthma remission persistence and relapse. Dark-grey represents persistent asthmatics (PersA), light-grey represents clinical asthma remission (ClinR), and the white-dotted pie piece represents complete asthma remission (ComR).

Table 2 shows results of the analyses of childhood factors associated with ComR versus PersA and ComR/ClinR versus PersA. The variables presented are childhood factors associated with asthma remission in previous studies [2, 4, 5, 7–9] and statistically significant factors in the present study (supplementary tables 3–6 for complete results). Three childhood factors were positively associated with ComR at visit 2, i.e. a family history of leukaemia, a higher FEV_1/IVC ratio and a positive skin prick test to mould. Acute lymphocytic leukaemia has been previously associated with a lower prevalence of atopic diseases [10, 11]. This inverse relationship might be caused by an imbalance between TH1 and TH2 cells, predisposing the patient to either autoimmune or atopic disease [12]. Although only five children had a family history of leukaemia in our cohort, results suggest that this phenomenon might also play a role in asthma remission.

Table 1: characteristics of subjects who were fully examined versus only filled in a questionnaire

Baseline characteristics	Visit 2 (n = 188)		P	Visit 3 (n = 102)		P
	Fully examined (n = 163)	Only questionnaires (n = 25)		Fully examined (n = 68)	Only questionnaires (n = 34)	
Age at visit 1 (years)	9.7 (1.4)	9.2 (1.4)	.16	9.6 (1.6)	9.8 (1.4)	.64
Male seks (%)	69.9	80.0	.35	76.5	63.2	.26
Height (cm)	143 (10)	140 (9)	.15	143 (10)	144 (10)	.47
FEV_1 % predicted (%)	77 (68-85)	73 (63-83)	.18	77 (65-84)	76 (68-80)	.60
FEV_1/IVC ratio (%)	74 (67-78)	67 (60-77)	.02	74 (66-79)	71 (67-77)	.30
Histamine threshold (mg/ml)	8 (4-16)	8 (4-16)	.18	12 (8-16)	8 (4-16)	.27
Eosinophil count x 10^6/L *	451 (231-715)	440 (336-605)	.53	462 (264-710)	402 (237-677)	.55

*Data are presented as mean (standard deviation), median [interquartile range], or percentage, P: p-value calculated with Fisher's exact test, Independent T-test and Mann-Whitney U test, *: for visits 1 and 2 eosinophils from a counting chamber.*

Table 2: Childhood factors associated with complete and clinical asthma remission

	Complete remission *versus* Persistent Asthma		Complete and clinical remission *versus* Persistent asthma	
	Visit 2: 25 years Remission (n = 14) Asthma (n = 154) OR (95% CI)	**Visit 3: 49 years Remission (n = 10) Asthma (n = 61) OR (95% CI)**	**Visit 2: 25 years Remission (n = 34) Asthma (n = 154) OR (95% CI)**	**Visit 3: 49 years Remission (n = 41) Asthma (n = 61) OR (95% CI)**
Age at 1st visit, years	1.3 (0.9-1.9)	1.0 (0.6-1.5)	1.0 (0.8-1.3)	1.0 (0.7-1.3)
Period between visits, years	1.3 (0.9-1.9)	1.0 (0.9-1.1)	1.1 (0.9-1.4)	1.0 (0.9-1.1)
Male sex	1.7 (0.4-6.3)	1.4 (0.3-6.0)	2.7 (.99-7.4)	1.9 (0.8-4.5)
Symptoms				
Sputum	0.6 (0.2-2.0)	0.4 (0.1-1.7)	0.8 (0.4-1.8)	**0.4 (0.1-0.8)**
Shortness of breath at rest	0.7 (0.2-2.5)	0.4 (0.1-2.3)	1.1 (0.5-2.6)	0.3 (0.1-0.9)
Attack of dyspnea at rest with wheezing	0.5 (0.2-1.8)	0.4 (0.1-2.0)	1.0 (0.4-2.6)	0.5 (0.2-1.2)
Wheeze during a cold	**0.2 (0.1-0.7)**	6.3 (0.7-56.4)	**0.2 (0.1-0.7)**	2.0 (0.8-5.0)
Cough	1.8 (0.4-8.6)	0.9 (0.2-4.7)	0.8 (0.3-1.8)	0.8 (0.3-2.4)
Peripheral blood				
Ln eosinophil count, x 10^6/L	0.9 (0.5-1.5)	1.0 (0.6-1.9)	0.8 (0.6-1.2)	1.2 (0.8-1.9)
Total proteins (<6.8 g/dL)	0.2 (0.03-1.8)	1.8 (0.4-8.2)	**0.2 (0.1-0.8)**	1.4 (0.5-3.8)
Peri- and postnatal factors				
Breastfeeding > 6 months	2.4 (0.2-28.1)	1.0*	**5.5 (1.1-27.1)**	0.3 (0.1-2.1)
History of pneumonia	0.2 (0.03-1.8)	0.5 (0.1-4.8)	**0.1 (0.01-0.9)**	0.7 (0.2-2.2)
Environmental exposures				
Pets in household	1.3 (0.4-4.1)	2.4 (0.4-12.5)	1.1 (0.5-2.5)	**3.1 (1.2-8.2)**
Firstborn child	1.5 (0.4-4.4)	0.5 (0.1-2.7)	0.7 (0.3-1.6)	0.9 (0.3-2.1)

Table 2: (continued)

	Complete remission *versus* Persistent Asthma		Complete and clinical remission *versus* Persistent asthma	
	Visit 2: 25 years Remission (n = 14) Asthma (n = 154) OR (95% CI)	**Visit 3: 49 years Remission (n = 10) Asthma (n = 61) OR (95% CI)**	**Visit 2: 25 years Remission (n = 34) Asthma (n = 154) OR (95% CI)**	**Visit 3: 49 years Remission (n = 41) Asthma (n = 61) OR (95% CI)**
Dusty/mouldy house[a]	2.9 (0.8-11.0)	1.7 (0.3-11.6)	**3.1 (1.2-8.2)**	1.6 (0.5-4.6)
Family history				
Atopic father	0.9 (0.2-3.6)	0.5 (0.1-3.0)	1.4 (0.6-3.5)	0.6 (0.2-1.5)
Atopic mother	**0.02***	0.18*	**0.1 (0.01-0.7)**	0.3 (0.1-1.1)
Cardiac disease in the family	3.2 (0.9-10.9)	2.0 (0.3-11.5)	**2.8 (1.1-6.8)**	2.0 (0.6-6.2)
Leukaemia in the family	**18.0 (2.6-124)**	1.0 *	**7.6 (1.2-49.0)**	1.2 (0.2-9.2)
Lung function				
FEV_1 % predicted	1.0 (0.99-1.1)	1.0 (0.96-1.1)	1.0 (0.99-1.1)	1.0 (0.97-1.1)
FEV_1 % predicted <80%	0.4 (0.1-1.1)	0.8 (0.2-3.2)	**0.5 (0.2-0.97)**	1.4 (0.6-3.2)
FEV_1 % IVC ratio	**1.1 (1.01-1.2)**	1.1 (0.97-1.2)	1.0 (0.97-1.1)	1.0 (0.96-1.1)
$FEF_{25-75\%}$ IVC ratio	1.0 (0.99-1.05)	1.0 (0.99-1.1)	1.0 (0.99-1.03)	1.0 (0.99-1.03)
Skin prick test				
House dust mite positive#	0.7 (0.2-2.4)	3.3 (0.8-14.2)	0.7 (0.3-1.6)	1.0 (0.4-2.2)
Mould positive#	**8.8 (1.7-46.1)**	9.4 (0.99-89.5)	3.6 (0.9-14.1)	3.1 (0.5-18.7)
Any skin prick test positive#	0.8 (0.2-2.3)	2.5 (0.6-11.0)	0.9 (0.4-1.9)	0.8 (0.3-1.8)
Hyperresponsiveness				
Histamine threshold, mg/ml	0.8 (0.2-2.8)	0.11*	1.1 (0.5-2.6)	1.5 (0.6-3.6)

*Data are presented as mean (SD), median (25th-75th percentile), or n (%). OR: odd ratio of the childhood factor, corrected for sex and age at visit 1 with 95% Confidence Interval, a: characteristics of house include ≥1 of the following: dusty/moisty vs. dry house, rotten floor and/or mould in house , *: p-value Fisher's exact test, #: times higher than the diameter of the positive histamine control.*

Two childhood factors that reduced the chance of ComR at visit 2 were symptoms of wheezing and an atopic mother. The finding of a lower frequency of ComR or ClinR at 25 years in the offspring of an atopic mother is consistent with the report of Burgess *et al.*, who found less frequent ClinR in similar subjects [3].

Four childhood factors increased the chances of ComR/ClinR at visit 2: being breastfed for more than 6 months; living in a dusty/damp house, with rotten floors and/or mould; and having a family member with cardiac disease, or with leukaemia. At visit 3, having pets during childhood was positively associated with ComR/ClinR. In addition, being breastfed for more than 6 months was associated with ComR/ClinR. A systematic review reported a reduced risk of asthma in breastfed children [13]. Furthermore, breastfed children are less susceptible to respiratory infections in early life, which are associated with the development and persistence of asthma [14]. Further studies are needed to assess whether prolonged breastfeeding also facilitates asthma remission when asthma has developed.

Five factors were found to reduce the chances of ComR/ClinR at visit 2, i.e. symptoms of wheezing during a cold; a total protein level <25th percentile; pneumonia during childhood; an atopic mother; and FEV_1 <80% predicted. At visit 3, sputum production in childhood was negatively associated with ComR/ClinR (table 1). Children of an atopic mother had less frequent ComR/ClinR and ComR at 25 years, whereas no association with atopy in the father was observed. This finding is consistent with those of another study that found less frequent ComR/ClinR in children of a mother with asthma [3].

Conclusion

Our study shows that over 39 years of follow-up, it is indeed possible to have persistent asthma remission after having childhood asthma, even though this was evident in only 11% of the subjects under investigation. Specifically, using the strict definition of complete asthma remission, we showed that when complete remission is present at age 25, 75% of this group have persistent remission to age 49. Several factors during childhood were found to be associated with a higher chance of asthma remission during adulthood, e.g. breastfeeding >6 months and a family history of leukaemia; and a lower chance of asthma remission, e.g. wheezing during a cold in childhood and an atopic mother. However, more research is needed to confirm our observations, using larger cohorts with a standard definition of asthma remission.

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Chapter 4

Assessing small airways dysfunction in asthma, asthma remission, and healthy controls using Particles in Exhaled Air (PExA)

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Introduction

Asthma is a chronic disease, characterized by variable airflow obstruction and airway inflammation [1]. Small airways are thought to be a major site of pathology in asthma [2,3]. There are different tools to assess small airways dysfunction (SAD), such as spirometry, body plethysmography, impulse oscillometry (IOS), multiple breath nitrogen wash-out (MBNW), alveolar fraction of exhaled nitric oxide (FeNO) and gas-trapping assessed by high-resolution CT (HRCT). However, there is no golden standard and some tests are difficult to perform [2,3]. PExA (Particles in Exhaled Air) is a recently developed technique with the potential to identify SAD phenotypes in asthma [4,5]. PExA measurements are non-invasive and easy to perform by subjects, even in severely obstructed patients. PExA captures the aerosol from exhaled breath, and specifically those endogenously generated particles in the size range 0.5-4 µm that are formed during airway closure and reopening. These particles contain water and non-volatile material originating from the respiratory tract lining fluid [6]. It is thought that SAD leads to impaired reopening of airways or altered composition of the respiratory tract lining fluid, causing less particles to be formed [7]. Therefore, severity of SAD is expected to be associated with a reduction of particles measured by PExA.

Some patients with asthma outgrow their disease and reach clinical asthma remission (ClinR); these individuals experience no asthma symptoms even without using asthma medication. Patients in ClinR, however, might still have (asymptomatic) bronchial hyperresponsiveness (BHR) or impaired lung function [8,9,10]. Broekema *et al.* demonstrated that subjects in ClinR still had ongoing airway inflammation [11]. In contrast, a smaller subset of asthma remission subjects may lack BHR and regain normal lung function, i.e. complete asthma remission (ComR) [10].

We hypothesized that more SAD leads to decreased exhalation of PExA particles and that this SAD is still present in ClinR-, but absent in ComR-subjects. Therefore, we compared exhaled PExA mass between ClinR- and ComR-subjects in relation to asthma patients and healthy controls. The second aim of this study was to investigate how PExA mass is associated with other measures of small and large airways function in these groups.

Methods

The study protocol was approved by the local ethical committee and all subjects gave informed consent (NL53173.042.15, Groningen). Included subjects were divided over four groups: subjects with childhood-onset asthma which persisted (PersA; subjects with wheezing and/or asthma attacks, asthma medication use, and a PC_{20} methacholine <8 mg/ml with 120s tidal breathing), or which had gone into clinical asthma remission (ClinR; subjects without wheezing/asthma attacks, no use of asthma medication in the last 3 years, with a documented history of asthma according to GINA guidelines, an FEV_1 % predicted <80% and/or PC_{20} methacholine <8 mg/ml), or into complete asthma remission (ComR; similar to ClinR, but with an FEV_1 % predicted ≥80%, PC_{20} methacholine ≥8 mg/ml and PC_{20} adeno-'5-monophosphate (AMP) ≥320 mg/ml), and healthy controls (Ctrl; similar to ComR, but without any history of asthma or use of asthma medication). All subjects were aged 40-65 years and were either never- or ex-smokers with a smoking history <10 pack years. Subjects were extensively characterized with the following tests: spirometry, body plethysmography, IOS, FeNO, MBNW, provocation tests, blood tests, sputum induction and CT-scans. PersA-subjects were withdrawn from inhaled corticosteroids six weeks prior to the clinical characterization.

PExA mass was collected using the PExA 2.0 device [5]. All subjects performed a similar breathing manoeuvre as described by Bake *et al.* [6]. To account for potential bias effects of circadian rhythm, all PExA measurements were performed in the morning.

Parametric Response Mapping (PRM) is a voxel-wise image analysis technique that was implemented on the CT-scans. PRM data was analysed according to the methods described in the literature [12,13].

Clinical characteristics and PExA mass in the subject groups were compared using independent sample T-test for normally distributed data (including log2-transformed variables), Mann-Whitney U tests for non-normally distributed data and Fisher's exact tests for categorical variables. Likewise, PExA mass was correlated with small and large airway parameters using either Pearson or Spearman. Last, a stepwise multivariate regression analysis was performed to assess independent associations with PExA mass.

Results and discussion

Clinical characteristics of the subject groups are presented in figure 1A. ComR-subjects were significantly younger than PersA-subjects (P=0.027). The FEV_1 was significantly higher in Ctrl- and ComR- compared to PersA-subjects, and higher in ComR- compared to ClinR-subjects.

Table 1: baseline characteristics

	Ctrl (n = 18)	ComR (n = 12)	ClinR (n = 16)	PersA (n = 18)	Kruskall-Wallis
Age (years)	56 [53 - 61]	46 [43 - 55]	54 [47 - 60]	60 [49 - 63]	0.044
Female (n, %)	6 (33.3)	4 (33.3)	7 (43.8)	7 (38.9)	0.918
Smoking pack years (min-max)	0 (0 - 5)	0 (0 - 6)	0 (0 - 1)	0 (0 - 2)	0.104
FEV_1 % predicted (%)	113.6 (12.0)	108.1 (9.5)	84.5 (23.1)	81.3 (17.2)	<0.001
PC_{20} methacholine threshold (mg/ml)	>8	>8	0.8 [0.12 - 2.83]	0.56 [0.27 - 2.16]	-
PExA mass (ng/L)	5.7 [3.0 - 9.6]	4.9 [2.9 - 6.5]	3.2 [0.7 - 5.6]	2.7 [0.5 - 4.0]	0.017

Ctrl: *healthy controls;* ***ComR:*** *complete remission subjects;* ***ClinR:*** *clinical remission subjects;* ***PersA:*** *persistent asthma patients;* ***FEV_1:*** *forced expiratory volume in one second;* ***PC_{20}:*** *substance provocative concentration causing a 20% drop of FEV_1. Age, pack years and PC_{20} methacholine presented as median, interquartile range. PExA mass presented as median, interquartile range and P-value based on ANOVA. FEV_1 presented as mean, standard deviation. Female presented as number, percentage and P-value based on Chi-Square test.*

PExA mass was significantly lower in PersA- compared to ComR- and Ctrl-subjects (P=0.028 and P=0.003 respectively). In addition, PExA mass was significantly lower in ClinR- compared to Ctrl-subjects (P=0.018). Comparison of particle size distribution per group did not yield additional information. This is the first study investigating exhaled particles in asthma remission subjects, showing a similar PExA mass in ComR compared to healthy controls and a decrease in PExA mass in ClinR compared to healthy controls, even though these individuals experience no wheeze or asthma attacks. Our findings are in concordance with the previously stated hypothesis that more SAD leads to decreased exhalation of particles. The fact that ClinR-subjects exhale less particles suggests that these subjects still have ongoing SAD similar to persistent asthmatics. In contrast, ComR-subjects exhale similar amounts of particles compared to healthy controls, possibly due to outgrown SAD.

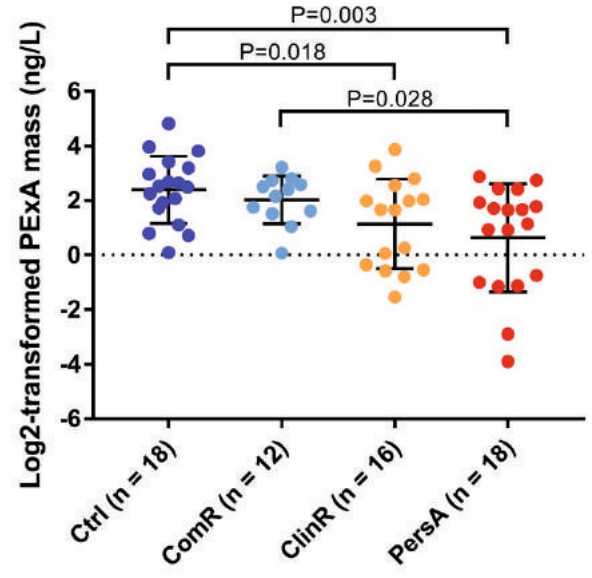


Figure 1: log2-transformed PExA mass in ng/L per subject group, with independent T-test P-values.

Next, we assessed the correlations between PExA mass and known small and large airway parameters. Results of these bivariate correlations are presented in figure 1B. Increased PExA mass was associated with less severe BHR and both parameters of large (higher FEV_1 % predicted and FEV_1/FVC ratio) and small (higher FEF25-75% % predicted, less hyperinflation as reflected by lower RV % predicted, lower IOS R5-R20 resistance and decreased MBNW Scond*VT) airway function. No correlation with PExA mass and PRM defined small airways disease (fSAD) was observed. Finally, a stepwise multiple regression analysis was performed including all variables significantly associated with PExA mass in the bivariate analysis (see figure 1B). This analysis showed that MBNW Scond*VT was independently associated with PExA mass.

Soares *et al.* found a correlation between mean number of particles per exhalation and FEV_1/FVC ratio (R=0.246, P=0.021), and between surfactant A PExA concentration and R5-R20 resistance (R=0.257, P<0.05)[4]. In accordance with the findings of Soares *et al.*, we show that increased PExA mass is associated with better function of both the large and the small airways.

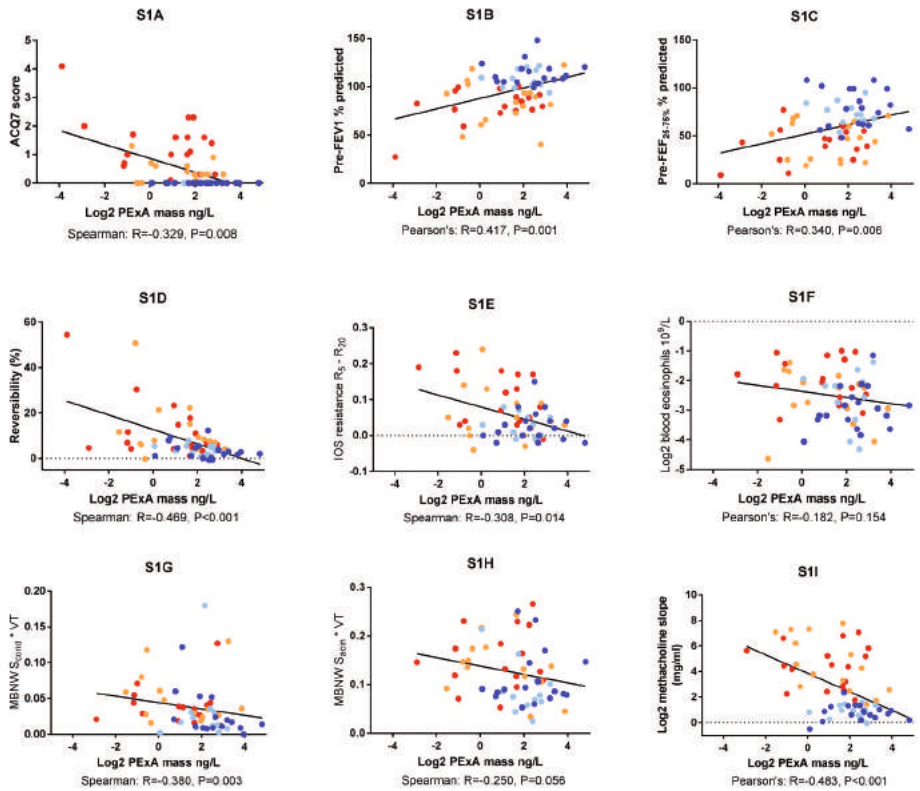
Table 2: PExA mass correlates with large and small airways parameters

Group	Parameter	Test	R-value	P-value
	Age (years)	Spearman	0.095	0.455
	Body Mass Index (kg/m^2)	Spearman	-0.203	0.107
Inflammatory	Blood eosinophils (10^9/L) #	Pearson	-0.182	0.154
	Sputum eosinophil differentiation (%)	Spearman	-0.449	0.013
Large	Reversibility pre-post (%)	Spearman	-0.469	<0.001
	PC_{20} methacholine slope (mg/ml) #	Pearson	-0.483	<0.001
	PC_{20} Adeno-'5-monophosphate slope (mg/ml)	Spearman	-0.441	0.001
	FEV_1/FVC ratio (pre-salbutamol) (%)	Spearman	0.355	0.004
	FEV_1 % predicted (pre-salbutamol) (%)	Pearson	0.417	0.001
	IOS R_{20} resistance (Hz)	Pearson	-0.386	0.002
Small	IOS R_5-R_{20} resistance (Hz)	Spearman	-0.308	0.014
	IOS AX (Hz kPa L^{-1})	Spearman	-0.342	0.006
	RV % predicted (%)	Spearman	-0.431	<0.001
	RV/TLC % predicted (%)	Spearman	-0.340	0.006
	MBNW S_{cond}*VT	Spearman	-0.380	0.003
	MBNW S_{acin}*VT	Spearman	-0.250	0.056
	$FEF_{25-75\%}$ % predicted (%)	Pearson	0.340	0.006
	Alveolar FeNO (parts per billion)	Spearman	-0.254	0.100
	CT PRM FSAD (%)	Spearman	-0.051	0.717
	CT PRM inferior-to-superior ventilation gradient (ΔHU)	Pearson	-0.197	0.152

#: log2-transformed, ***PC_{20}****: substance provocative concentration causing a 20% drop of* FEV_1*;* ***FEV_1****: forced expiratory volume in one second;* ***FVC****: forced vital capacity;* ***R_5****: resistance at 5 Hz;* ***R_{20}****: resistance at 20 Hz;* ***AX****: area of reactance;* ***RV****: residual volume;* ***TLC****: total lung capacity;* ***S_{cond}****: conductive ventilation heterogeneity;* ***S_{acin}****: acinar ventilation heterogeneity;* ***VT****: tidal volume;* ***$FEF_{25-75\%}$****: forced expiratory flow at 25-75% of the pulmonary volume;* ***FSAD****: functional small airways disease*

Conclusion

In conclusion, PExA mass can distinguish asthmatics from healthy individuals. In addition, we show that subjects with complete, but not clinical, asthma remission exhale more PExA mass compared to asthma. Our findings are in concordance with previous studies showing that decreased PExA mass is associated with more severe obstructive pulmonary disease [7,14]. These results reinforce the theory that clinical asthma remission subjects still have ongoing small airways disease and that subjects in complete asthma remission have completely outgrown their disease [10]. Our observations demonstrate that higher PExA mass is not only related to better large airway function, but also independently associated with small airways disease as reflected by Scond. This indicates that PExA mass could potentially be used as a tool to assess small airways dysfunction. Future research should focus on exploring the composition of exhaled particles to gain more insight on the pathophysiology of small airways dysfunction in asthma persistence and remission.



Supplemental figure 1: correlation between PExA mass and large or small airways parameters, red dots: asthmatics, yellow dots: clinical asthma remission subjects, light blue: complete asthma remission subjects, dark blue: healthy controls, **S1A:** log2-transformed PExA mass and Asthma Control Questionnaire Score, **S1B:** correlation between log2-transformed PExA mass and FEV_1 % predicted, **S1C:** correlation between log2-transformed PExA mass and $FEF_{72-75\%}$ % predicted, **S1D:** correlation between log2-transformed PExA mass and reversibility after 400mcg salbutamol, **S1E:** correlation between log2-transformed PExA mass and impulse oscillometry $R_5 - R_{20}$ in Hz, **S1F:** correlation between log2-transformed PExA mass and log2-transformed blood eosinophils in 10^9/L, **S1G:** correlation between log2-transformed PExA mass and Multiple Breath Nitrogen Wash-out S_{cond} * VT, **S1H:** correlation between log2-transformed PExA mass and Multiple Breath Nitrogen Wash-out S_{acin} * VT, **S1I:** correlation between log2-transformed PExA mass and log2-transformed methacholine slope in mg/ml.

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Chapter 5

Small airways disease and airway inflammation in asthma remission

Orestes A. Carpaij, Susan Muiser, Alex J. Bell, Huib A.M. Kerstjens, Craig J. Galban, Aleksa B. Fortuna, Salman Siddiqui, Anna-Carin Olin, Martijn C. Nawijn, Maarten van den Berge

Manuscript in preparation

Introduction

Asthma is a chronic disease, characterized by variable airflow obstruction and airway inflammation [1]. To date, there is no cure for this disease. Nevertheless asthma can go into spontaneous remission [2,3], meaning that some asthma patients no longer experience symptoms and do no longer require any asthma medication. These subjects might still have (asymptomatic) bronchial hyperresponsiveness or a sub-normal lung function, and then are considered to be in clinical remission [4]. In fewer cases, asthma patients go into complete remission, which means that they no longer have any pulmonary function impairment or bronchial hyperresponsiveness [2,3].

Although asthma affects the entire bronchial tree, small airways - defined as those with an internal diameter of <2 mm - are thought to be a major site of pathology in asthma [6]. The small airways comprise 90% of the total airway volume and significantly contribute to total airway resistance in patients with obstructive pulmonary disease [7,8]. Previous studies have investigated lung function and airway inflammation in asthma remission, but whether and to what extent small airways disease is still present in clinical and complete remission is yet unknown.

The aim of the current study was to more closely investigate the phenotypes of clinical and complete asthma remission. To this end, we extensively characterized subjects with complete and clinical asthma remission and compared them to both patients with asthma and healthy controls. We assessed parameters of large and small airways function next to the presence and extent of markers for Th2 inflammation in blood, sputum and bronchial biopsies.

Methods

The local ethics committee approved the study protocol and all subjects gave their written informed consent (NL53173.042.15, Groningen). Enrolled subjects were aged 40 - 65 years and either never- or ex-smokers with a smoking history <10 pack years. Participants were divided in four groups; subjects with childhood-onset asthma that persisted (PersA; subjects with wheezing and/or asthma attacks, asthma medication use, and a PC_{20} methacholine <8 mg/ml with 120s tidal breathing), individuals who had gone into clinical asthma remission (ClinR; subjects without wheezing/asthma attacks, no use of asthma medication in the last 3 years, with a documented history of asthma according to GINA guidelines, an FEV_1 % predicted <80% and/or PC_{20} methacholine <8 mg/ml), individuals into complete asthma remission (ComR; similar to ClinR, but with an FEV_1 % predicted ≥80%, PC_{20} methacholine ≥8 mg/ml and PC_{20} adenosine 5'-monophosphate (AMP) ≥320 mg/ml), and healthy controls (Ctrl; no history of asthma or use of asthma medication and lung function as in the ComR group). Inhaled corticosteroids were withdrawn six weeks prior to enrollment. Subjects underwent spirometry, body plethysmography, provocation tests, impulse oscillometry (IOS), multiple breath nitrogen wash-out, fractional nitric oxide (FeNO), skin prick tests, and computed tomography (CT)-scans. Parametric Response Mapping was applied on in- and expiratory CT-scans, quantifying functional small airways disease and inferior-to-superior ventilation gradients, both small airways parameters as described previously [10,11].

The four groups were compared using independent sample T-tests for normally distributed data, Mann-Whitney U tests for non-normally distributed data and Fisher's exact tests for categorical variables. Second, a regression analysis was performed to identify and correct for confounders.

Results and discussion

Clinical characteristics of the four groups are presented in table 1. Apart from ComR-subjects being younger than the PersA-patients and healthy controls, no significant differences were seen in demographical characteristics. ComR-subjects had significantly better large and small airways function and less Th2 inflammation as reflected by sputum eosinophils and alveolar nitric oxide than individuals with ClinR. ComR-subjects were similar to healthy controls in this respect, whereas subjects with ClinR were intermediate between PersA and Ctrl, in terms of large and small airways- and inflammatory parameters (table 1). Our results confirm previous studies showing that the asthma remission with absence of bronchial hyperresponsiveness, ComR, is different from ClinR with the level of lung function and Th2 inflammatory markers being reverted towards normal [2,5].

We extend these findings by showing that in addition, complete remission patients have lost all features of small airways disease, both defined by physiological parameters and by in- and expiratory HRCT. Small airway function of complete remission patients is the same as that of healthy controls without a history of asthma. We found that peripheral airways dysfunction as reflected by R_5-R_{20} resistance and AX reactance, was significantly less in asthma remission, both ComR and ClinR, subjects compared to PersA-patients. The Dunedin birth cohort assessed IOS measurements using the forced oscillation technique in healthy individuals, asthmatics and asthma remission subjects at age 38 [12]: the FEV_1 and FEV_1/FVC ratio of the remission subjects were similar to those of healthy controls, suggesting a higher complete remission prevalence. These results are in agreement with ours: asthma remission was associated with a similar R_5-R_{20} resistance and AX reactance to that of healthy controls, whereas persistent asthmatics had increased resistance and reactance. Overall, both associations were stronger in men, and more apparent in childhood-onset asthma, which is in concordance with our data.

We observed that the inferior-to-superior ventilation gradient from HR-CT PRM was positive in persistent asthmatics, while it was negative in all other groups, suggesting that a higher proportion of the asthmatics' ventilation takes place in the superior part of the lung. This is in line with earlier research showing that small airways dysfunction is

lower lobe-predominant in asthmatics [10]. This observation may support the hypothesis that subjects in asthma remission regain their lower lobe ventilation, which might be associated with improvement in symptoms and bronchial hyperresponsiveness. Perhaps the more distal lower lobes are affected most by bronchial hyperresponsiveness due to more central bronchoconstriction. We expect that if the ClinR-group was bigger, the ventilation gradient would also be positive. Future automated CT-scan analyses may incorporate this gradient, illustrating ventilation shifts in disease progression or remission.

Finally, multiple breath nitrogen wash-out, S_{cond} and S_{acin} were significantly higher in persistent asthmatics and ClinR-subjects, compared to healthy subjects and individuals who were into ComR. There were no studies investigating the nitrogen washout parameters in adult subjects in remission, but Steinbacher *et al.* did measure S_{cond} and S_{acin} in children who were in remission for more than one year and either normoresponsive or hyperresponsive to cold dry air challenge during the study [13]. In that study, hyperresponsive children with asthma remission showed a significant increase and decrease in S_{cond} after cold dry air challenge and salbutamol inhalation, respectively. This indicates that small airways dysfunction may still be present in asthma remission subjects that still react to exogenous triggers.

Our study provides evidence that subjects in clinical but not complete asthma remission still have a degree of small airways disease and presence of inflammatory markers. Second, subjects in complete remission are very similar to healthy subjects. Thus, we propose that in order to elucidate the pathophysiology of asthma remission, future studies should focus on the biological pathways responsible for the induction of complete asthma remission. Further work is needed to establish histological differences in airway wall remodeling and inflammatory parameters in complete remission, supplemented by single-cell RNA sequencing [14]. We believe this research in subjects in complete remission to be a promising route of research for elucidating new pathways and perhaps new treatments.

Table 1: clinical features of healthy controls, complete remission, clinical remission, and persistent asthmatic subjects

	Healthy controls (Ctrl, n = 22)	Complete remission (ComR, n = 14)	Clinical remission (ClinR, n = 17)	Persistent asthma (PersA, n = 24)
Demographics				
Sex, male (n, %)	13 (59.1%)	10 (71.4%)	10 (58.8%)	15 (65.2%)
Age (years) [B,E]	57 [51 – 61]	48 [44 – 56]	52 [46 – 58]	58 [49 – 63]
Never/past-smoker ratio (n, %)	8 (63.3%)	12 (85.7%)	15 (88.2%)	20 (87.0%)
Pack years (min-max) [A]	0 [0 – 5]	0 [0 – 6]	0 [0 – 1]	0 [0 – 2]
Age of symptom onset (years)	NA	4 [2 – 5]	5 [0 – 7]	5 [4 – 9]
Asthma-free years [#]	NA	30 [12 – 33]	15 [8 – 33]	NA
BMI (kg/m^2)	24.3 [22.5 – 28.1]	25.3 [22.5 – 27.8]	25.3 [22.8 – 28.4]	26.0 [23.7 – 30.8]
Large airways parameters				
FEV_1 % predicted (%) [A,B*,D]	113.4 (±10.9)	108.4 (±9.1)	85.1 (±22.4)	83.6 (±14.4)
FEV_1/FVC ratio (%) [A,B*,D]	79.5 (±5.4)	78.3 (±5.3)	67.0 (±8.3)	66.7 (±9.3)
Reversibility after salbutamol 400µg (%) [A,B*,D]	2.4 [0.9 – 5.0]	3.4 [1.8 – 5.3]	7.9 [5.0 – 13.1]	6.5 [3.2 – 14.0]
PC_{20} methacholine (mg/ml)	>9.8	>9.8	1.1 [0.3 – 3.4]	0.7 [0.3 – 2.6]
PC_{20} adenosine 5'-monophosphate (mg/ml)	>320	>320	16.4 [2.2 – 65.9]	1.3 [4.0 – 27.4]
Small airways parameters				
$FEF_{25-75\%}$ % predicted (%) [A,B*,D]	79.6 (±19.9)	77.4 (±14.5)	46.5 (±18.0)	42.5 (±17.6)
RV/TLC ratio (%) [A,B*,C]	29.7 (±4.8)	27.6 (±4.9)	31.5 (±6.7)	37.1 (±7.0)
Exhaled particle mass (ng/L) [A,B*]	5.7 [3.0 – 9.6]	4.9 [2.9 – 6.3]	3.1 [0.7 – 5.4]	3.2 [0.6 – 4.3]
IOS R_5-R_{20} resistance (Kpa x s x L^{-1}) [A,B*]	0.02 [-0.01 – 0.05]	0.02 [-0.04 – 0.05]	0.03 [0.003 – 0.11]	0.08 [0.03 – 0.18]
IOS AX reactance (Kpa/L) [A,B*,C]	0.21 [0.08 – 0.40]	0.20 [0.10 – 0.30]	0.24 [0.14 – 1.00]	0.69 [0.35 – 2.20]
MBNW S_{cond} x VT [A,B,D]	0.01 [0.009 – 0.02]	0.01 [0.007 – 0.03]	0.04 [0.03 – 0.08]	0.04 [0.02 – 0.05]

Table 1: (continued)

	Healthy controls (Ctrl, n = 22)	Complete remission (ComR, n = 14)	Clinical remission (ClinR, n = 17)	Persistent asthma (PersA, n = 24)
MBNW S_{acin} x VT [A,B*,D]	0.08 [0.07 – 0.13]	0.08 [0.06 – 0.10]	0.14 [0.11 – 0.17]	0.13 [0.11 – 0.19]
Alveolar FeNO (ppb) [B,D]	2.7 [1.7 – 9.5]	2.6 [0.4 – 5.1]	5.8 [3.9 – 10.4]	4.1 [3.1 – 8.1]
TH2 markers				
FeNO at 50ml/s (ppb) [B]	20.4 [13.5 – 26.7]	18.2 [13.0 – 23.9]	21.5 [16.3 – 41.2]	32.9 [17.0 – 48.7]
Blood eosinophils (10^9/L) [A,C]	0.12 [0.09 – 0.16]	0.20 [0.15 – 0.24]	0.15 [0.11 – 0.26]	0.23 [0.16 – 0.42]
Blood eosinophils >0.3 x 10^9/L (n, %) [A]	1 (4.5%)	2 (14.3%)	3 (17.6%)	8 (33.3%)
Sputum eosinophils (%) [A,B]	0.5 [0.2 – 1.0]	0.2 [0.0 – 0.7]	0.8 [0.3 – 9.0]	3.0 [1.0 – 9.2]
Sputum eosinophils >3% (n, %) [A,D]	0 (0.0%)	0 (0.0%)	4 (80.0%)	6 (50.0%)
Atopy parameters				
Self-reported allergic symptoms (n, %) [A]	2 (9.1%)	5 (38.5%)	10 (58.8%)	15 (62.5%)
Inhalation/total IgE ratio (%) [A,B,D]	0.4 [0.1 – 3.4]	1.7 [0.2 – 18.8]	15.0 [5.4 – 22.0]	13.0 [2.0 – 32.8]
Any skin prick test positive (n, %) [A,D]	8 (38.1%)	7 (50.0%)	16 (100.0%)	17 (77.3%)
HR-CT of the thorax				
PRM functional small airways disease (%)	0.6 [0.3 – 4.5]	0.4 [0.1 – 10.6]	0.6 [0.4 – 20.0]	3.0 [0.7 – 9.7]
PRM interstitial lung density (%) [B]	10.3 [9.5 – 17.4]	8.2 [7.4 – 10.1]	10.6 [8.2 – 13.8]	13.2 [9.9 – 19.0]
PRM inferior-to-superior ventilation (ΔHU) [A,B*]	-2.0 (±2.8)	-2.8 (±3.2)	-0.5 (±2.9)	0.4 [-1.3 – 3.5]

Variables are presented in either mean with standard deviation (± ...), median with interquartile range [... - ...], or number with percentage within group (...%). ***#:*** *years without symptoms of wheeze/asthma attacks and asthma medication use,* ***BMI:*** *body mass index,* ***PC_{20}:*** *dosage of agent that causes a 20% FEV_1 decrease,* ***$FEF_{25-75\%}$:*** *forced expiratory flow at 25% to 75% of forced vital capacity,* ***RV/TLC:*** *residual volume / total lung capacity ratio,* ***IOS:*** *impulse oscillometry* ***MBNW****: multiple breath nitrogen wash-out,* ***S_{cond}****: conductive ventilation heterogeneity,* ***S_{acin}****: acinar ventilation heterogeneity;* ***VT****: tidal volume,* ***FeNO****: fractional nitric oxide,* ***HR-CT:*** *high-resolution computed tomography,* ***PRM****: parametric response mapping,* ***A****: P<0.05 difference between Ctrl and PersA using independent T-, Mann Whitney U-, of Fisher's test,* ***B****: P<0.05 difference between ComR and PersA,* ***B*:*** *<0.05 difference between ComR and Persa after correcting for age,* ***C****: P<0.05 difference between ClinR and PersA,* ***D****: P<0.05 difference between ClinR and ComR,* ***E****: P<0.05 difference between ComR and Ctrl.*

Table 2: significantly different clinical features between complete asthma remission subjects either persistent asthmatics

	ComR *versus* PersA B and P-value	
FEV_1 % predicted (%)	0.02	**<0.001**
FEV_1/FVC ratio (%)	0.03	**0.002**
Reversibility after salbutamol 400µg (%)	-0.02	**0.038**
$FEF_{25-75\%}$ % predicted (%)	0.02	**<0.001**
RV/TLC ratio (%)	-0.04	**0.001**
Exhaled particle mass (ng/L)	0.08	**0.030**
Impulse oscillometry R_5-R_{20} (Kpa x s x L^{-1})	-1.70	**0.037**
Impulse oscillometry AX (Kpa/L)	-0.13	**0.035**
MBNW S_{cond} x VT	-2.59	0.257
MBNW S_{acin} x VT	-2.91	0.060
Blood eosinophils (10^9/L)	-0.97	0.113
Sputum eosinophils (%)	-0.02	0.200
Inhalation/total IgE ratio (%)	-0.01	0.159
PRM interstitial lung density (%)	-0.02	0.066
PRM inferior-to-superior ventilation (ΔHU)	-0.06	**0.036**

Variables are presented B coefficients with P-value, ***FEF***$_{25-75\%}$*: forced expiratory flow at 25% to 75% of forced vital capacity,* ***RV/TLC****: residual volume / total lung capacity ratio,* ***MBNW****: multiple breath nitrogen wash-out,* S_{cond}*: conductive ventilation heterogeneity,* S_{acin}*: acinar ventilation heterogeneity;* ***VT****: tidal volume,* ***PRM****: parametric response mapping.*

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Chapter 6

Applying the CAMP trial asthma remission prediction model to the Dutch asthma remission studies

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Introduction

A small subset of patients with asthma can go into spontaneous remission later in life [1, 2]. Predicting this clinical trajectory would be of great interest, because these asthma remission subjects are not burdened by symptoms anymore and no longer require any medication. Wang *et al.* [3] created a prediction model that could predict asthma remission outcome. They showed that the combination of normal FEV_1/forced vital capacity (FVC) ratio, less severe bronchial hyperresponsiveness, and blood eosinophil counts of less than 500 cells/μL at age 8 years yields more than 80% probability to achieve asthma remission by adulthood.

Methods

We were interested in the generalizability of predicting remission in childhood and applied this prediction model on our Dutch asthma remission cohorts. Children included in these cohorts described by Vonk et al (cohort 1, n = 94) and Carpaij et al (cohort 2, n = 157) had doctor-diagnosed asthma and were bronchial hyperresponsive (i.e. substance provocative concentration causing a 20% drop in FEV_1 [PC_{20}] ≤16 mg/mL histamine)[1,2]. Similar to the definition used by the *Childhood Asthma Management Program* (CAMP), we defined asthma remission at follow-up as no wheeze or asthma attacks in the last year, having an FEV_1/inspiratory vital capacity (IVC) ratio of greater than or equal to 80%, and no use of asthma-related medication. We used a different measure for airway obstruction, that is, FEV_1/IVC, because no data on FVC were available. Subjects with missing data were excluded. Normally and non-normally distributed variables were compared with t test and Mann-Whitney U test, respectively. We constructed 6 groups on the basis of baseline criteria provided in Wang et al and calculated the prevalence of subjects in remission for each group.

Results and discussion

After combining cohorts 1 and 2, the clinical and complete remission rate was 10.0% compared with 26.1% in CAMP (table 1). Like Wang et al, we observe an increase if the prevalence of remission as baseline FEV_1/IVC% is higher. In subjects with an FEV_1/IVC ratio of greater than or equal to 85%, PC_{20} value of greater than or equal to 1 mg/mL, and an eosinophil level of less than 500 cells/μL has additional value to predict asthma remission (table 2). In this group, the prevalence of remission was 40%, whereas those with greater than or equal to 500 eosinophils/μL had a 10% prevalence of remission. In accordance to the CAMP study, children in cohorts 1 + 2 had a significantly higher FEV_1, FEV_1/IVC%, and PC_{20} threshold and significantly lower blood eosinophils in the asthma remission group compared with the persistent asthma group. These are known clinical features associated with asthma remission [4-6]. The FEV_1/FVC% measured in CAMP was higher than in cohorts 1 + 2, resulting in a higher proportion of subjects subdivided in group 2. The definition for airway obstruction is not expected to be the cause, because the difference between FEV_1/FVC% and FEV_1/IVC% is marginal in children and young adults with mild to moderate asthma [7].

Conclusion

Taking this into account, we show that the model proposed by Wang et al can correctly predict future development of asthma remission in up to 40% of cases. Although usable, more research is needed to disentangle the pathophysiology of asthma remission, which is a highly relevant yet poorly understood outcome of childhood asthma.

Table 1: baseline clinical characteristics of three prospective childhood cohorts and application of the prediction model

	Persistent asthma			Asthma remission		
	Cohort 1 Vonk *et al.* 2004 (n = 79)	**Cohort 2** Carpaij *et al.* 2017 (n = 147)	**CAMP** Wang *et al.* 2018 (n = 650)	**Cohort 1** Vonk *et al.* 2004 (n = 15) (15%)	**Cohort 2** Carpaij *et al.* 2017 (n = 10) (6.4%)	**CAMP** Wang *et al.* 2018 (n = 229) (26.1%)
Enrollment year range	1966 - 1969	1972 – 1976	1993 - 1995	1966 - 1969	1972 – 1976	1993 - 1995
Mean age at baseline (SD)	9.9 (2.0)	9.7 (1.4)	8.8 (2.1)	9.6 (2.0)	10.2 (1.2)	8.6 (1.9)
Mean follow-up (years)	16	15	12	16	15	12
Male sex (N, %)	58 (73.4%)	105 (71.4%)	407 (62.6%)	9 (60.0%)	7 (70.0%)	115 (50.2%)
Mean FEV_1 % pred. (SD)	82.1% (16.5)*	75.4% (14.3)*	92.2% (14.1)*	85.7% (17.2)*	82.0% (10.6)*	99.0% (12.7) *
Mean FEV_1/VC % (SD)#	75.0% (12.2)*	72.3% (7.9)*	77.9% (7.9)*	78.1% (12.0)*	79.7% (7.1)*	85.6% (6.3) *
Median PC_{20} threshold in mg/ml [IQR] #	2.0 [7.0]*	4.0 [6.0]*	0.9 [1.6]*	8.0 [30.0]*	8.0 [4.0]*	1.7 [3.6] *
Median blood eosinophil count in cells/µL [IQR]	462.0 [495.0]*	385.0 [396.0]*	422.0 [493.5]*	220.0 [297.0]*	286.0 [236.5]*	320.5 [327.3] *

*NA: not applicable, #: FEV_1/IVC and PC_{20} histamine threshold on cohort 1 and 2, FEV_1/FVC and PC_{20} methacholine threshold in CAMP. *: either significant difference (P<0.05) between asthma remission and persistent asthma within cohort 1+2 or CAMP.*

Table 2: implementing the prediction model

	Group 1	**Group 2**	**Group 3**	**Group 4**	**Group 5**	**Group 6**
	FEV_1/FVC% ≤75%	FEV_1/FVC% 75-79%	FEV_1/FVC% 80-84%	FEV_1/FVC% ≥85% PC_{20} <1mg/ml	FEV_1/FVC% ≥85% PC_{20} ≥1mg/ml blood eosinophils ≥500 cell/µL	FEV_1/FVC% ≥85% PC_{20} ≥1mg/ml blood eosinophils <500 cell/µL
CAMP* (n = 876)	9.5% (n = 199)	27.6% (n = 190)	53.8% (n = 228)	58.3% (n = 71)	65.4% (n = 49)	82.6% (n = 139)
Cohort 1+2 (n = 251)	5.5% (n = 3/55)	5.6% (n = 7/126)	15.4% (n = 6/39)	0.0% (n = 0/1)	10.0% (n = 1/10)	40.0% (n = 8/20)

**: predicted probability*

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Chapter 7

The asthma-obesity relationship: underlying mechanisms and treatment implications

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Abstract

Obesity is a worldwide epidemic with a prevalence that has tripled in the last two decades. Worldwide, more than 1.5 billion adults are overweight and more than 500 million obese. Obesity has been suggested to be a risk factor for the development of more difficult-to-control asthma. Although the mechanisms underlying the asthma-obesity relationship are not fully understood, several possible explanations have been put forward. These will be reviewed in this manuscript as well as the implications for the treatment of overweight and obese asthma patients.

Insulin resistance is a possible factor contributing to the asthma-obesity relationship and the effect is independent of other components of the metabolic syndrome such as hypertriglyceridemia, hypertension, hyperglycaemia, and systemic inflammation. Obesity has important effects on airway geometry, by especially reducing expiratory reserve volume causing obese asthmatics to breathe at low lung volumes. Furthermore, obesity affects the type of inflammation in asthma and is associated with reduced inhaled corticosteroids treatment responsiveness.

Obesity induces the development of asthma with a difficult-to-control phenotype. Treatment targeting insulin resistance may be beneficial in obese asthma patients, especially when they have concomitant diabetes. Systemic corticosteroids should be avoided as much as possible as they are not very effective in obese asthma and associated with side-effects like diabetes, weight gain, and osteoporosis.

Introduction

Obesity is a worldwide epidemic with a prevalence that has tripled in the last two decades [1]. Worldwide, more than 1.5 billion adults are overweight (BMI 25 kg/m^2), and more than 500 million obese (BMI 30kg/m^2) [1]. In parallel, the prevalence of asthma has increased during the last 20 years [2]. Obesity has been reported to be a risk factor for the development of asthma and affects its clinical expression toward a more severe and difficult-to-control phenotype [3,4]. In this manuscript, the asthma–obesity relationship and its clinical implications will be reviewed focusing on literature in PubMed of the last 5 years including the most clinically relevant manuscripts in the view of the authors.

Increased risk to develop asthma in overweight and obese study participants

Several studies have shown that overweight (BMI 25 kg/m^2) and obesity (BMI 30 kg/m^2) are associated with a 1.5–2.5-fold increase in the risk to develop asthma [5–7,8,9&]. Rönmark *et al.* [5] investigated 309 cases of new-onset asthma in the Obstructive Lung Disease of Northern Sweden studies and compared them to 309 non-asthmatic controls selected from the Swedish population register. Their data show that overweight (BMI 25 kg/m^2) and obesity (BMI 30 kg/m^2) increase the risk of new-onset asthma by two and 2.7-fold respectively, the risk factor pattern being independent of the presence of allergy. In line with this, Chen *et al.* [6] showed obesity (BMI 30 kg/m^2) to be a risk factor for both allergic and non-allergic asthma. However, some studies were unable to find a link between obesity and the risk of asthma, particularly those investigating children and male individuals [10–13]. A possible explanation may be that the asthma–obesity relationship has a U-shaped pattern with both underweight (BMI<18.5 kg/m^2) and overweight increasing the risk (Figure 1) [14]. Furthermore, an increased BMI does not necessarily reflect a higher fat mass, but could also be because of increased muscle mass, especially in men. Thus, BMI may not be the best way to define obesity. Indeed, some studies found abdominal obesity, defined as a waist circumference more than 88cm in women and more than 102cm in men, respectively, to be a stronger risk factor for asthma than BMI-defined obesity [15–17]. Finally, it should be noted that many epidemiologic studies investigating the obesity-asthma relationship have relied on a doctor's diagnosis of asthma or self-reported asthma. This may have led to an incorrect diagnosis in up to 30% of cases as demonstrated in studies using extensive

lung function testing after withdrawal of inhaled corticosteroids (ICS) [18,19]. However, the percentage of misdiagnosed cases for asthma in these studies was similar in obese and non-obese study participants, making a bias toward an asthma–obesity relation unlikely [18,19].

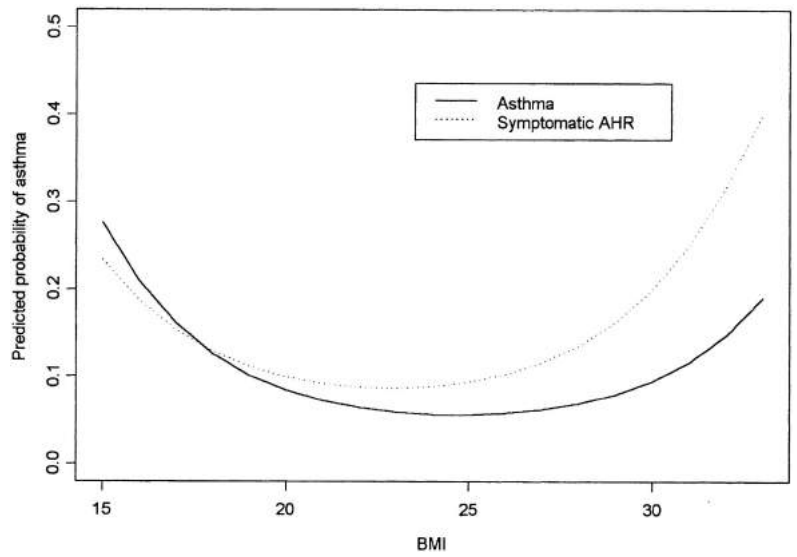

Figure 1: the relation between BMI and risk of asthma in 3386 men in Anqing, adjusted for age, intensity of cigarette smoking, skin, test reactivity to one or more allergens, and familial correlations. Reprinted with permission (Celedón JC et al. Am J Respir Crit Care Med. 2001 Nov 15;164(10 Pt 1):1835-40).

Physical activity, obesity, and the risk to develop asthma

Reduced physical activity has been put forward as a possible mechanism to explain the relationship between obesity and asthma [20]. A systematic review and meta-analysis concerning the association between physical activity and asthma identified 39 cross-sectional studies (n = 661 222) and five longitudinal studies (n = 85,117) [21]. Indeed, the cross-sectional studies observed an association between high physical activity and low asthma prevalence suggesting a protective effect of physical activity. However, this might also be because of reverse causality as asthmatics may be less likely to exercise regularly than non-asthmatics. The five longitudinal studies are more convincing in that respect as they all measured physical activity levels before asthma was diagnosed. The meta-analysis was not able to assess the independent contribution of physical inactivity and overweight to asthma development, which is important as they are closely

associated [21]. In this respect, the findings of Egan *et al.* in 1596 Norwegian adolescents are important. They showed that the presence of general or abdominal obesity is a risk factor for the development of asthma during 11 years follow-up after adjusting for current sports participation as a proxy for physical activity [9]. Another interesting observation is the association between asthma and television watching, which may be an indirect marker of physical inactivity. In a cross-sectional study in 20.016 children at 6–7 years of age, a high body weight, spending a lot of time watching television, and a salty diet each independently increased the risk of asthma [22]. The effect of television watching was also investigated in a longitudinal study in 3065 children who did not have symptoms of wheeze at an age of 3.5 years, but from whom 6% developed asthma at the age of 11.5 years, as demonstrated by a positive methacholine provocation test and children who watched television for more than two hours/day were almost twice as likely to develop asthma, irrespective of their BMI [23]. Different hypotheses have been put forward to explain the possible negative effects on asthma development [21,24]. First, physical inactivity may increase systemic and local airway inflammation [25], second, it may decrease mucociliary clearance and the patency of the bronchioles [21], and third, decreased deep inspiration and sigh rate during physical inactivity like television watching could result in a latch state of the smooth muscles and increased risk of bronchial hyperresponsiveness (BHR) [26].

Link between obesity and airway inflammation

Obesity is well known to be associated with a chronic low-grade systemic inflammation, as reflected by blood leukocytosis and increased serum levels of C-reactive protein [27,28]. Adipocytes produce and store several pro-inflammatory mediators such as leptin, tumor necrosis factor (TNF)a, monocyte chemotactic protein-1, and Interleukin (IL)-6, also called adipokines [29,30,31]. These Adipokines have the ability to modulate the adaptive and immune system in several ways including activation of T helper cells particularly those of the T helper 1 phenotype. In addition to the pleiotropic pro-inflammatory effects of TNFa and IL-6, leptin and monocyte chemotactic protein-are known to induce chemotaxis and activation of leukocytes and monocytes [32,33]. Furthermore, IL-6 has been implicated in the shift toward T helper 17 differentiation that has been observed in T cells from obese mice [34]. In obese individuals, adipose tissue derived dendritic cells, expressing high levels of IL-6 [35], have been shown to promote T helper 17 differentiation [36]. Of interest, T helper 17 cells have been associated with

neutrophilic airway inflammation in asthma [37]. Finally, increased blood levels of markers for oxidative stress have been reported in obese asthma patients [38]. These increased features of systemic inflammation and oxidative stress may also affect the type and severity of inflammatory process within the airways of patients with asthma [38]. Indeed, overweight and obese asthma patients have been reported to present with a different type of airway inflammation, with less eosinophils and more neutrophils in their induced sputum [3,39,40]. This is compatible with the findings by Haldar *et al.* [41] using a cluster analysis to identify an asthma subphenotype characterized by obese patients with an increased percentage of sputum neutrophils, high levels of symptoms, and poor responsiveness to treatment with inhaled or oral corticosteroids. Telenga *et al.* [3] also found obesity to be associated with a higher percentage of sputum neutrophils and a lower percentage of sputum eosinophils. Taken together, obesity may affect the type of airway inflammation in asthma leading to more neutrophils and less eosinophils in sputum. However, this contrasts with the findings from Desai *et al.* [42] investigating the association between obesity (BMI 30 kg/m²) and airways inflammation in both sputum and bronchial biopsies from patients with severe asthma. They did not find the number of blood or sputum eosinophils to be increased, yet found significantly higher IL-5 levels in sputum of obese asthmatics compared with both overweight (BMI 25–30 kg/m²) and lean (BMI <25 kg/m²) asthmatic controls. Interestingly, higher numbers of eosinophils were found in the airway walls of obese asthma patients as compared with the same control groups. This suggests a discrepancy between inflammatory cell findings in sputum and airway wall biopsies in obese and non-obese study participants with asthma. The latter was recently confirmed by van der Wiel *et al.* [43] in mild-to-moderate asthma patients. They found the percentage of sputum eosinophils to be lower in obese compared with non-obese asthma patients, which is compatible with the previously reported non-eosinophilic phenotype of obese asthma based on sputum analysis [3,39–41]. However, similar to the findings of Desai *et al.* [42] submucosal eosinophil numbers were found to be increased in obese compared with non-obese asthma patients [43]. The above-described findings show that, despite the absence of blood eosinophilia, eosinophilic airway inflammation can be present in obese asthma patients and possibly even to a higher extent than in non-obese asthmatics. However, sputum may not be the appropriate compartment to investigate this.

Mechanical effects of obesity

Spirometry, lung volumes, and airway resistance

Obesity has been associated with reduced lung function. An increased abdominal and thoracic adipose tissue mass inhibits an optimal inflation of the lungs. In line with this, several studies have reported a higher BMI to be associated with both a lower forced expiratory volume in 1 second (FEV_1) and a lower forced vital capacity (FVC) [44–46]. However, the effect was found to be small and even undetectable in some studies (figure 2) [3,47–49]. As both FEV_1 and FVC are similarly affected by obesity, the FEV_1/FVC ratio usually remains unaltered in obese asthmatics.

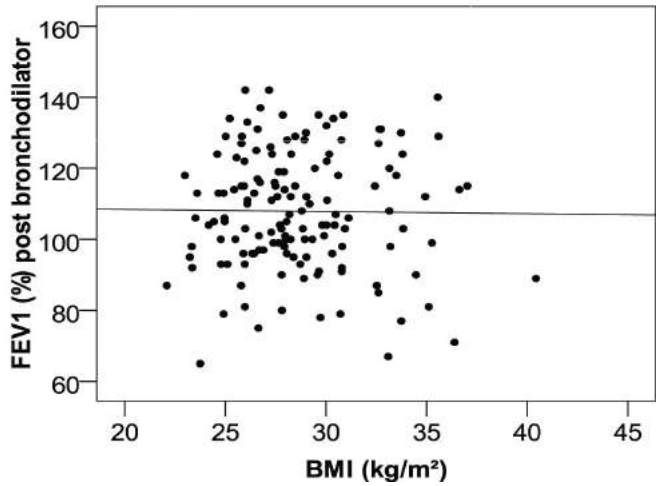

Figure 2: effects of BMI on FEV_1. This study found no significant correlation. (Pearson correlation coefficient: 0.027, P=0.41). FEV_1, forced expiratory volume in 1 second.
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Jones *et al.* [50] investigated how obesity affects lung volumes in study participants without obstructive or interstitial lung disease. Obesity was found to weakly affect the residual volume and total lung capacity, the decrease being approximately 10% in study participants with morbid obesity (BMI 40 kg/m²) compared with lean (BMI <25 kg/m²) controls. In contrast to the relatively small effect on residual volume, and total lung capacity, the impact of obesity on functional residual capacity and expiratory reserve volume (ERV) was found to be much larger with a clear dose–response relationship. As an example, the ERV was only 34% of the predicted normal value in study participants with morbid obesity (BMI 40 kg/m²; Figure 3). Importantly, the dramatic decrease of

ERV may cause morbidly obese study participants to perform tidal breathing at very low lung volumes. As a consequence, non-cartilaginous small airways are more likely to collapse at the end of expiration, leading to their cyclical opening and closure during tidal breathing. The latter renders obese study participants more prone to develop hyperinflation during bronchoconstriction [51,52]. In addition, these sequelae may damage the airway epithelium, thereby inducing pro-inflammatory response in the airways [53].

Bronchial hyperresponsiveness

The relationship between obesity and BHR has been the subject of debate with some studies reporting a significant association [11,54,55], whereas other studies found no relationship [3,56,57]. One very large study by Chinn *et al.* [58] investigating 11 277 adults from the European Community Survey II, reported that a higher BMI was associated with a more severe BHR measured with Provocative dose of methacholine causing a 20% drop in FEV_1 (PC_{20} methacholine) [58]. However, the magnitude of the effect reported in this study was small: an increase in BMI of 10 kg/m² would be needed to decrease the PC_{20} methacholine by only 0.3 doubling doses. Taken together, it can be concluded that obesity only weakly affects the presence and severity of BHR in asthma patients.

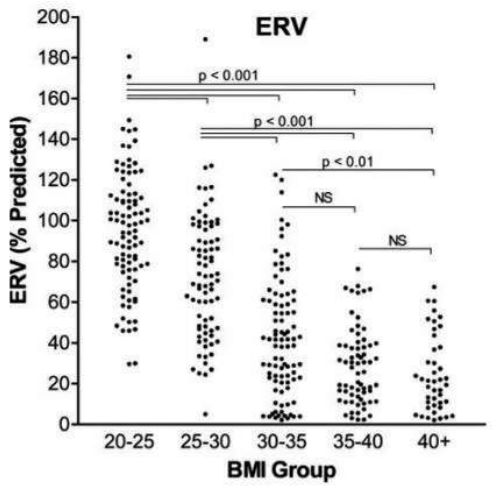

Figure 3: effects of BMI on expiratory reserve volume. Obesity has a dramatic and dose-dependent effect on the ERV such that study participants with morbid obesity perform tidal breathing at very low lung volumes close to their residual volume. ERV, expiratory reserve volume. Reproduced with permission (Jones RL, et al. Chest 2006; 130:827–833).

Obesity affects asthma symptoms and response to anti-inflammatory therapy

Obesity is associated with reduced asthma control and more frequent exacerbations in patients with asthma, even after adjusting for age, sex, and level of FEV_1 [59–62]. In addition, obese asthmatics are more difficult to control by anti-inflammatory treatment with ICS [3,48,49,63,64]. Anderson *et al.* [65] performed a post hoc analysis of a cross-over study investigating the efficacy of inhaled budesonide in 72 patients with asthma divided into two groups: lean (BMI <25 kg/m², n = 25) and overweight (BMI 25 kg/m², n = 47). Patients were treated for 4 weeks with either 200 mg/day or 800mg/day budesonide separated by a washout period of 1 – 2 weeks [65]. Although no differences with respect to change in FEV_1 and BHR were found between lean and overweight asthma patients, inhaled budesonide was less effective in improving symptoms in overweight asthma patients [65]. As budesonide also induced less cortisol suppression in overweight compared with lean asthma patients, it was suggested that airway geometric factors leading to a lower peripheral lung deposition may play a role in the reduced ICS treatment responsiveness [65]. In another study, Peters- Golden *et al.* [49] investigated how obesity affects treatment responsiveness to either inhaled beclomethasone 200mg twice daily or montelukast 10mg once daily in 3,073 patients with asthma. In lean asthma patients, inhaled beclomethasone improved the percentage of asthma control days to a higher extent than montelukast. However, overweight and obesity decreased treatment responsiveness to inhaled beclomethasone, whereas responsiveness to montelukast was not affected. As a consequence montelukast was found to be as effective as inhaled beclomethasone in overweight and obese asthma patients [49]. However, these findings are in contrast with those of Sutherland *et al.* [66] who performed a post hoc analysis in 1.052 asthma patients and found the ICS fluticasone proprionate to be more effective in improving FEV_1, peak-flow, beta-agonist use, and symptom scores than Montelukast not only in lean, but also in overweight and obese asthma patients. In a further study, Farah *et al.* [46] investigated the association between obesity and asthma symptoms before and after treatment with 1500 mg beclomethasone daily (or equivalent) in 49 asthma patients, a total of 14 out of 49 being overweight and 15 out of 49 obese. Although no correlation was found at baseline, a higher BMI strongly correlated with residual asthma symptoms that remained present despite intensive treatment with high doses of ICS for 3 months [Fig. 4] [46]. These findings suggest that both corticosteroid responsive and unresponsive factors contribute to the poorer asthma control generally observed in obese patients. The latter may be important, especially given the recent findings from

Gibeon *et al.* [67] comparing the clinical characteristics of obese (BMI 30) and non-obese (BMI 18–25) patients with severe asthma. Despite a similar degree of airflow obstruction and eosinophilic sputum inflammation, obese asthma patients more often received maintenance treatment with a high dose of oral prednisolone [67]. This approach is unlikely to improve corticosteroid unresponsive factors linked to reduced asthma control, but may lead to side-effects which can be quite severe, among others the fact that it drives insulin resistance and, consequently, obesity.

Effects of weight loss in obese asthma patients

Weight loss achieved by either caloric restriction and/or exercise has been shown to improve asthma symptoms and lung function [68–71]. Scott *et al.* [71] showed that even a modest weight loss of 5–10% improves asthma control as reflected by asthma control questionnaire scores. Although a larger weight loss did not further improve asthma control, it did induce further lung function improvements, particularly with respect to functional residual capacity and ERV values. In the study by Scott *et al.*, caloric restriction was more effective in reducing body weight than exercise alone.

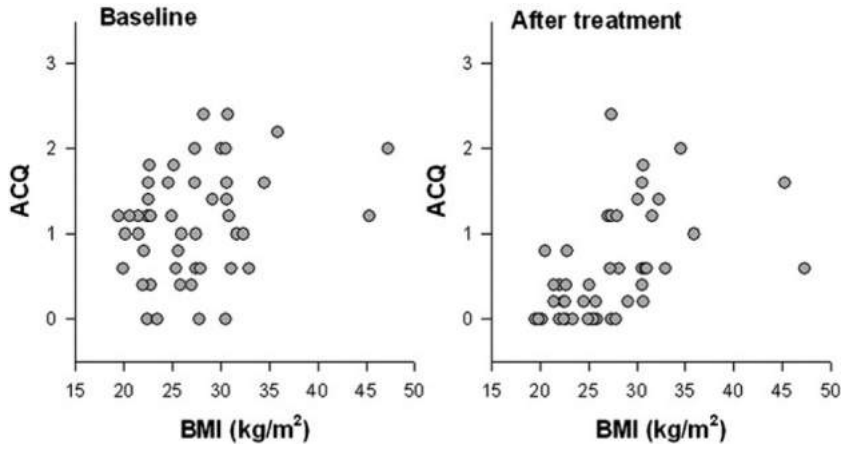

Figure 4: there was no correlation between the ACQ scores and BMI at baseline (Spearman correlation coefficient: 0.22, P=0.14). However, ACQ scores strongly correlated with BMI after 3 months of intensive treatment with a high dose inhaled corticosteroids (Spearman correlation coefficient: 0.58, P<0.001), suggesting that obesity contributes importantly to the corticosteroid-unresponsive component of symptoms in asthma patients. ACQ, asthma control questionnaire.
Reproduced with permission (Farah CS, et al. Chest. 2011 Sep;140(3):659-666).

Studies investigating the effects of bariatric surgery in morbidly obese asthma patients consistently report improvements of asthma control. In one retrospective study, 257 patients using asthma medication were followed up 1-year post-bariatric surgery. Among these 257 patients, 13/28 did not need oral corticosteroids any longer to control their asthma, whereas the overall use of ICS decreased from 50 to 30%. Importantly, patients who underwent laparoscopic gastric banding, which resulted in less weight loss than other surgical treatments, were significantly less likely to stop their oral or ICS. In addition to improvements of asthma control, bariatric surgery has been shown to improve small and large airway function and BHR [72-74]. Few studies have investigated the effects of weight loss on measures of airway inflammation. In a study by Dixon *et al.* [72] bariatric surgery did not improve airways inflammation as reflected by inflammatory cell numbers in bronchoalveolar lavage fluid. Van Huisstede *et al.* [74] investigated the effects of profound weight loss after bariatric surgery on lung function and on systemic inflammation as well as on inflammatory cell counts in bronchial biopsies in 27 asthmatics and 39 non-asthmatics with morbid obesity. As anticipated, improvements in symptoms and large and small airway function were observed both in obese study participants with and without asthma. In addition, BHR markedly improved in obese asthma patients after bariatric surgery and BHR (i.e., methacholine PC_{20} <1.8mg) could no longer be detected in 13 out of 25 patients despite lower doses ICS. Finally, bariatric surgery was found to decrease systemic inflammation and the number of mast cells in bronchial biopsies, whereas other inflammatory cell counts in bronchial biopsies did not change.

Asthma-related comorbidities and obesity

Comorbid conditions such as obstructive sleep apnea (OSA), gastroesophageal reflux disease (GERD), metabolic syndrome, and cardiovascular diseases are more prevalent in obese study participants and may also contribute to the clinical expression of asthma. Obesity is a well-known risk factor for OSA. This may have implications for asthma, as the presence of OSA has been associated with worse asthma control and its treatment with continuous positive airway pressure improves asthma symptoms, peak-flow, and BHR. On the other hand, it is important to understand that asthma itself is also a risk factor for OSA, irrespective of BMI. In a study by Julien *et al.* [75] OSA was found to be more frequently present in severe asthma (23 out of 26 patients) and moderate asthma (15 out of 26 patients) when compared with non-asthmatic controls (eight out

of 26) with a similar age and BMI. GERD is another frequently occurring comorbidity in asthma that is related to obesity [18] and poor asthma control. However, treatment of asymptomatic GERD with a proton-pump inhibitor did not improve asthma control even in a subgroup of obese asthma patients [76]. In addition, adjustment for both OSA and GERD did not change the asthma–obesity relationship In two large epidemiological studies [77,78]. Further, cardiopulmonary deconditioning is more frequently observed in obese than lean asthma patients [79]. Finally, insulin resistance or elevated blood glucose, one of the components of the metabolic syndrome, is another possible factor contributing to the asthma–obesity relationship. In a study by Brumpton *et al.* [80] an elevated fasting glucose or established diabetes was found to be associated with the presence of asthma even after adjustment for obesity. This is compatible with the findings of Thuesen *et al.* [81] showing insulin resistance to be associated with the development of asthma symptoms during a follow-up period of 5 years. Cardet *et al.* [82] showed that the association between insulin resistance and asthma is robust and remains present after adjusting for other components of the metabolic syndrome like hypertriglyceridemia, hypertension, hyperglycaemia, and systemic inflammation. The latter may be important, as it has been suggested that treatments targeting insulin resistance may be beneficial in obese asthma patients. In a recent retrospective study, Li *et al.* [83] found metformin, a drug that recovers peripheral insulin sensitization, to reduce the number of asthma exacerbations in obese patients with asthma and diabetes.

Conclusion

Obesity increases the risk for asthma development and affects its clinical expression toward a difficult-to-control phenotype. Although the mechanisms underlying the asthma–obesity relationship are not fully understood, several possible explanations have been put forward. First, obesity considerably reduces the ERV causing obese study participants to perform tidal breathing at low lung volumes, making them more susceptible to develop hyperinflation during bronchoconstriction. Second, obesity in asthma is associated with comorbidities such as OSAS and GERD, enhancing treatment complexity. Third, it affects the type and severity of the inflammatory process occurring in the airways in obese and overweight asthmatics characterized by more neutrophils and less eosinophils in sputum. Finally, it has been proposed that obesity is associated with a reduced corticosteroid treatment response. Nevertheless, obese asthma patients are often prescribed maintenance treatment with systemic corticosteroids. It is questionable whether this approach improves asthma control. To the opinion of the authors, systemic corticosteroid treatment should be avoided as much as possible even more in overweight and obese asthma patients, also because it further worsens obesity itself.

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Chapter 8

A cellular census of human lungs identifies novel cell states in health and in asthma

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Abstract

Human lungs enable efficient gas exchange and form an interface with the environment, which depends on mucosal immunity for protection against infectious agents. Tightly controlled interactions between structural and immune cells are required to maintain lung homeostasis. Here, we use single-cell transcriptomics to chart the cellular landscape of upper and lower airways and lung parenchyma in healthy lungs, and lower airways in asthmatic lungs. We report location-dependent airway epithelial cell states and a novel subset of tissue-resident memory T cells. In the lower airways of patients with asthma, mucous cell hyperplasia is shown to stem from a novel mucous ciliated cell state, as well as goblet cell hyperplasia. We report the presence of pathogenic effector type 2 helper T cells (TH2) in asthmatic lungs and find evidence for type 2 cytokines in maintaining the altered epithelial cell states. Unbiased analysis of cell-cell interactions identifies a shift from airway structural cell communication in healthy lungs to a TH2-dominated interactome in asthmatic lungs.

Main

The lung plays a critical role in both gas exchange and mucosal immunity, and its anatomy serves these functions through: (1) the airways that lead air to the respiratory unit, provide mucociliary clearance and form a barrier against inhaled particles and pathogens; and (2) the alveoli, distal saccular structures where gas exchange occurs. We set out to profile lung-resident structural and inflammatory cells and their interactions by analyzing healthy human respiratory tissue from four sources: nasal brushes, bronchial biopsies and brushes from living donors, tissue samples from lung resections and transplant donor lungs. Our single-cell analysis identifies differences in the proportions and transcriptional phenotype of structural and inflammatory cells between upper and lower airways and lung parenchyma. We identify a novel tissue-resident CD4 T cell subset that harbors features of both circulating memory cells and of tissue-resident memory (TRM) cells. We demonstrate that many disease-associated genes have highly cell type-specific expression patterns.

In addition, we evaluate the altered cellular landscape of the airway wall in asthmatic lungs. We identify a novel epithelial cell state highly enriched in asthmatic lungs. These mucous ciliated cells represent a transitioning state of ciliated cells and contribute to mucous cell hyperplasia in this chronic disease. Other asthma-associated changes include increased numbers of goblet cells, intraepithelial mast cells, and pathogenic effector type 2 helper T cells (TH2) in airway wall tissue. Analysis of intercellular communications in healthy and asthmatic airway walls reveals a remarkable loss of structural cell communication and a concomitant increase in TH2 cell interactions. We generate novel insights into epithelial cell changes and altered communication patterns between immune and structural cells of the airways that underlie asthmatic airway inflammation.

Results

Human lung cell census identifies macro-anatomical patterns of epithelial cell states across the human respiratory tree

The cellular landscape along the 23 generations of the airways in human lung is expected to differ both in terms of relative frequencies of cell types and their molecular phenotype [1]. We used 10x Genomics Chromium droplet single-cell RNA sequencing (scRNA-seq) to profile a total of 36,931 single cells from upper and lower airways and lung parenchyma (Fig. 1a,b and Supplementary Table 1). We profiled nasal brushes, bronchial brushes, and airway wall biopsies (third to sixth generation) from healthy volunteers. For parenchyma (small respiratory airways and alveoli), we obtained lung tissue from deceased transplant donors.

Our analysis reveals a diversity of epithelial, endothelial, stromal, and immune cells, with approximately 21 coarse-grained cell types in total (Figs. 1 and 2 and Extended Data Fig. 1), that can be explored in a user-friendly web portal (www.lungcellatlas.org). We further confirmed our observations by comparing our dataset with parenchymal lung tissue from resection material analyzed on a bespoke Drop-seq-like microfluidics platform [2] (Extended Data Fig. 2). We observed extensive overlap in cell-type identities (Extended Data Fig. 2). In our analysis below, we first concentrate on epithelial cells (Fig. 1) and then focus on the stromal and immune compartments (Fig. 2).

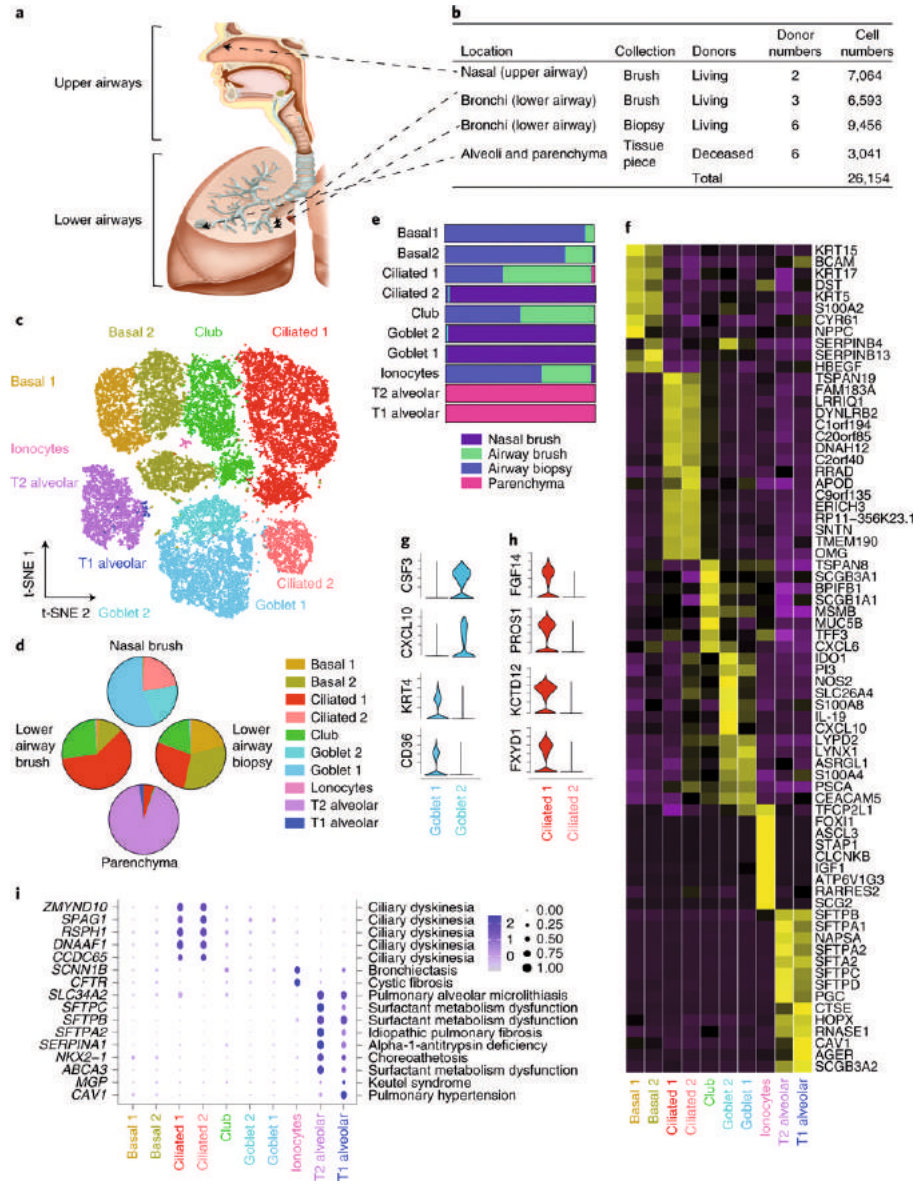

Figure 1: A human lung cell census identifies zonation of novel epithelial cell states across macro-anatomical location. **a:** schematic depicting anatomical regions analyzed in this study, **b:** table with the details of anatomical region, tissue source, donors, and cell numbers present in this figure, **c:** t-SNE displaying the major epithelial clusters present in the full extent of the human respiratory tree. T1, type 1; T2, type 2, **d:** Pie charts depicting the cellular composition by anatomical region, **e:** Horizontal slice bar depicting the anatomical distribution of each cell type identified, **f:** Heat map depicting the average expression levels per cluster of the top differentially expressed markers in each cluster, **g:** Violin plots of selected markers identified by differential expression analysis comparing the two goblet subsets to each other, **h:** Violin plots of selected markers identified by differential expression analysis of ciliated 1 versus ciliated 2 clusters, **i:** Dot plot depicting gene expression levels and percentage of cells expressing genes associated with specific lung phenotypes according to the OMIM database. All the differential expression analyses in f–i were performed using the non-parametric two-sided Wilcoxon rank sum test in Seurat. All panels depict the number of cells and individuals described in b.

a

Location	Collection	Donors	Donor numbers	Cells
Nasal (upper airway)	Brush	Living	2	23
Bronchi (lower airway)	Brush	Living	3	172
Bronchi (lower airway)	Biopsy	Living	6	652
Alveoli and parenchy	Tissue piece	Deceased	6	9,930
		Total		10,777

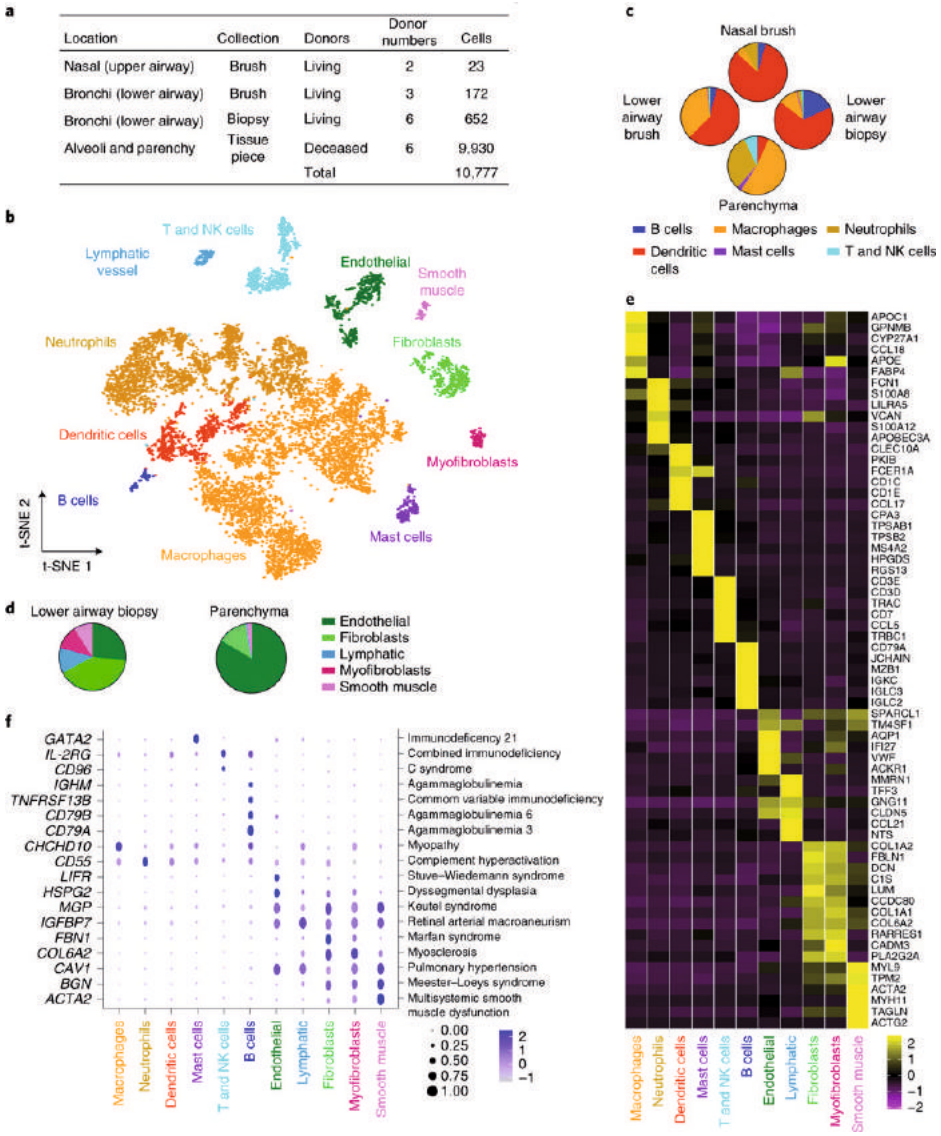

Figure 2: A cellular and molecular map of the stromal and immune components of across the upper and lower human respiratory airways. **a:** Table with details of anatomical region, tissue source, donors, and cell numbers present in this figure, **b:** t-SNE displaying the major immune and mesenchymal clusters present in the full extent of the human respiratory tree. NK, natural killer, **c:** Pie charts depicting the cellular composition of immune cells by anatomical region, **d:** Pie charts depicting the cellular composition of stromal cells in lower airway biopsies and parenchyma tissue, **e:** Heat map depicting the average expression levels per cluster of the top differentially expressed markers in each cluster, **f:** Dot plot depicting gene expression levels and percentage of cells expressing genes associated with lung phenotypes according to the OMIM database. All the differential expression analyses in e and fwere performed using the non-parametric two-sided Wilcoxon rank sum test in Seurat. All panels depict the number of cells and individuals described in a.

In the epithelial lineage, we identified at least ten cell types across the upper and lower airways and lung parenchyma (Fig. 1c and Extended Data Fig. 1). We detected multiple basal, club, ciliated, and goblet cell states, as well as type 1 and type 2 alveolar cells, and the recently described ionocyte [3,4] (Fig. 1d and Extended Data Fig. 3). We did not identify specific clusters of tuft cells or neuroendocrine cells. Supervised analysis using neuroendocrine (CHGA, ASCL1, INSM1, HOXB5) and tuft (DCLK1, ASCL2)[3] cell marker genes identified a small number of cells with neuroendocrine-like features present only in lower airways (Extended Data Fig. 4). Tuft cell marker genes did not identify a unique cell population.

We identified two discrete cell states in each of basal, goblet, and ciliated epithelial cells. Basal cells were present in upper and lower airways, although at relatively low frequency in upper airways (Fig. 1e). The two basal cell states corresponded to differentiation stages, with the less-mature basal 1 cell state expressing higher levels of TP63 and NPPC compared with more-mature basal 2 cells (Fig. 1f and Extended Data Fig. 1), which were more abundant in bronchial brushes, suggesting a more apical localization (Fig. 1d,e). Goblet 1 and 2 cells were both characterized by high expression of CEACAM5, S100A4 and MUC5AC, and lack of MUC5B (Fig. 1f and Extended Data Figs. 1 and 4). Goblet 1 cells specifically express KRT4 and CD36 (Fig. 1g and Extended Data Fig. 4). Genes involved in recruitment of neutrophils, monocytes, dendritic cells, and T cells5, such as IDO1, NOS2, IL19, CSF3 (granulocyte-colony stimulating factor), and CXCL10 are expressed at high levels in goblet 2 cells (Fig. 1f,g and Extended Data Fig. 4). Both goblet cell states are present in upper airway epithelium. In contrast, only the goblet 2 cell state was present in lower airways, albeit at low abundance (Fig. 1e).

Ciliated cells were also zonated in terms of their presence across macro-anatomical locations, with a discrete ciliated cell state more abundant in upper airways (ciliated 2) compared with lower airways and lung parenchyma. Nasal epithelial ciliated 2 cells express proinflammatory genes, such as CCL20, and higher levels of metabolic (ATP12A and COX7A1) and vesicle transport (AP2B1 and SYT5 [6]) genes compared with the ciliated 1 cells (Extended Data Fig. 4). In contrast, the ciliated 1 cells from lower airways specifically expressed genes involved in cytoprotection (PROS1 [7]) and fluid reabsorption (FXYD1 8]) (Fig. 1h and Extended Data Fig. 4). We detected a location-

specific transcriptional signature specific for the upper airways in both ciliated and goblet cells (Extended Data Fig. 4b).

Next, we assessed the contribution of specific epithelial cell types to Mendelian disease. Cell type-specific expression patterns of genes associated with Mendelian disorders (based on the Online Mendelian Inheritance in Man (OMIM) database) confirmed ionocytes as particularly high expressors of the CFTR gene, mutated in cystic fibrosis (Fig. 1i and Supplementary Table 2). These cells also express SCNN1B, mutations of which can cause bronchiectasis, another feature of cystic fibrosis, suggesting a pathological role for ionocytes in both bronchiectasis and cystic fibrosis. In addition, expression of SERPINA1 (Fig. 1i) was found to be enriched in type 2 alveolar epithelial cells, underscoring their role in alpha-1-antitrypsin deficiency [9].

Differential anatomical distribution of the stromal and immune components in the human respiratory tree

Next, we analyzed the single-cell transcriptomes of immune and stromal cells from the upper airways, lower airways, and the lung parenchyma (Fig. 2a). We identified immune clusters of myeloid (macrophages, neutrophils, dendritic cells, and mast cells) and lymphoid cells (T and natural killer cells, B cells; Fig. 2b and Extended Data Fig. 5). Immune and stromal cell numbers and composition varied greatly across different anatomical regions (Fig. 2a,c). Nasal brushes contained only a small number of immune cells, with the large majority being dendritic cells. In the lower airways, the fraction of inflammatory cells was much larger and relatively enriched for macrophages (Fig. 2c and Extended Data Fig. 5), which was directly confirmed by cell composition comparison of upper versus lower airway brushes obtained from the same donor (Extended Data Fig. 5e). The stromal component of the parenchyma region was dominated by vascular endothelial cells with a small number of fibroblasts, while the upper airways had a richer stromal compartment, with fibroblasts, myofibroblasts, smooth muscle, vascular, and lymphatic endothelial cells (Fig. 2d).

Macrophage transcriptional phenotypes showed large donor variation (Extended Data Fig. 5), but they all shared high expression of MARCO, CCL18, and genes involved in apolipoprotein metabolism (APOC1 and APOE) (Fig. 2e and Extended Data Fig. 5). Lung neutrophils express high levels of the granulocyte markers S100A8, S100A12 [10], and

LILRA5, a receptor poorly characterized in the lungs that has been shown to have a proinflammatory function in synovial fluid macrophages [11] (Fig. 2e and Extended Data Fig. 5). Dendritic cells were mostly myeloid, with high expression of CD1E, CD1C, and CLEC10A (Fig. 2e and Extended Data Fig. 5) and of FCER1A (IgE receptor) and CCL17, which have a key role in inflammatory conditions such as asthma [12].

In the scRNA-seq datasets, we could not distinguish T cells and natural killer cells from each other (Fig. 2b). The B cells in our dataset were mostly plasma cells, expressing high levels of JCHAIN. IgM^+ (IGHM) B cells were enriched in the bronchial brushes and in the lung parenchyma, while $IgG3^+$ (IGHG3) B cells were enriched in airway biopsy samples and were virtually absent from the bronchial brushes, suggesting a micro-anatomical segregation of B cell subsets (Extended Data Fig. 5f). The immune and stromal lung components also displayed cell type-specific expression patterns of genes associated with Mendelian disorders. However, in contrast to epithelial cell associated syndromes, syndromes associated with immune and stromal cells were largely systemic in nature (with lung involvement [13]) (Fig. 2f).

Molecular features of mucous cell metaplasia in asthma

Asthma is a complex and heterogeneous disease [14], where epithelial [15,16], stromal [17], and immune cells18,19 contribute to a spectrum of clinical phenotypes. We analyzed bronchial biopsies from six volunteers with chronic, childhood-onset asthma (Fig. 3a and Supplementary Table 3) and compared them with bronchoscopy samples obtained from healthy sex- and age-matched volunteers (Supplementary Table 3). The Global Initiative for Asthma (GINA) score at time of recruitment indicated patients had either mild or moderate asthma (Supplementary Table 3). Withdrawal of inhaled corticosteroids was a minimum of six weeks before sample collection for all patients. Most patients had controlled asthma on inhaled corticosteroid withdrawal (asthma control questionnaire score < 1.5). The combined airway wall dataset reveals a cellular landscape dominated by epithelial cells, with minor contributions from endothelial, mesenchymal, and immune cells (Extended Data Fig. 6a,b and Supplementary Tables 4 and 5).

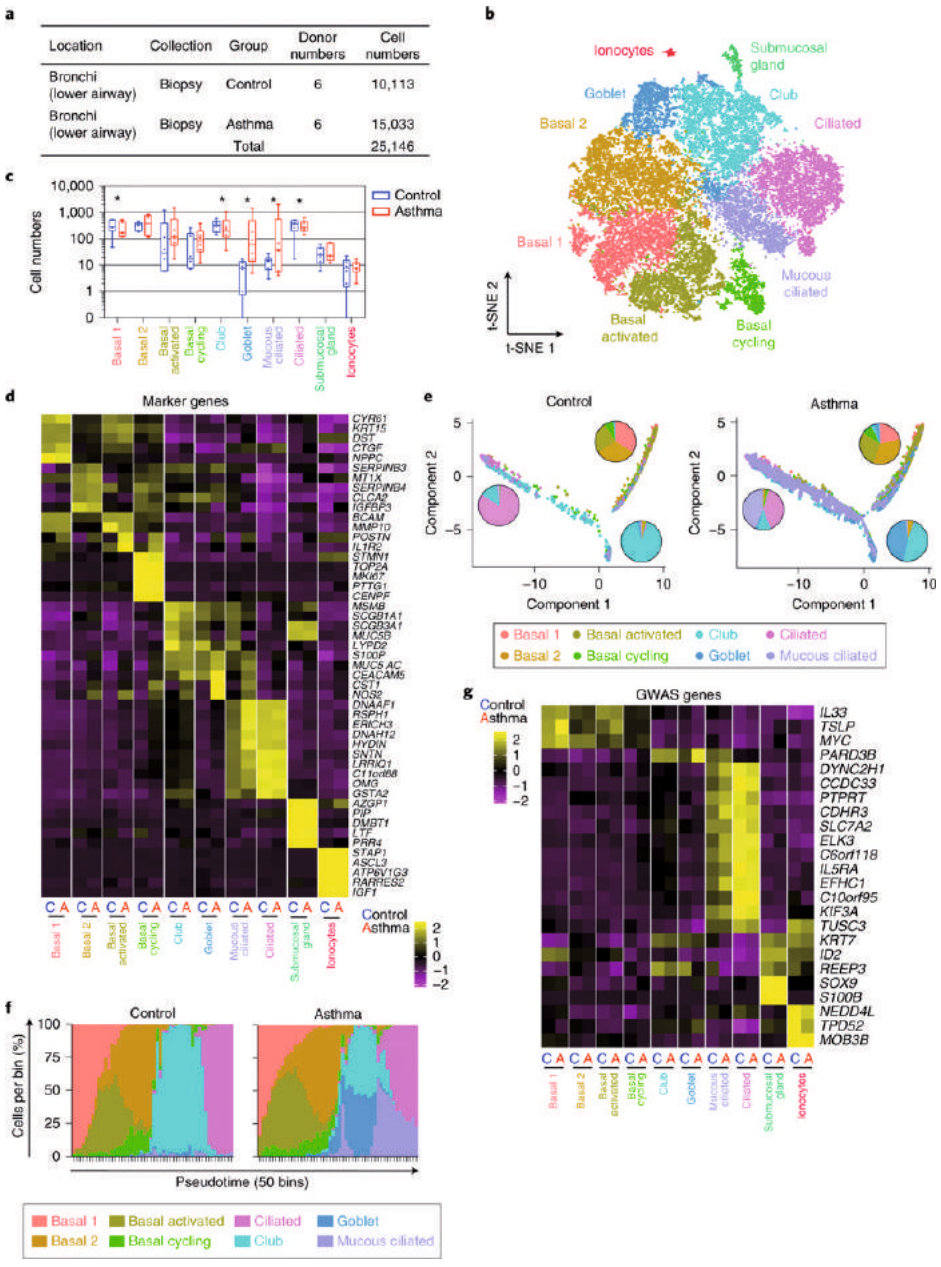

Figure 3: distinct programs of epithelial cell differentiation in asthmatic versus healthy airways, **a:** Table with overview and cell numbers for healthy control volunteers and volunteers with asthma analyzed in this figure, **b:** t-SNE displaying all epithelial cells analyzed colored by their specific cluster assignment, **c:** Box and whisker plots depicting cell numbers of healthly control patients and patients with asthma in each cluster, **d:** Heat map displaying the top five differentially expressed genes per cluster, **e:** Pseudotime developmental trajectory analysis from Monocle2 depicting how each of the basal, secretory, and ciliated subsets relate to each other, **f:** Binned pseudotime analysis displaying how each subset is ordered in a one-dimensional continuous space, **g:** Heat map displaying the expression of asthma genes from GWAS. Only genes present in our list of differentially expressed genes are depicted for each cell cluster. Significance analysis in c calculated using Fisher's exact test corrected for multiple comparison using the Bonferroni method. Significance was calculated using all the clusters present in **Figs. 3 and 4**, which were derived from the same set of samples. *P < 0.001. A full list of P values is given in **Supplementary Table 5**. In box and whisker plots in c, all points are shown, and the box represents the second and third quartiles and median. All the differential expression analyses in d and g were performed using the non-parametric two-sided Wilcoxon rank sum test in Seurat. **Panels b–d** and **g** depict the number of cells and individuals described in **a; e** and **f** depict the number of individuals described in a.

Clustering of the EPCAM+ cells identified ten sub-clusters representing the six epithelial cell types observed in healthy airway wall (Fig. 1c), as well as four additional cell states: mucous ciliated cells, activated basal cells, cycling cells, and serous cells from the submucosal glands (Fig. 3b).

Activated basal cells closely resemble basal 1 cells, but also express proinflammatory genes such as POSTN (Fig. 3d). Cycling basal cells are characterized by expression of canonical marker genes of proliferating cells (MKI67 and TOP2A) (Fig. 3d), and this is the only cluster of airway epithelial cells expressing the squamous cell marker KRT13 (Extended Data Fig. 6).

We observe mucous cell hyperplasia in asthma, with a marked increase in goblet cell numbers (Fig. 3c), which are very rare in healthy airway wall biopsies (Fig. 1e). Moreover, the goblet cell transcriptional phenotype is altered in asthma, with strong up regulation of proinflammatory and remodeling genes NOS2, CEACAM5, and CST1 (Fig. 3d). In addition, we identified a strong increase in mucous ciliated cells, a novel cell state highly similar to ciliated cells, but co-expressing a number of mucous genes also observed in goblet cells, including MUC5AC and CEACAM5 and the ciliated genes FOXJ1 and PIFO (Fig. 3d and Extended Data Fig. 6).

To further dissect the inferred differentiation trajectories of epithelial cells in healthy and asthmatic airway walls, we performed pseudotime analysis [20]. This reveals a trajectory starting with basal cell subsets, bifurcating into either a secretory lineage (mainly club cells) and a ciliated lineage in healthy airway wall (Fig. 3e). In asthma, the secretory lineage is a mix of club and goblet cells, while the mucous ciliated cell state is mapped to the ciliated differentiation trajectory (Fig. 3e,f).

Next, we further analyzed the transcriptional profiles of the mucous ciliated and goblet cells. As both Notch and interleukin-4 (IL-4)/IL-13 signaling by TH2 cells contribute to mucous cell differentiation [21], we analyzed expression of both Notch [22,23] and IL-4/IL-13 target genes [24]. Expression of IL-4/IL-13-induced genes [24] is highest in activated basal cells, goblet cells, and mucous ciliated cells (Extended Data Fig. 7a) and prominent in asthma in club, goblet, and mucous ciliated cells (Extended Data Fig. 7b). In club cells, expression of Notch target genes [22,23] does not differ between asthma- and healthy-derived cells. In contrast, in goblet cells, the Notch target gene signature is retained only in cells from healthy airway wall, and is lost in asthma. Interestingly, mucous ciliated cells also lack expression of Notch target genes in asthma (Extended Data Fig. 7). Hence, we postulate that mucous ciliated cells represent a transition cell state in the ciliated lineage—induced by IL-4/IL-13 signaling—leading to a mucous cell phenotype that contributes to mucous cell metaplasia in asthma [21]. Similar to goblet cells, mucous ciliated cells express key asthma genes such as CST1 [25] and POSTN (Fig. 3d), indicating that these cells can contribute to airway inflammation and remodeling.

Analysis of asthma genome-wide association study (GWAS) gene expression in our epithelial scRNA-seq data revealed a broad contribution of airway epithelial cell types to asthma susceptibility (Fig. 3g), with high expression of asthma GWAS genes in ciliated and mucous ciliated cells. This includes genes involved in ciliary function (DYNC2H1 and KIF3A), cell adhesion (ELK3, CDHR3 and PTPRT), and IL-5-induced mucus metaplasia (IL-5RA) [26], further suggesting a direct link between mucous ciliated cells and TH2 cells.

Remodeling of the stromal and immune compartments in asthmatic airways

Asthma is associated with chronic inflammation and remodeling of the airway wall [27]. Analysis of the immune and stromal cell populations (Fig. 4a) in the bronchial biopsies by reveals the presence of B and T cells, neutrophils, macrophages, dendritic cells, mast cells, fibroblasts, smooth muscle cells, and endothelial cells (Fig. 4b and Extended Data Fig. 8). We did not detect innate lymphoid, basophil, or eosinophil clusters (Extended Data Fig. 8). Analysis of bulk transcriptome data of matched airway wall biopsies before and after tissue dissociation identified very low expression levels of the eosinophil marker gene CLC, indicating that these cells are rare in the samples we analyzed (Extended Data Fig. 9).

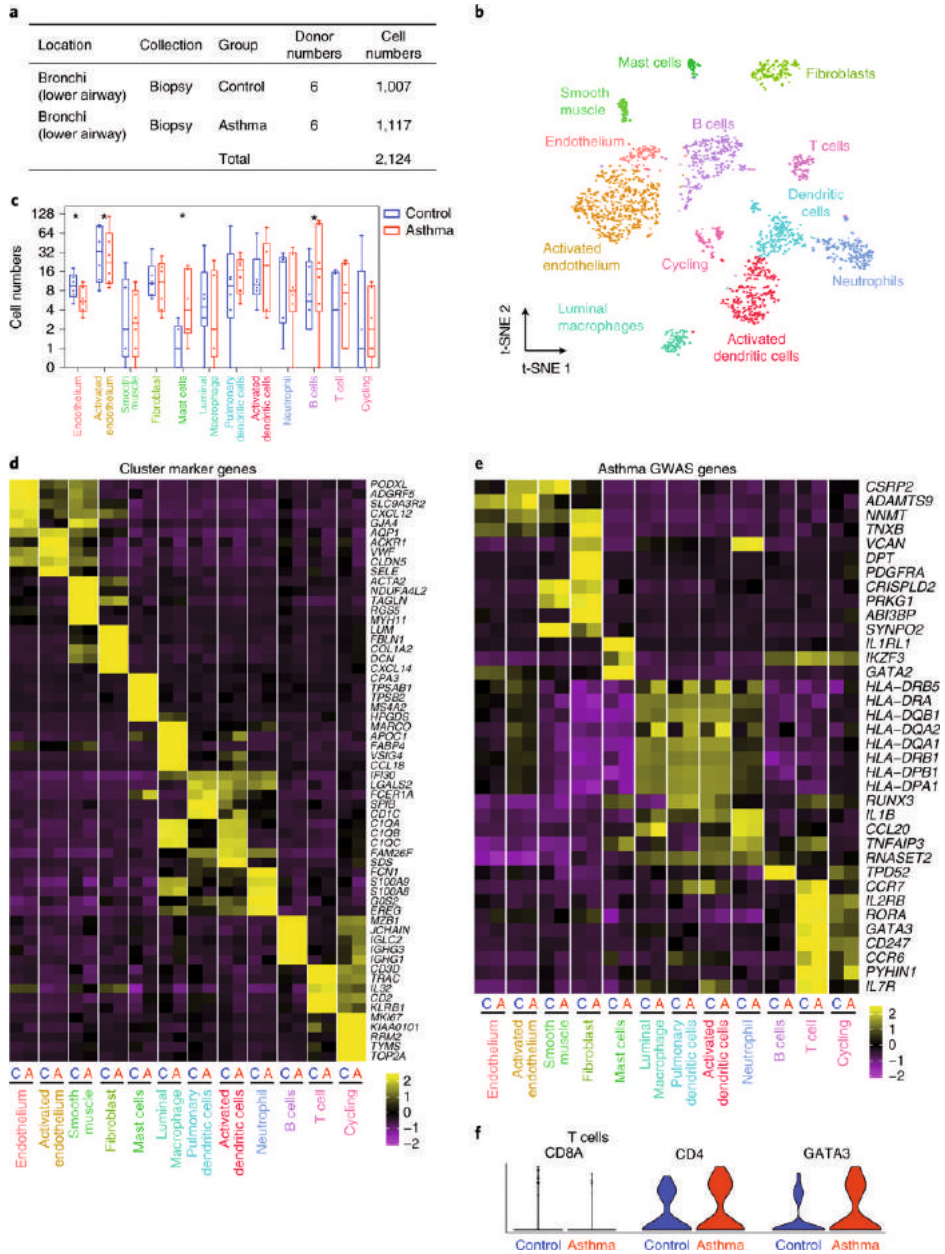

Figure 4: remodeling of the stromal and immune compartments in asthmatic airways, **a:** Table with the number of donors and cells per volunteer group included in this figure, **b:** t-SNE depicting the immune and stromal cell types identified in the human airway combined dataset of healthy controls and patients with asthma, **c:** Box and whisker plots depicting the cell numbers of healthy and asthmatic cells in each cluster, **d:** Heat map displaying gene expression levels of the top five differentially expressed genes per cluster, **e:** Heat map displaying asthma GWAS gene expression per cluster. Only genes present in the top 50 (per cluster) of our list of differentially expressed genes are shown, **f:** Violin plots of selected T cell markers in patients with asthma. Significance was calculated using all the clusters present in **Figs. 3 and 4**, which were

derived from the same set of samples. *P < 0.001. A full list of P values is given in **Supplementary Table 5**. All the differential expression analyses in d and f were performed using the non-parametric two-sided Wilcoxon rank sum test in Seurat. In the box and whisker plot in **c**, all points are shown, and the box represents the second and third quartiles and median. **Panels b–f** depict the number of cells and individuals described in **a**.

Mast cell numbers were increased in asthma (Fig. 4c). Mast cells in asthmatic airways lack chymase 1 expression (CMA1) and express high levels of tryptase genes (TPSB2, TPSAB1) and prostaglandin synthetase genes PTGS2 and HPGDS (Fig. 4d and Extended Data Fig. 8d). PTGS2 (cyclooxygenase-2), also known as inflammatory cyclooxygenase, converts the precursor arachidonic acid to prostaglandin endoperoxide H2 (PGH2). HPGDS (hematopoietic prostaglandin D synthase) catalyzes the conversion of PGH2 to prostaglandin D2 (PGD2). PGD2 activates TH2 cells [28], innate lymphoid cells 2 [29], basophils, and neutrophils [28] and plays a key role in asthma pathology. Expression of all PGD2 biosynthesis enzymes is a unique feature of mast cells (Extended Data Fig. 8d), suggesting that mast cells are a major source of PGD2 in asthma patients. These cells are most likely intraepithelial mast cells, previously shown to accumulate in TH2-high asthmatic airway epithelium [30], and reported to increase [31] with disease severity [18].

Asthma GWAS genes show cell-type restricted expression (Fig. 4e). When excluding the widely expressed HLA genes from the analysis, fibroblasts and T cells express the highest number of asthma GWAS genes (Fig. 4f), which are also mostly upregulated in asthma (Fig. 4f). GATA3 expression is restricted to T cells (Fig. 4f) and increased in patients with asthma (Fig. 4f). We detected up-regulation of CD4 (but not CD8A) in the T cell cluster, suggesting an increase in TH2 $CD4^+$ T cells (Fig. 4f). Therefore, we investigated the $CD4^+$ T cell compartment in airway wall biopsies in more detail.

Pathogenic effector T helper 2 cells are enriched in asthmatic airways

In line with the increased GATA3 and CD4 expression mentioned above, TH2 cells are known to be key drivers of asthma [14,32]. To assess the presence of TH2 effector cells in the airways of patients with asthma (Fig. 4f), we single-cell sorted $CD4^+$ T cells followed by in-depth transcriptional phenotyping by SmartSeq2 profiling (Fig. 5a and Methods). We analyzed cells from both peripheral blood and airway wall biopsies (Fig. 5a) from a larger cohort of patients with asthma and healthy controls (Fig. 5b and Supplementary Table 6). Unbiased clustering reveals six major populations of $CD4^+$ T cells (Fig. 5c and Extended Data Fig. 10) with no differences in their relative abundance between asthma and healthy airway wall (Fig. 5d).

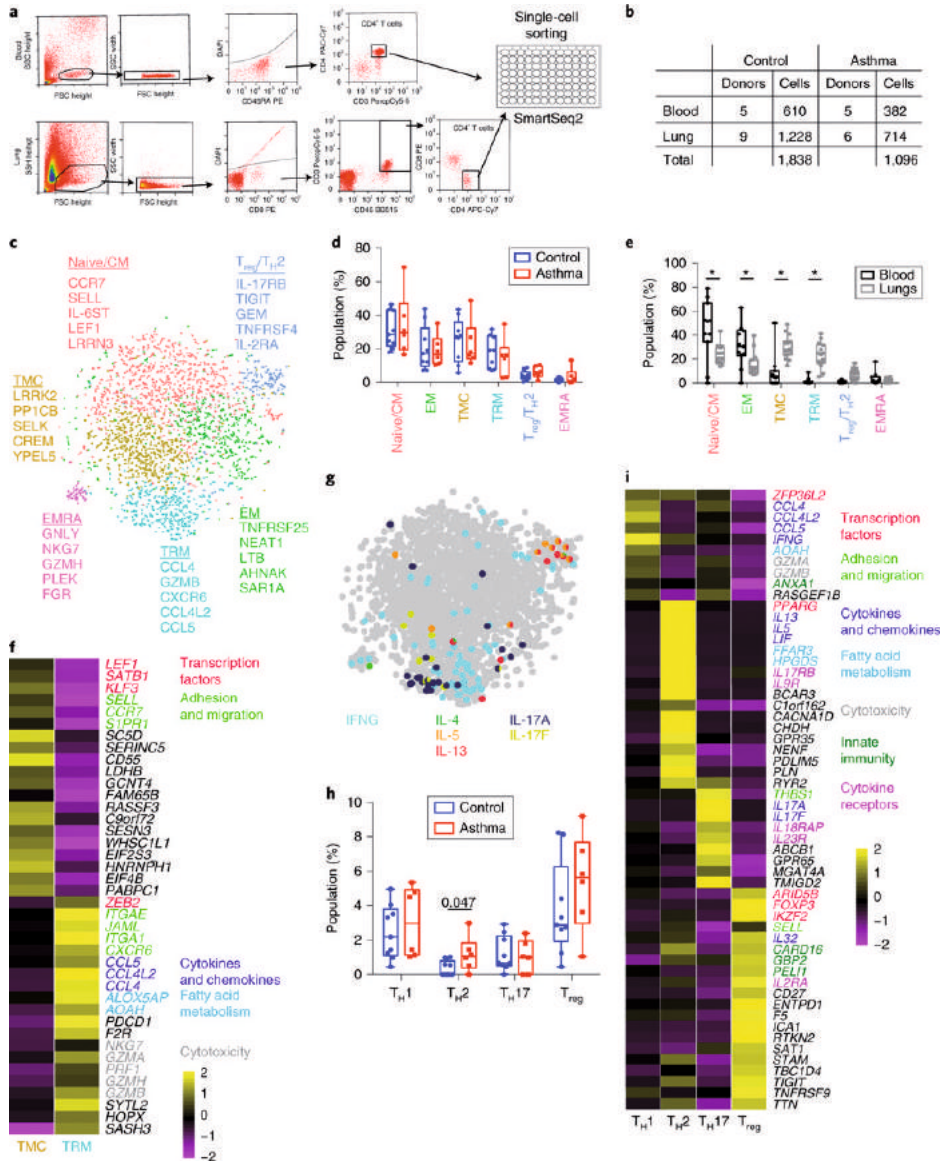

Figure 5: Pathogenic effector TH2 cells are enriched in asthmatic airways, **a:** Schematic depicting experimental layout of single-cell sorting of CD4 T cells from blood and lung airway biopsies, **b:** Table with the number of donors by anatomical location for healthy control and asthma groups, **c:** t-SNE displaying clusters of T cells identified by analyzing the combined cells from blood and lung from healthy control and asthma groups, **d:** Box and whisker plots showing the cluster cell distributions from healthy control patients and patients with asthma, **e:** Box and whisker plots depicting the cluster composition per donor according to the tissue source from which the cells were isolated, **f:** Heat map showing the average expression per cluster of genes differentially expressed between the two lung specific CD4 T cell populations. Gene names colored according to functional categories, **g:** t-SNE depicting canonical cytokines from TH1, TH2, and TH17 cells,

h: Box and whisker plots showing the number of TH1, TH2, and TH17 cells defined by canonical cytokines expression and T_{reg} cells identified by unbiased clustering, **i:** Heat map of average cluster gene expression of markers differentially expressed between TH1, TH2, TH17, and T_{reg} cells. Gene names colored according to functional categories. In **d, e** and **h**, box and whisker plots show all data points, and the box represents the second and third quartiles and median. Significance was analyzed using unpaired multiple t-tests assuming no consistent s.d. and corrected for multiple comparison using the Holm–Sidak method. All the differential expression analyses in **f** and **i** were performed using the non-parametric two-sided Wilcoxon rank sum test in Seurat. Panels **c–g** depict the number of cells and individuals described in **b**. Panels **h** and **i** depict the number of individuals described in **b**.

Comparative analysis of $CD4^+$ T cells isolated from paired blood and lung samples allowed us to differentiate between tissue-resident and circulating T cells in an unbiased way (Fig. 5e and Methods). We identified two $CD4^+$ T cell subsets highly enriched in airway wall: the classical TRM $CD4^+$ T cells, and a novel subset, which we named the tissue migratory $CD4^+$ T cell (TMC) subset (Fig. 5e). Naive/central memory (CM), effector memory (EM), and effector memory reexpressing CD45RA (EMRA) $CD4^+$ T cells, as well as a mixed regulatory T cell (Treg)/TH2 cluster, are either enriched in blood or present in both blood and airway wall biopsies (Fig. 5e).

To better understand the two distinct lung airway-resident $CD4^+$ T cell subsets, we performed differential expression analysis between TRM and TMC cells (Fig. 5f). TRM cells lack S1PR1 and CCR7 expression. TRM cells in airway wall also expressed high levels of CXCR6 and ITGA1, chemokines (CCL4, CCL4L2, CCL5) and effector molecules (PRF1, GZMB, GZMA, GZMH) (Fig. 5f and Extended Data Fig. 10), indicating they are in a primed state capable of direct effector function, as recently shown for TRMs from lung parenchyma [33]. TMC cells expressed the tissue egression markers S1PR1, CCR7, and SELL (CD62L) (Fig. 5f) as well as several transcription factors highly expressed in circulating cells such as LEF1, SATB1, and KLF3. Small numbers of TMC cells were present in peripheral blood $CD4^+$ T cells (Fig. 5e), suggesting that these cells might have the potential to transit between lung, lymph, and blood.

$CD4^+$ effector T cells are classically divided into distinct functional subsets based on their cytokine profile [14]. We manually annotated clusters of TH1 ($IFNG^+$), TH2 ($IL4^+$, $IL5^+$ or $IL13^+$), and TH17 ($IL17A^+$ or $IL17F^+$) cells based on their cytokine expression profiles (Fig. 5g and Extended Data Fig. 10). Although rare overall (Supplementary Table 6), TH2 cells were significantly increased in the airway wall in patients with asthma, with no difference in the relative proportions of the other TH subsets (Fig.

5h). In addition to the signature cytokines IL4, IL5, and IL13 and the transcription factor GATA3 (Extended Data Fig. 10), airway wall TH2 cells express HGPDS, identifying them as pathogenic effector TH2 (peTH2) cells, previously associated with eosinophilic inflammation of the gastrointestinal tract and skin [34]. Airway TH2 cells also express the transcription factor PPARG and the cytokine receptors IL17RB and IL9R (Fig. 5i). IL17RB has been reported to be upregulated in pathogenic allergen-specific TH2 cells (coined TH2A cells), which are present in allergic disease [35] as well as in chronic rhinosinusitis with nasal polyps [36], suggesting airway wall TH2 cells share features with both TH2A and peTH2 cells.

Asthma is characterized by specific signaling networks

Asthma is characterized by remodeling of the airways, which depends on complex interactions between structural and inflammatory cells [14], both via direct physical interactions and secreted proteins and small molecules. We used our recently developed receptor/ligand database and statistical inference framework CellPhoneDB [37] (www.cellphonedb.org) to identify potential cell–cell interactions in the airway wall, and define their changes in asthma. While most interactions are unchanged, some were specific to the diseased or healthy states (Supplementary Table 7).

In healthy controls, the cell–cell interaction landscape of the airway wall was dominated by lung structural cells (mesenchymal and epithelial cell types) communicating with each other, and with both TRM and TMC $CD4^+$ T cells (Fig. 6a,b, left panels). In the asthmatic airway wall, the number of predicted interactions between epithelial and mesenchymal cells was strongly reduced. Instead, the cell–cell communication landscape is dominated by TH2 cells in asthma. TH2 cells have increased predicted interactions with other immune cells, epithelial cells, and especially mesenchymal cells, both fibroblasts and smooth muscle cells (Fig. 6a,b, right panels).

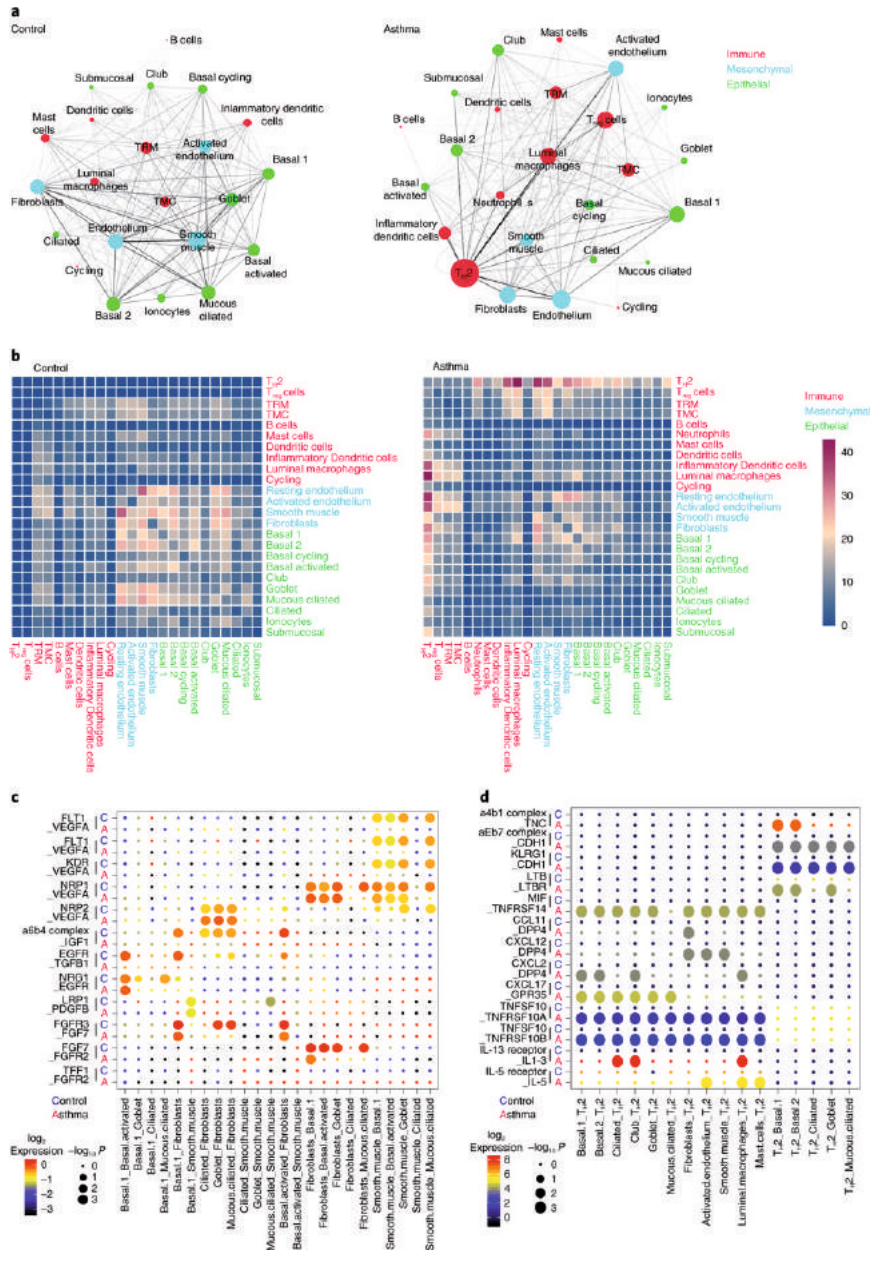

Figure 6: asthma is characterized by unique cell-to-cell signaling networks. We quantified the predicted cell interactions in healthy and asthmatic airways between all the epithelial and non-epithelial cell clusters identified in Figs. 3 and 4, plus the lung airway-enriched populations of CD4 T cells (TH2, T_{reg}, TMC, and TRM), **a:** Networks depicting cell types as nodes and interactions as edges. Size of cell type is proportional to the total number of interactions of each cell type, and edge thickness is proportional to the number of interactions between the connecting types, **b:** Heat map depicting the number of all possible interactions between the clusters analyzed. Cell types grouped by broad lineage (epithelia, mesenchymal, or immune), **c:** Dot plot depicting selected epithelial–epithelial and epithelial–mesenchymal interactions enriched in healthy airways but absent in asthmatic airways, **d:** Dot plot depicting selected epithelial–immune and mesenchymal–immune interactions highly enriched in asthmatic airways but absent in healthy airways. Panels **a–d** depict nine healthy individuals and six individuals with asthma, as shown in Fig. 5b.

Analysis of the predicted cell–cell interactions between structural cells in healthy airway wall revealed a wealth of growth factor signalling pathways including the fibroblast growth factor (FGF), epidermal growth factor receptor (EGFR), insulin-like growth factor (IGF), transforming growth factor (TGF), platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF) pathways (Supplementary Table 7). Cell–cell interactions unique to asthma included TH2–epithelial cell contacts (for example, KLRG1 and CD103/E-cadherin; integrin/tenascin-C). Epithelial expression of alarmins and cytokines, such as IL-33, TSLP, and TNFSF10/TRAIL (Fig. 6d), all of which are known to play a role in asthma [38-40], might activate TH2 cells expressing their receptors.

In addition to validating these well-known interactions, which for IL-33 and TSLP failed to reach statistical significance in our unbiased cell–cell interaction analysis, we identified novel epithelial–TH2 cell interactions in asthma: the interactions between epithelial chemokines CXCL2, CXCL17, and their TH2-expressed receptors (Fig. 6d). Interestingly, mesenchymal cells share some of these interactions, such as expression of TNFSF10/TRAIL and MIF. Predicted mesenchymal–TH2 cell interactions in asthma are CXCL12 and CCL11, expressed by fibroblasts and smooth muscle cells. Airway wall TH2 cells in asthma express IL-5 and IL-13 (Fig. 5i), with receptor expression by immune cells and epithelial cells, respectively, in line with the observed IL-13-driven gene signature in mucous ciliated and goblet cells in asthma (Extended Data Fig. 7). In addition to these classical TH2 cytokines, TH2 cells express LTB for which basal epithelial cells express the receptor.

Discussion

We describe the cellular landscape of human lung tissue at the single-cell level, charting differences in frequencies and molecular state of lung structural and inflammatory cells between upper and lower airways and parenchyma. We provide a first detailed molecular description of two separate tissue-resident subsets of $CD4^+$ T cells in airway wall, one of which was hitherto unknown. We also conclusively show the presence of the recently identified [34-36] pathogenic effector TH2 cells in the airway wall in asthma, as evidenced by the combined expression of IL5, IL13, HPGDS, PPARG, and IL17RB.

We identify a novel mucous ciliated cell state in asthmatic airway epithelium that contributes to mucous cell metaplasia. The goblet-like gene expression profile in $FOXJr^+$ cells with a full-blown ciliated cell transcriptional phenotype strongly indicates that this molecular state is induced in ciliated cells by type 2 cytokines. Mucous metaplasia of ciliated cells and goblet cell hyperplasia both contribute to the increase in mucin-producing cells in asthma.

These changes in airway epithelium in asthma differ from those in patients with chronic rhinosinusitis with polyps [24]. In this type 2 inflammatory upper airway disease, IL-4/IL-13-driven gene transcription was observed in basal cells, which were arrested in differentiation and increased in frequency24. In asthma, basal cell numbers are not strongly increased. Instead, we observe expression of the IL-4/IL-13-driven gene signature mainly in goblet and mucous ciliated cells (Extended Data Fig. 7). Hence, chronic type 2 inflammation has divergent effects on the epithelia of the upper versus the lower airways. In contrast, the changes in the eicosanoid pathway observed in chronic type 2 inflammation of the upper24 and lower (Extended Data Fig. 8) airways are very similar, underscoring the presence of common cellular mechanisms between these two anatomical locations.

Finally, comprehensive analysis of the cell–cell interactions in airway wall in asthma identifies dominance of TH2 cells interacting with structural and inflammatory cells. The extensive growth factor signaling between epithelial cells and mesenchymal cells observed in healthy airway wall is largely lost in asthmatic airway wall, at odds with a reactivation of the epithelial–mesenchymal trophic unit [41]. Instead, our data support a

shift in cellular phenotypes in airway wall due to the local production of TH2 cytokines in our patient cohort with mild to moderate childhood-onset asthma. This global view of the airway wall cellular landscape opens up new perspectives on lung biology and molecular mechanisms of asthma.

Methods

Patient recruitment and ethical approval

Bronchoscopy biopsy (10X and SmartSeq2 analysis)

Cohort inclusion criteria for all subjects were: age between 40 and 65 years old and a history of smoking <10 pack-years. For the patients with asthma, inclusion criteria were: age of onset of asthmatic symptoms ≤12 years old, documented history of asthma, use of inhaled corticosteroids with(out) β2-agonists due to respiratory symptoms, and a positive provocation test (that is, PC_{20} methacholine (concentration of methacholine needed to produce a 20% fall in the forced expiratory volume in the first second (FEV_1)) ≤ 8 mg ml–1 with 2 min protocol). For the non-asthmatic controls, the following criteria were essential for inclusion: absent history of asthma, no use of asthma-related medication, a negative provocation test (that is PC_{20} methacholine >8 mg ml, and adenosine 5′-monophosphate >320 mg ml with 2 min protocol), no pulmonary obstruction (that is, FEV_1/forced vital capacity (FVC) ≥ 70%) and absence of lung function impairment (that is FEV_1 ≥ 80% predicted).

Patients with asthma stopped inhaled corticosteroid use six weeks before all tests. All subjects were clinically characterized with pulmonary function and provocation tests, blood samples were drawn, and finally subjects underwent a bronchoscopy under sedation. If a subject developed upper respiratory symptoms, bronchoscopy was postponed for ≥6 weeks.

Fibreoptic bronchoscopy was performed using a standardized protocol during conscious sedation [42]. Six macroscopically adequate bronchial biopsies were collected for this study, located between the third and sixth generation of the right lower and middle lobe. Extracted biopsies were processed directly thereafter, with a maximum of 1 hour delay.

The medical ethics committee of the Groningen University Medical Center Groningen approved the study, and all subjects gave their written informed consent. Detailed patient information is given in Supplementary Table 3.

Lung resection (Drop-seq analysis)

Fresh resected human lung tissue (parenchymal lung and distal airway specimens) was obtained via the CPC BioArchive at the Comprehensive Pneumology Center Munich (CPC-M). In total, we analyzed parenchymal tissue of uninvolved areas of tumor resection material from four patients. All participants gave written informed consent and the study was approved by the local ethics committee of the Ludwig-Maximilians University of Munich.

For transport from the surgeon to the laboratory, lung tissue samples were stored in ice-cold DMEM-F12 media and packed in thermo stable boxes. Tissue was processed with a maximum delay of 2 hours after surgery. On delivery to the lab, tissue samples were assessed visually for qualification for the study.

Donor information is given in Supplementary Table 8.

Lung transplant tissue (10X analysis)

Human lung tissue was obtained from deceased organ donors from whom organs were being retrieved for transplantation. Informed consent for the use of tissue was obtained from the donors' families (REC reference: 15/EE/0152 NRES Committee East of England—Cambridge South).

Fresh tissue from the peripheral parenchyma of the left lower lobe or lower right lobe of the lung was excised within 60 min of circulatory arrest and preserved in University of Wisconsin (UW) organ preservation solution (Belzer UW Cold Storage Solution, Bridge to Life) until processing.

Donor 284C. Gender: male. Age band: 55–60. BMI: 25.83. Cause of death: hypoxic brain damage. Smoking history: smoked 20 per day for 25 yr. Stopped: 08/2000. Respiratory related information: chest X-ray normal on admission. No pleural effusion or pneumothorax. Not diagnosed with asthma, but inhalers for possible seasonal

wheeze. Family report only using inhaler approximately five times per year. No recent peak flow on record last one in 2008 when it was 460, predicted is 611. Time from death to cell lysis: 12 h.

Donor 290B. Gender: female. Age band: 60–65. BMI: 27.77. Cause of death: hypoxic brain damage. Smoking history: smoked 15 per day for 7 yr. Stopped: no details. Respiratory related information: respiratory tests all normal on admission; maintaining own airway. GP notes report acute bronchitis in 1994. Time from death to cell lysis: 2 h 27 min.

Donor 292B. Gender: male. Age band: 55–60. BMI: 27.44. Cause of death: intracranial hemorrhage. Smoking history: smoked 20 per day for 46 yr. Stopped: no details. Respiratory related information: chest X-ray normal on admission, lungs appear clear. Bronchoscopy results show global inflamed mucosa. No other history of respiratory issues. Time from death to cell lysis: 18 h 50 min,

Donor 296C. Gender: female. Age band: 30–35. BMI: 20.9. Cause of death: intracranial hemorrhage. Smoking history: smoked 20 per day for 19 yr. Stopped: no details. Respiratory related information: chest X-ray shows collapsed left lobe on admission due to consolidation. Right lobe looks normal. No history or record of respiratory issues. Time from death to cell lysis: 15 h 30 min.

Donor 298C. Gender: male. Age band: 50–55. BMI: 24. Cause of death: intracranial hemorrhage. Smoking history: not available. Stopped: no details. Respiratory related information: no details. Time from death to cell lysis: 15 h 30 min.

Donor: 302C. Gender: male. Age band: 40–45. BMI: 34.33. Cause of death: known or suspected suicide. Smoking history: smoked 20 per day for 25 yr. Stopped: no details. Respiratory related information: chest X-ray shows reduced volume in right lung due to collapsed right lower lobe on admission. No history or record of respiratory issues. Time from death to cell lysis: 13 h 30 min.

Archived formalin-fixed paraffin-embedded lung blocks

Left-over frozen peripheral lung tissues from six current smokers and four non-smokers who underwent lung resection surgery. These subjects did not have a history of lung

disease, apart from lung cancer for which the patients underwent surgery. Lung tissue samples were taken as distant from the tumor as possible. Thus, any possible effect of the tumor on the lung tissue was minimized. All samples were obtained according to national and local ethical guidelines and the research code of the University Medical Center Groningen. Sample information in given in Supplementary Table 9.

Blood processing

Lithium heparin-anticoagulated whole blood (500 µl) was lysed using an ammonium chloride-potassium solution (155 mM ammonium chloride (NH_4Cl), 10 mM potassium bicarbonate ($KHCO_3$), 0,1 mM EDTA). Cells were centrifuged for 5 min at 4 °C, 550g, after which the cell pellet was washed twice with PBS containing 1% BSA, followed by staining for cell surface markers.

Lung tissue processing

Bronchoscopy biopsy

A single-cell solution was obtained by chopping the biopsies finely using a single edge razor blade. The chopped tissue was then put in a mixture of 1 mg ml^{-1} collagenase D and 0.1 mg ml^{-1} DNase I (Roche) in HBSS (Lonza). This was then placed at 37 °C for 1 h with gentle agitation. The single-cell suspension was forced through a 70 µm nylon cell strainer (Falcon). The suspension was centrifuged at 550g, 4 °C for 5 min and washed once with a PBS containing 1% BSA (Sigma-Aldrich). The single-cell suspensions used for 10x Genomics scRNA-seq analysis were cleared of red blood cells by using a red blood cell lysis buffer (eBioscience) followed by staining for cell surface markers.

Lung tissue resection

For each sample, 1.0–1.5 g of tissue was homogenized by mincing with scissors into smaller pieces (~0.5 mm2 per piece). Before tissue digestion, lung homogenates were cleared from excessive blood by addition of 35 ml of ice-cold PBS, followed by gentle shaking and tissue collection using a 40 µm strainer. The bloody filtrate was discarded. The tissue was transferred into 8 ml of enzyme mix consisting of dispase (50 caseinolytic U ml^{-1}), collagenase (2 mg ml^{-1}), elastase (1 mg ml^{-1}), and DNase (30 µg ml^{-1}) for mild enzymatic digestion for 1 h at 37 °C while shaking. Enzyme activity was inhibited by adding 5 ml of PBS supplemented with 10% FCS. Dissociated cells in suspension were passed through a 70 µm strainer and centrifuged at 300g for 5 min at 4 °C. The cell pellet

was resuspended in 3 ml of red blood cell lysis buffer and incubated at room temperature for 2 min to lyse remaining red blood cells. After incubation, 10 ml of PBS supplemented with 10% FCS was added to the suspension and the mix was centrifuged at 300g for 5 min at 4 °C. The cells were taken up in 1 ml of PBS supplemented with 10% FCS, counted using a Neubauer chamber, and critically assessed for single-cell separation. Dead cells were counted to calculate the overall cell viability, which needed to be above 85% to continue with Drop-seq. Two-hundred and fifty thousand cells were aliquoted in 2.5 ml of PBS supplemented with 0.04% of bovine serum albumin and loaded for Drop-seq at a final concentration of 100 cells µl^{-1}.

Rejected lung transplant

For each sample, 1–2 g of tissue was divided in 5 smaller pieces then transferred to 5 ml eppendorfs containing 1.5 ml 0.5 mg ml^{-1} collagenase D and 0.1 mg ml^{-1} DNase I (Sigma) in RPMI. Samples were then finely minced using scissors. Minced tissue was then transferred to a Petri dish and extra digestion medium added to completely cover the tissue. Samples were incubated 30 min at 37 °C. Cells were then passed up and down through a 16-gauge needle 10 times. Samples were incubated for an additional 15 min at 37 °C. Cells were filtered a 70 µm filter, then spun down for 6 min 1,400 r.p.m. One milliliter of red blood cell lysis (eBioscience) was added to the pellet during 5 min. Cells were resuspended in RPMI^{+} 10%FCS and counted. Dead cells were removed using the Dead Cell Removal Kit (Miltenyi Biotec). In brief, cells were incubated with anti-annexin V beads for 15 min. The cell suspension was then passed through a magnetic column and dead annexin V^{+} cells remained in the column, while live cells were collected. Viability was then estimated via trypan blue. More than 99% of cells were viable.

Flow cytometry

Blood leukocytes were stained with CD4 APC-Cy7, CD3 PerCP Cy5.5, CD8 APC, and CD45RA-PE (eBioscience) for 30 min at 4 °C and washed twice with PBS containing 1% BSA. Propidium iodide was added 5 min before sorting.

Airway wall biopsy single-cell suspensions were stained for 30 min at 4 °C with CD3 PerCP Cy5.5, CD45 BB515, CD4 APC-Cy7 (BD), and CD8 PE and washed twice with PBS containing 1% BSA. Propidium iodide was added 5 min before sorting.

Cell sorting

Lymphocytes were selected in the FCS/SSC plot. These were then selected on single, live cells for blood or single, live, $CD45^+$ for lung. The sorted cells were positive for CD3 and CD4 as shown in Fig. 5a. All cells were sorted in a MoFlo Astrios (Beckman Coulter) using Summit Software (Beckman Coulter).

Immunohistochemical staining

Human lung tissue containing large airways were collected from archival formalin-fixed paraffin-embedded blocks (n = 10, 6 smokers and 4 non-smokers). Serial sections (~4 µm) were cut for immunohistochemistry (IHC) and immunofluorescent (IF) staining.

Serial sections from formalin-fixed paraffin-embedded lung tissue were stained for using standard protocols, with antibodies specified in the figures. Briefly, serial sections were deparaffinized in xylene, rehydrated, and immersed in 10 mM sodium citrate buffer (pH 6.0). Antigen retrieval was performed by boiling the sections in a pressure cooker at 120 °C for 20 min.

IHC and IF staining was performed as described previously [42,43]. For the IHC staining cells were stained with a primary antibody (see below for antibody details) and visualized with diaminobenzidine (DAB, Sigma) solution. For the IF staining, cells were stained with primary antibody. Secondary antibodies conjugated to fluorophores (donkey anti-rabbit-488, donkey anti-mouse-555) were used at a dilution of 1:100. DAPI, dissolved in Dako Fluorescence Mounting Medium (Dako S3023) at a dilution of 1:1,000, was used as a nuclear stain.

Chromium 10X Genomics library and sequencing

Airway biopsy

Single-cell suspensions were manually counted using a haemocytometer and concentration adjusted to a minimum of 300 cells μl^{-1}. Cells were loaded according to standard protocol of the Chromium single-cell 3′ kit to capture between 2,000 and 5,000 cells per chip position. All the following steps were performed according to the standard protocol. Initially, we used one lane of an Illumina Hiseq 4000 per 10x Genomics chip

position. Additional sequencing was performed to obtain coverage of at least mean coverage of 100,000 reads per cell.

Lung transplant

Single-cell suspensions were manually counted using a haemocytometer and concentration adjusted to 1,000 cells μl^{-1}. Cells were loaded according to standard protocol of the Chromium single-cell 3′ kit to capture between 2,000 and 5,000 cells per chip position. All the following steps were performed according to the standard manufacturer protocol. Initially, we used one lane of an Illumina Hiseq 4000 per 10x Genomics chip position. Additional sequencing was performed to obtain coverage of at least mean coverage of 100,000 reads per cell.

Antibody list

A full antibody list is given in Supplementary Table 10.

SmartSeq 2 library preparation and sequencing

Library preparation was performed with minor modifications from the published SmartSeq2 protocol [44]. In short, single cells were flow sorted onto individual wells of 96 or 384 wells containing 4 µl (96 wells) or 1 µl (384 wells) of lysis buffer (0.3% triton plus DNTPs and OligoDT). After sorting, plates were frozen and stored at –80 °C until further processing. PCR with reverse transcription (25 cycles) and Nextera library preparation performed as described in ref. [44].

Drop-seq library preparation and sequencing

Drop-seq experiments were performed largely as described previously [2] with few adaptations during the single-cell library preparation. Briefly, using a microfluidic polydimethylsiloxane device (Nanoshift), single cells (100 μl^{-1}) from the lung cell suspension were co-encapsulated in droplets with barcoded beads (120 μl^{-1}, purchased from ChemGenes) at rates of 4,000 µl h^{-1}. Droplet emulsions were collected for 15 min each before droplet breakage by perfluorooctanol (Sigma-Aldrich). After breakage, beads were collected and the hybridized mRNA transcripts reverse transcribed (Maxima RT, Thermo Fisher). Unused primers were removed by the addition of exonuclease I (New England Biolabs), following which beads were washed, counted, and aliquoted for pre-amplification (2,000 beads per reaction, equals ~100 cells per reaction) with 12 PCR

cycles (primers, chemistry, and cycle conditions identical to those previously described). PCR products were pooled and purified twice by 0.6x clean-up beads (CleanNA). Before tagmentation, cDNA samples were loaded on a DNA High Sensitivity Chip on the 2100 Bioanalyzer (Agilent) to ensure transcript integrity, purity, and amount. For each sample, 1 ng of pre-amplified cDNA from an estimated 1,000 cells was tagmented by Nextera XT (Illumina) with a custom P5 primer (Integrated DNA Technologies). Single-cell libraries were sequenced in a 100 bp paired-end run on the Illumina HiSeq4000 using 0.2 nM denatured sample and 5% PhiX spike-in. For priming of read 1, 0.5 μM Read1CustSeqB (primer sequence: GCCTGTCCGCGGAAGCAGTGGTATCAACGCAGAGTAC) was used.

Bulk transcriptome

Biopsies were fresh frozen in liquid nitrogen and stored at −80 °C. RNA was extracted after a few weeks using a combination of Trizol and the RNeasy MinElute Clean Up kit from Qiagen. RNA was prepared from sequencing using the TruSeq RNA Library Prep Kit v2. Samples were then sequenced inn a Hiseq 4000.

Single-cell RNA sequencing data alignment

For SmartSeq2 raw sequencing data, paired-end reads were mapped to the human genome (GRCh38) using GSNAP with default parameters [45]. Then, uniquely mapped reads were counted using htseq-count (http://www-huber.embl.de/users/anders/HTSeq/). Low-quality cells were filtered out using the outlier detection algorithm in R Scater package based on a cut-off of 2 × median absolute deviation.

10x Genomics raw sequencing data were processed using CellRanger software version 2.0.2 and the 10x human genome GRCh38 1.2.0 release as the reference.

The Drop-seq core computational pipeline was used for processing next generation sequencing reads of the Drop-seq scRNA-seq data, as previously described [2]. Briefly, STAR (version 2.5.2a) was used for mapping [46]. Reads were aligned to the human reference genome hg19 (provided by Drop-seq group, GSE63269).

Bulk transcriptome computational analysis

The bulk samples were aligned using STAR 2.5.1b, using the STAR index from the GRCh38 reference that was used when mapping 10x data, and quantified using HTSeq.

The data were then processed using the Seurat-inspired workflow within Scanpy, adding a number of 'pseudo-bulks' obtained by taking 10x data from donors matching the bulk samples and summing expression across all cells.

Data quality control

General strategy for 10x datasets

Optimal tissue dissociation conditions are cell-type dependent, resulting in a certain degree of cell lysis when working with a mixed tissue sample. This results in substantial background levels of ambient RNA in the single-cell suspension that vary with cell-type composition, so we applied SoupX for background correction (see below). We analyzed each donor sample separately and excluded cells with a number of genes higher than the median + 2 s.d. for that donor. We further excluded cell with high number of unique molecular identifiers (UMIs) and high percentage of mitochondrial reads (see below).

In parallel, we used scrublet (see below) to infer the number of the doublets in the dataset before applying the filters previously described and excluded any remaining cells predicted to be doublets that were still present in the dataset. We normalized and scaled our data (see below), performed clustering (see below), and identified and subset the data into epithelial and non-epithelial cell groups (as shown in Extended Data Figs 1 and 5). After separation between epithelial and non-epithelial, we clustered the cells and performed curated doublet removal (see below) based on known lineage restricted markers.

General strategy for Drop-seq data

We normalized and scaled the data, then performed filtering based on the number of genes and percentage of mitochondrial reads.

General strategy SmartSeq2 data

We normalized and scaled the data, then performed filtering based on the number of genes and percentage of mitochondrial reads. To avoid potential batch effects from the lung digestion protocol, we corrected the gene expression of the CD4 SmartSeq2 dataset using a small subset of genes, the expression of which has been recently shown to be highly responsive to enzymatic digestios [47]: *FOS*, *ZFP36*, *JUN*, *FOSB*, *HSPA1A*, *JUNB*, *EGR1*, *UBC*.

Ambient RNA correction (SoupX)

Different batches can be affected by different levels of ambient RNA. To take this into account, we used the recently developed SoupX method [48]. Briefly, ambient RNA expression is estimated from the empty droplet pool (10 UMI or less). Expression of these genes in each cell is then calculated and compared with their proportion in the ambient RNA profile. Transcripts with a bimodal profile (that is, that characterize specific groups of cells but are also highly abundant in empty droplets) are then grouped based on their function. The contamination fraction derived from the expression of these genes is then used to calculate the fraction of each droplet's expression corresponding to the actual cell. Finally, this fraction and the ambient profiles are subtracted from the real expression values.

UMI and number of genes filtering

10x data (after SoupX correction)

nUMI: minimum 1000/maximum 60000. percent.mito, minimum 0/maximum = 3%.

SmartSeq2 data

nGene: minimum 1000/maximum 4000. percent.mito, minimum 0/maximum = 15%.

Drop-seq data

nGene: minimum 200/maximum 4000. percent.mito, minimum 0/maximum = 20%.

Scrublet

We used Scrublet [49] for unbiased computational doublet inference. Doublets were identified in each 10x sample individually using Scrublet, setting the expected doublet rate to 0.03 and keeping all other parameters at their default values. Cells were excluded when they had a score higher than 0.1 for upper and lower airway samples or higher than 0.05 for parenchyma samples.

Normalization and scaling

Downstream analyses including, normalization, scaling, clustering of cells, and identifying cluster marker genes were performed using the R software package Seuray [50] version 2.1 (https://github.com/satijalab/seurat).

Samples were log normalized and scaled for the number of genes, number of UMIs, and percentage of mitochondrial reads. The epithelial biopsy dataset comparing healthy and asthma was also scaled for *XIST* expression, as we observed some gender specific clusters of cells that shared lineage markers with the other observed clusters.

Curated doublet removal

In addition to the general quality control described above, we combined literature knowledge about cell lineages with computational clustering to identify clusters enriched in potential doublets. The strategy for each dataset is described below.

Lung atlas epithelial dataset

We removed cells with expression level higher than 0.5 for any of the following markers: PTPRC (immune), FCER1G (immune), PDGFRA (fibroblast), or PECAM1 (endothelial) (Fig. 1 and associated Extended Data figures).

Lung atlas non-epithelial dataset

We removed cells with expression level higher than 0.5 for any of the following markers: *EPCAM* (epithelial), *KRT5* (basal), *FOXJ1* (ciliated), or *MUC5AC* (secretory). We then performed first clustering round (7 PCs, resolution 2) and excluded clusters that expressed combinations of the following lineage specific markers: *MARCO* (macrophage), *CCL21* (lymphatic endothelial), *TPSB2* (mast cell), or *CD3D* (T cell). We performed a second clustering round and exclude a cluster formed by cells from one donor that had low expression *TPSB2*, while lacking markers for all other immune lineages (Fig. 2 and associated Extended Data figures).

Asthma biopsy epithelial cells

Owing to the smaller number of cells, in addition to the general quality control metrics, we only performed cluster-based doublet exclusion, without cell filtering. We performed one round of clustering and removed one clusters with high expression of PECAM1 (endothelial marker) (Fig. 3and associated Extended Data figures).

Asthma biopsy non-epithelial cells

In addition to the general quality control metrics, we performed three rounds of clustering where we excluded clusters with high levels of *EPCAM* or *KRT5* expressed

in much higher levels than immune lineage markers (Fig. 4 and associated Extended Data figures).

Dimensionality reduction

We performed principal component analysis (PCA) dimensionality reduction with the highly variable genes as input. We then used the PCs to calculate *t*-distributed stochastic neighbor embedding (t-SNE) for each dataset, using a perplexity value of 50.

Data clustering

We used the function 'FindClusters' from Seurat. In brief, this method uses a shared nearest neighbor (SNN) modularity optimization-based clustering algorithm to identify clusters of cells based on their PCs. Before constructing the SNN graph, this function calculates *k*-nearest neighbors (we used $k = 30$) and then it constructs the SNN graph. The number of PCs used for each clustering round was dataset dependent and they were estimated by the elbow of a PCA scree plot, in combination with manual exploration of the top genes from each PC.

matchSCore

We used matchSCore [51] to quantify the overlap of cell-type marker signatures between experiments, which is based on the Jaccard index. Only marker genes with adjusted *P* value < 0.1 and average log fold change > 1 were considered.

CellPhoneDB

We developed a manually curated repository of ligands, receptors and their interactions called CellPhoneDB (www.cellphonedb.org) [37], integrated with a statistical framework for predicting cell–cell communication networks from single-cell transcriptome data. Briefly, the method infers potential receptor–ligand interactions based on expression of a receptor by one cell type and a ligand by another cell type. Only receptors and ligands expressed in more than 30% of the cells in the specific cluster were considered. To identify the most relevant interactions between cell types, the method prioritizes ligand–receptor interactions that have cell type-specific expression. To this end, pairwise cluster–cluster interaction analyses were performed by randomly permuting the cluster labels of each cell 1,000 times. For each permutation, the total mean of the average receptor expression level of a cluster and the average ligand expression level of

the interacting cluster is calculated, and a null distribution is derived for each receptor–ligand pair in each cluster–cluster interaction. An empirical *P* value is calculated from the proportion of the means which are 'as or more extreme' than the actual mean. For the multi-subunit heteromeric complexes, the member of the complex with the minimum average expression is used for calculating the mean.

Network visualization was done using Cytoscape (version 3.5.1). All the interaction pairs with collagens were removed from the analysis. The networks layout was set to force-directed layout.

Trajectory analysis

Trajectory analysis was performed using Monocle version 2.2.0 (ref. 20). We ordered the cells onto a pseudotime trajectory based on the union of highly variable genes obtained from all cells, as well as those from only healthy or asthmatic donors.

Supervised analyses using GWAS genes

Asthma-associated GWAS gene list was collected using the GWAS Catalog of EMBL-EBI searching for the term asthma (https://www.ebi.ac.uk/gwas/). The list was downloaded on 8 February 2018. We took the genes that are in the top 50 hits of our single-cell differential expression marker list (either epithelial or non-epithelial) and asthma-associated GWAS list (the 'matched' gene list). We then hierarchically clustered the expression matrix of the matched gene list along its rows (genes) and columns (single cells) and represented this as a heat map.

Neuroendocrine cell identification

Neuroendocrine cells were identified by the expression of *CHGA*. Any cell expressing any amount of *CHGA* was classified as a neuroendocrine cell.

OMIM search for lung diseases

We searched the clinical synopses with known molecular basis in the OMIM database for the following terms: 'pulm*' or 'bronchi*' or 'alveol*' or 'surfactant' and retrieved 337 entries. These terms were chosen to minimize the return of genetic conditions causing respiratory insufficiency as a consequence of neuromuscular dysfunction, skeletal dysplasia (small rib cage), or lung segmentation defects arising in early embryogenesis.

These 337 entries were then manually curated to identify those conditions with features affecting the bronchial tree, alveoli, lung parenchyma, and pulmonary vasculature. On manual review, entries containing terms such as 'alveolar ridge' of the jaw and 'pulmonary valve stenosis' and 'pulmonary embolism', but no terms related to primary pulmonary disorders, were excluded from further consideration. Syndromes caused by chromosomal disorder or contiguous gene deletion were excluded.

Cluster specific marker expression were generated by comparing this list to the genes present in the top 50 (per cluster) of our list of differentially expressed genes.

Statistical methods

For 10x samples comparing healthy versus asthma, we used Fisher's exact test corrected for multiple testing with Bonferroni method. Normalized CD4 cluster proportions (percentage of total cells) were analyzed using unpaired multiple *t*-tests assuming no consistent s.d. and corrected for multiple comparison using the Holm–Sidak method. We used a non-parametric two-sided Wilcoxon rank sum test in Seurat to identify differentially expressed genes in all the comparisons discussed.

Data availability

Data requests for raw and analyzed data and materials will fall under two categories. Datasets from healthy live volunteers and live volunteers with asthma will be promptly reviewed by the University of Groningen. Any data and materials that can be shared will be released via a Material Transfer Agreement. These datasets can be found on European Genome–phenome Archive (https://www.ebi.ac.uk/ega/home) EGAS00001001755. Datasets generated from lung resection samples using Drop-seq can be accessed in GSE130148. Datasets generated from deceased donors fall under Open Access Policies of the Human Cell Atlas (https://www.humancellatlas.org for details). This data can be accessed at European Genome–phenome Archive (https://www.ebi.ac.uk/ega/home) EGAS00001002649. Interactive exploration tool: www.lungcellatlas.org.

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Chapter 9

Optical coherence tomography intensity correlates with extra-cellular matrix components in the airway wall

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Introduction

Obstructive pulmonary diseases are characterised by structural airway remodeling, including alterations in the extracellular matrix (ECM) [1]. Studies have shown that the ECM profile differs in asthmatic airways compared to non-asthmatics, with less elastin, and higher abundance of collagen I and fibronectin in asthma [2]. Additionally, increased airway wall collagen deposition is associated with more severe disease in asthma [3]. In COPD, alterations in collagen and elastin affect the mechanical properties of the lung, subsequently decreasing the lung elasticity and contributing to emphysema [1]. Currently, two diagnostic tools are available to assess airway remodeling: (high resolution) computed tomography (HRCT) of the chest and immunohistochemistry in endobronchial biopsies. While bronchial wall thickness and lumen area can be assessed by HRCT, the resolution is not sufficient to assess separate airway wall layers and ECM components leaving the pathophysiology of airway wall remodeling unclear. Biopsies are the gold standard for determining airway remodeling; however, the applicability is limited due to its invasiveness, small sample area and elaborative histology processing.

Optical coherence tomography (OCT) generates high resolution, real-time, near-infrared-based cross-sectional images of the airway wall [4,5], with potential for visualizing airway remodeling and enabling three-dimensional airway wall reconstructions. Several studies found an increased airway wall thickness and decreased lumen area in asthma and COPD patients using OCT [5,6]. Furthermore, OCT imaging was able to identify and quantify mucosal and submucosal airway layers [4]. To the best of our knowledge, no study has linked OCT imaging to ECM protein deposition in the airway wall. We hypothesized that the ECM deposition within the airway wall can be detected using OCT. The aim of this study was to relate the OCT scattering characteristics with ECM deposition in the airway wall.

Methods

Data were acquired as previously described [4]. The local medical ethics committee approved the protocol (NL51605.018.14) and informed consent was obtained. In brief, five patients scheduled for a lobectomy were included. From these five lobectomy specimens, thirteen airways were dissected and marked with needles to match ex-vivo OCT images with 51 histological sections. Ex-vivo OCT imaging was performed immediately after resection, using a C7 Dragonfly catheter from St Jude Medical (St Paul, MN, USA). The OCT images were analysed using Matlab software (Natick MA, USA), which enabled roll-off correction and point spread function as previously described [7]. Three sequential frames were combined to minimize noise. Sheath and lumen segmentation was applied according to Adams *et al.* to minimize the influence of scattering intense components in the lumen [8]. OCT mm^2 areas were calculated using a threshold in light scattering intensity, illustrated in figure 1A. To correct for probe optics and the imperfect sampling of the OCT system in depth, the fixed threshold was adjusted for the distance between the lumen and the airway wall in each axial line: a lower threshold was used in larger distances as compared with a higher threshold in shorter distances. For each calculated OCT area, the median OCT intensity (arbitrary units) was measured.

The histological sections were stained with the following biochemical or antigen stainings: Picosirius Red (TC; total collagen), Masson's Trichrome blue (MT blue; total collagen and bone), anti-collagen A1 antibody (CA1; collagen type 1 A1), Verhoeff's (EL; elastin) and anti-fibronectin antibody (FN; fibronectin). The region of interest (ROI) was defined as the area of the airway wall between the epithelium and the outer border of the desmin-positive smooth muscle. Sections without distortions within the ROI were included. The stained sections for each airway were aligned and colour deconvoluted using ImageJ software [9]. Thereafter, the positive mm^2 area (threshold >150 of 255 max grayscale intensity) and the mean grayscale (0-255: lowest-highest intensity) of the stained airway wall were calculated. Correlations were calculated using the Spearman's rank correlation coefficient.

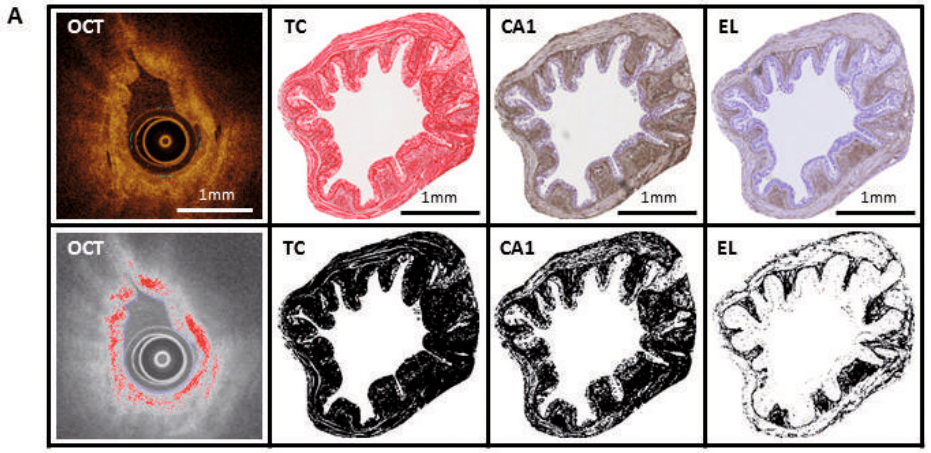


Figure 1: illustration of methods for correlating OCT image with ECM staining sections. **1A:** Three airway wall ECM staining sections aligned to the paired OCT-image, **OCT:** optical coherence tomography image with the area above the threshold (threshold per axial line for the distance between the lumen and the airway wall) in red, **TC:** total collagen, **CA1:** collagen A1, **EL:** elastin, all three stained sections with the area above the threshold (i.e. >150 of 255 grayscale) in black. **1B:** Spearman's correlation of OCT area with TC area in mm², CA1 area in mm² and EL area in mm² respectively. **1C:** Spearman's correlation of OCT intensity with TC mean grayscale, CA1 mean grayscale, and EL mean grayscale respectively.

Results and discussion

A total of 36 from the 51 OCT-histology pairs from the right upper lobe and left lower lobe were analysed. Reasons for exclusion were damaged histology sections (7 pairs), unavailability of histology for additional staining (2 pairs) and when the OCT image was taken at a bifurcation (2 pairs) (supplementary table 1). The mean lumen area of the included sections was 2.38 (±2.06) mm². ECM component stained areas showed a similar spatial pattern as the OCT threshold measured area (figure 1A). Quantification of ECM component stained areas in mm² were significantly positively correlated with the OCT area, while total collagen, MT blue, and collagen A1 mean grayscale correlated with OCT intensity as well (table 1).

Table 1: Spearman's correlations paired OCT-histology areas and intensities

	Optical Coherence Tomography Area (mm²)		Optical Coherence Tomography Intensity (arbitrary unit)	
	R-value	P-value	R-value	P-value
Total collagen area (mm²)	**0.554**	**<0.001**	-	-
Total collagen mean grayscale	-	-	**0.395**	**0.017**
Masson's Trichrome blue area (mm²)	**0.427**	**0.012**	-	-
Masson's Trichrome blue mean grayscale	-	-	**0.466**	**0.005**
Collagen A1 area (mm²)	**0.537**	**0.001**	-	-
Collagen A1 mean grayscale	-	-	**0.427**	**0.010**
Elastin area (mm²)	**0.639**	**<0.001**	-	-
Elastin mean grayscale	-	-	0.254	0.168
Fibronectin area (mm²)	**0.622**	**<0.001**	-	-
Fibronectin mean grayscale	-	-	0.138	0.468

This study shows for the first time that OCT is able to detect and quantify ECM protein deposition in the airway wall. In other research areas focusing on skin and ovarian tissue, an association has been made between collagen deposition and OCT imaging [10,11]. In the airways however, OCT imaging studies have mainly focused on the identification and quantification of the airway wall structure. Intriguingly, elastin and fibronectin area correlated the strongest with OCT area, yet no significance was found between intensity parameters. Further research is required to determine light scattering properties of these ECM components separately.

Our findings that OCT may directly reflect collagen deposition, without the need of extracting endobronchial biopsies, is of specific interest in obstructive lung diseases in which airway remodeling plays an important role. Furthermore, by assessing not only the thickness but also ECM content of the airway wall, it might be possible to monitor treatments targeting airway remodeling in more detail such as bronchial thermoplasty and liquid nitrogen spray.

An achievement of this study is the development of an automated analysis of the OCT image and light scattering intensity areas by threshold and segmentation technique. While in previous studies OCT areas were drawn manually, this study shows that by using a light scattering –based intensity threshold, it is possible to automatically identify and quantify ECM structures. Additionally, by combining this method with innovative polarization sensitive - OCT systems, it may be possible to identify and quantify individual structural components of the airway wall with even greater accuracy [12].

Several limitations need to be addressed. First, in order to make a comparison between OCT and histology we used ex-vivo material. However, by using this approach we were able to assess ECM structures of the entire airway wall in a cross sectional manner, while endobronchial in-vivo biopsies only give superficial mucosal information from one specific location in the airways. Second, we were not able to use all OCT images or histological sections due to damage or artifacts. Despite these, a strong correlation between OCT light scattering areas and ECM stained components within the airways was found.

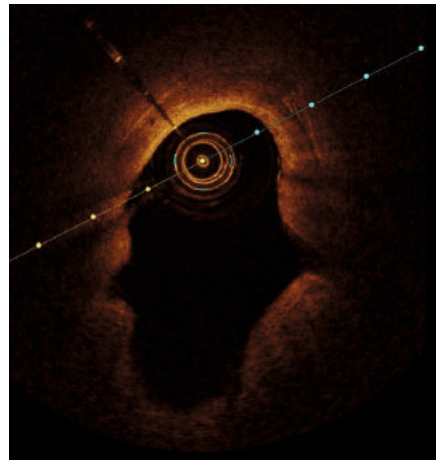
Conclusion

In conclusion, our data shows that increased OCT intensity area locations correspond and correlate with higher collagen, elastin and fibronectin areas in the airway wall. This suggests that it is now possible to directly measure airway remodeling in vivo, in a minimally invasive, real-time manner.

Supplementary table 1: distribution of lung segments and excluded samples

	Segment	**Excluded in the present study**
Patient 1	RB3: 4 pairs	All (4 pairs): no histology available
Patient 2	LB7 anterior subsegment: 2 pairs	
	LB7 posterior subsegment: 2 pairs	All (2 pairs): OCT images taken on a bifurcation
	LB10: 1 pair	
Patient 3	LB10 left subsegment: 3 pairs	1 pair excluded: damaged histology
	LB10 right subsegment: 7 pairs	
Patient 4	LB8 right subsegment: 4 pairs	1 pair excluded: damaged histology
	LB9: 5 pairs	1 pair excluded: no histology available
	LB10: 6 pairs	2 pairs excluded: damaged histology
Patient 5	RB3: 6 pairs	1 pair excluded: damaged histology
	RB2: 3 pairs	1 pair excluded: damaged histology
	RB1 lateral subsegment: 4 pairs	
	RB1 medial subsegment: 4 pairs	2 pairs excluded: damaged histology

A



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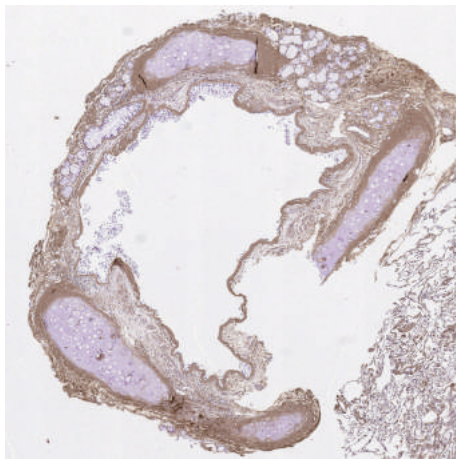

Supplementary figure 1: examples of excluded images and histological sections. **1A:** example of an excluded OCT image taken from a bifurcation, **1B:** example of an excluded damaged histological section.

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Chapter 10

Serum periostin does not reflect type 2-driven inflammation in COPD

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Abstract

Although TH2 driven inflammation is present in COPD, it is not clearly elucidated which COPD patients are affected. Since periostin is associated with TH2 driven inflammation and inhaled corticosteroid (ICS)-response in asthma, it could function as a biomarker in COPD.

The aim of this study was to analyze if serum periostin is elevated in COPD compared to healthy controls, if it is affected by smoking status, if it is linked to inflammatory cell counts in blood, sputum and bronchial biopsies, and if periostin can predict ICS-response in COPD patients.

Serum periostin levels were measured using Elecsys Periostin immunoassay. Correlations between periostin and inflammatory cell count in blood, sputum, and bronchial biopsies were analyzed. Additionally, the correlation between serum periostin levels and treatment responsiveness after 6 and 30 months was assessed using i.e. ΔFEV_1% predicted, ΔCCQ score and ΔRV/TLC ratio.

Forty-five COPD smokers, 25 COPD past-smokers, 22 healthy smokers and 23 healthy never-smokers were included. Linear regression analysis of serum periostin showed positive correlations age (B = 0.02, 95% CI 0.01 - 0.03) and FEV_1 % predicted (B = 0.01, 95% CI 0.01 - 0.02) in healthy smokers, but not in COPD patients

In conclusion, COPD -smokers and -past-smokers have significantly higher periostin levels compared to healthy smokers, yet periostin is not suitable as a biomarker for TH2-driven inflammation or ICS-responsiveness in COPD.

Introduction

Recent research suggests that type 2-driven eosinophilic inflammation is present in a subset of COPD patients [1]. This is important as it may predict responsiveness to anti-inflammatory treatment with inhaled corticosteroids (ICS) and possibly also targeted therapies like interleukin-5 monoclonal antibodies [2].

Periostin is an extracellular matrix protein that has been proposed as biomarker for type 2-driven inflammation [3]. While the majority of studies so far investigated the clinical implication of circulating periostin levels in asthma, data regarding COPD is scarce [3–5].

The aim of this study was to investigate whether serum periostin levels are different in COPD patients compared to healthy controls and whether they are affected by smoking. In addition, we assessed to what extent serum periostin levels reflect inflammatory cell counts in blood, sputum and bronchial biopsies in COPD and whether serum periostin levels predict airway wall remodeling and ICS responsiveness following treatment of 6 or 30 months.

Methods

We included COPD patients who participated in the Groningen and Leiden Universities study of Corticosteroids in Obstructive Lung Disease (GLUCOLD) as described previously [6,7]. Patients were 45–75 years, Caucasian, had an FEV_1/FVC ratio <70%, ≥10 pack years, and no history of asthma. Subjects were randomly assigned to receive long-term ICS with or without an added long-acting beta2-agonist (LABA) or placebo-treatment. Healthy subjects were 40-75 years, Caucasian, had an FEV_1/FVC ratio ≥70% and PC_{20} methacholine >19.6 mg/mL [8], and were divided into smokers (i.e. smoking ≥10 cigarettes/day and ≥10 pack years) and never-smokers.

From the previous studies [6,8], COPD patients and healthy controls underwent the following tests: pulmonary function tests, peripheral blood tests, sputum induction, a bronchoscopy and filled in questionnaires. From the present study, serum periostin levels were measured using the clinical trial version of the Elecsys Periostin immunoassay (Roche Diagnostics, Penzberg Germany) [9]. The local ethics committee approved both study protocols and all subjects gave written informed consent.

First, demographic and clinical variables in COPD patients and healthy controls were compared using independent sample t-tests for normally distributed data, Mann-Whitney U tests for non-normally distributed data and chi-square tests for categorical variables. To assess possible confounders of log2 transformed periostin values, a univariate analysis was performed. Next, a linear regression model was used to assess the association between serum periostin levels and inflammatory cell counts in blood, sputum and bronchial biopsies at baseline, with correction for significant confounders. A linear regression was used to analyze serum periostin levels in association with ICS treatment responsiveness (i.e. change in FEV_1, Clinical COPD Questionnaire (CCQ)-total score and RV/TLC after 6 and 30 months treatment) and airway wall remodelling in COPD patients. Airway wall remodeling in bronchial biopsies was measured by dividing immunohistochemical stained area for elastic fibers, versican, decorin, collagen I and III by the total selected lamina propria area as described previously [7].

Results and discussion

Of the 114 COPD patients enrolled in GLUCOLD, 70 subjects had available measured serum periostin level at baseline. COPD smokers (n=45/70) were 60.3 (SD±7.9) years, smoked 46.7 (SD±19.8) pack years, had a % FEV_1 predicted of 63.8% (SD±7.8%) and a serum periostin level of 51.8ng/ml [IQR 48.4-59.8ng/ml]. COPD former-smokers (n=25/70) had a mean age of 64.7 (SD±7.3), smoked 45.6 (SD±27.7) pack years, % FEV_1 predicted of 60.9% (SD±10.5%) and a serum periostin level of 54.8ng/ml [IQR 47.8-62.2ng/ml]. The healthy smokers (n=22) were 52.1 (SD±7.5) years, smoked 29.0 (SD±11.6) pack years, had a mean % FEV_1 predicted of 104.0% (SD±11.2%) and a serum periostin of 44.6ng/ml [IQR 39.8-51.2ng/ml]. The healthy never-smokers (n=23) had a mean age of 58.4 (SD±9.1), mean FEV_1 % predicted of 108.6% (SD±13.9%) and serum periostin of 49.7ng/ml [IQR 41.8-54.7ng/ml].

Serum periostin was significantly higher in COPD smokers (P=0.009) as well as COPD former-smokers (P=0.001) compared to the healthy smoker-group. Serum periostin was similar between COPD smokers and COPD former-smokers. In agreement with our findings, Golpe *et al.* also found higher significantly periostin levels in both tobacco smoke- as well as biomass cooking-induced COPD compared to healthy controls [10]. However, the latter study only investigated never-smoking controls and did not include matched current- and former-smoking controls. In addition, they used another method to analyse serum periostin and found undetectable levels in the never-smoking controls. Two other studies did not find a difference in serum periostin levels between COPD and predominantly never-smoker controls [4,5].

In our study, healthy never-smokers tended to have higher periostin levels compared to healthy smokers (P=0.08), which was also seen in other studies [11,12]. Caswell-Smith *et al.* saw a significantly higher periostin level in 312 never-smokers compared to 22 healthy smokers [12]. Taking together, there is evidence that current smoking is associated with lower serum periostin levels in healthy controls. It is interesting to note that studies from our laboratories using cultured human bronchial epithelial cells did not provide evidence for induction of epithelial periostin expression by cigarette smoke exposure, an even showed that type 2 cytokine (IL-13) induced periostin expression was suppressed by cigarette smoke [13].

Next, we assessed the correlations between serum periostin levels and clinical and inflammatory characteristics in COPD patients and healthy smokers and never-smokers. Results of the univariate and linear regression analyses are presented in Table 1. In COPD patients, no correlation was found between periostin and age, lung function and inflammatory cell counts in blood, sputum and biopsies. In the healthy smoker-group, periostin levels were significantly positively associated with age (B=0.02, 95%CI 0.01-0.03) and post-bronchodilator FEV_1 % predicted (B=0.01, 95%CI 0.01-0.02). After adjusting the data for the last mentioned possible confounders, no further correlations were found in the healthy smokers. In the healthy never-smoker group, periostin was associated with higher percentages of sputum lymphocytes (B=0.3, 95%CI 0.1-0.5). Our finding is that serum periostin levels do not reflect type 2-driven inflammation in COPD, is in agreement with the findings of Konstantelou *et al.* who measured serum periostin in 155 COPD patients admitted for a COPD exacerbation and found no correlations with severity of airflow obstruction or eosinophilic inflammation measured in blood [14].

To our analysis, baseline periostin levels did not predict ICS responsiveness in COPD; there was no correlation with improvement in lung function, decrease in hyperinflation or CCQ-total score after either 6 or 30 months of ICS-treatment. Studies investigating periostin as biomarker for ICS treatment in COPD patients are limited. In this context, the findings of Park *et al.* are of interest [15]. They studied 130 COPD patients before and after three months of ICS/LABA treatment and found that a combination of high plasma periostin levels (>23ng/mL) and high blood eosinophil counts (>260/µL) could predict a better improvement in FEV_1. However, it is important to note that patients with this combination of high periostin and blood eosinophils already had a higher bronchodilator response at baseline and therefore the better improvement might have been due to the LABA component alone.

Table 1: regression analysis of baseline log2-transformed periostin with baseline characteristics in COPD-patients, healthy smokers and never-smokers

	ICS naive COPD (n = 70)	Healthy smoker (n = 22)	Healthy never-smoker (n = 23)
Univariate regression	**B_{exp} (95% CI)**	**B_{exp} (95% CI)**	**B_{exp} (95% CI)**
Sex, male (%)	4.7 (0.6 – 36.9)	0.2 (0.004 – 11.4)	1.9 (0.1 – 29.7)
Smokers (%)	0.4 (0.1 – 2.0)	NA	NA
Linear regression	**B (95% CI)**	**B (95% CI)**	**B (95% CI)**
Pack years (years)	7.0×10^{-5} (-0.004 – 0.004)	NA	NA
Age (years)	0.01 (-0.003 – 0.2)	0.02 (0.01 – 0.03) *	0.01 (-0.01 – 0.03)
BMI, (kg/m^2)	0.2 (-0.1 – 0.1)	-0.1 (-0.3 – 0.1)	0.04 (-0.1 – 0.2)
% predicted FEV_1 post-bronchodilator	-0.03 (-0.01 – 0.01)	0.01 (0.04 – 0.02) *	0.01 (-0.01 – 0.02)
FEV_1/IVC ratio (%)	0.01 (-0.004 -0.01)	0.0 (-0.02 – 0.02)	0.013 (-0.02 – 0.04)
RV/TLC ratio (%)	0.003 (-0.01 – 0.01)	0.02 (-0.02 – 0.1)	0.02 (-0.01 – 0.04)
Fractional exhaled Nitric Oxide (ppb)	0.001 (-0.01 – 0.01)	NA	NA
Total IgE (IU/L)	4.2×10^{-5} (0.0 – 0.0)	NA	NA
PC_{20} methacholin threshold (mg/ml) #	0.1 (-0.03 – 0.1)	NA	NA
Blood eosinophils (%) #	0.1 (-0.03 – 0.1)	-0.07 (-0.2 – 0.1)	0.03 (-0.2 - 0.2)
Blood basophils (%) #	0.04 (-0.04 – 0.1)	-0.1 (-0.2 – 0.1)	0.1 (-0.1 – 0.2)
Blood neutrophils (%)	-0.001 (-0.01 – 0.01)	-0.004 (-0.02 – 0.01)	-0.02 (-0.04 – 0.01)
Blood monocytes (%)	0.03 (-0.01 – 0.1)	0.01 (-0.1 – 0.1)	0.02 (-0.08 -0.1)
Blood lymphocytes (%)	-0.003 (-0.01 – 0.01)	0.01 (-0.01 -0.02)	0.02 (-0.01 – 0.04)
Sputum eosinophils (%)	-0.01 (-0.1 – 0.04)	-0.01 (-0.2 – 0.1)	0.1 (-0.1 – 0.2)
Sputum neutrophils (%)	-7.7×10^{-6} (-0.01 – 0.01)	0.002 (-0.006 – 0.01)	-0.004 (-0.01 – 0.01)
Sputum macrophages (%)	-0.001 (-0.01 – 0.01)	-0.002 (-0.01 -0.01)	0.001 (-0.01 – 0.01)
Sputum lymphocytes (%)	0.1 (-0.01 - 0.1)	0.108 (-0.2 – 0.4)	0.3 (0.1 – 0.5) *

Table 1: (continued)

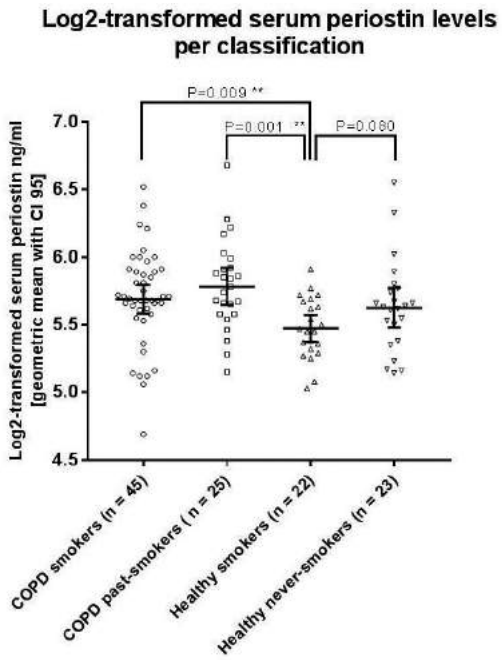
	ICS naive COPD (n = 70)	Healthy smoker (n = 22)	Healthy never-smoker (n = 23)
Biopsy eosinophils (count/0.1mm²)	-0.001 (-0.003 – 0.002)	-0.03 (-0.1 – 0.06)	0.01 (-0.03 - 0.04)
Biopsy neutrophils (count/0.1mm²) #	0.02 (-0.03 – 0.08)	-0.002 (-0.07 – 0.07)	-0.08 (-0.2 – 0.04)
Biopsy macrophages (count/0.1mm²) #	0.045 (-0.03 – 0.1)	-0.03 (-0.1 – 0.1)	0.03 (-0.1 - 0.1)
Biopsy lymphocytes (count/0.1mm²)	0.000 (-0.001 – 0.001)	-0.01 (-0.01 – 0.002)	-0.02 (-0.1 – 0.1)
Biopsy elastic fibers area (%)	0.002 (-0.01 – 0.01)	NA	NA
Biopsy elastic fibers density (gray value)	0.002 (-0.01 – 0.02)	NA	NA
Biopsy versican area (%)	-0.001 (-0.01 – 0.01)	NA	NA
Biopsy versican density (gray value)	-0.01 (-0.03 – 0.01)	NA	NA
Biopsy decorin area (%)	-8.15×10^{-6} (<0.001 – <0.001)	NA	NA
Biopsy decorin density (gray value)	-0.003 (-0.1 – 0.045)	NA	NA
Biopsy collagen I area (%)	-0.003 (-0.01 – 0.01)	NA	NA
Biopsy collagen I density (gray value)	0.003 (-0.02 – 0.03)	NA	NA
Biopsy collagen III area (%)	-0.004 (-0.01 – 0.03)	NA	NA
Biopsy collagen III density (gray value)	-0.01 (-0.02 – 0.01)	NA	NA
Biopsy mean number of ki-67⁺ cells (count/0.1mm²)	0.001 (-0.001 – 0.003)	NA	NA
Biopsy PAS pos. area epithelium (%)	0.000 (-0.01 – 0.01)	NA	NA
Biopsy EGFR pos. epithelium area (%)	0.002 (-0.01 – 0.01)	NA	NA
Biopsy EGFR pos. epithelium density (gray value)	5.8×10^{-6} (<0.001 – <0.001)	NA	NA

*#: log2 transformed variable, *: statistically significant P <0.05,* **BMI***: Body Mass Index.* **NA***: not available, area (%): the percentage stained area for a specific extracellular matrix component was calculated dividing the stained area by the total selected area, density (gray value): staining intensity was analyzed by densitometry (weighted mean per biopsy) and presented as gray value (black: gray value: 0, white: gray value: 255).*

Finally, no correlation was detected between baseline periostin and change in extracellular matrix (lamina propria components stained area or density) after 30 months on ICS or placebo treated COPD patients.

Conclusion

In conclusion, we show that smoking and former-smoking COPD patients have significantly higher serum periostin values compared to healthy smoking controls, yet periostin levels do not reflect type 2-driven inflammation, airway remodeling, or ICS treatment responsiveness and is thus not a good biomarker in this population.



Supplementary figure 1: significant differences in log2-transformed serum periostin levels per study group.

Supplementary table 1: baseline characteristics of COPD smokers, COPD former-smokers, healthy smokers and healthy never-smokers

		COPD smokers (n = 45)	COPD former-smokers (n = 25)	Healthy smokers (n = 22)	Healthy never-smokers (n = 23)
	Sex, male (%)	37 (82.2%)	24 (96.0%)	13 (59.1%)	16 (69.6%)
	Age (years)	60.3 ± 7.9 [a b]	64.7 ± 7.3 [a]	52.1 ± 7.5 [b c]	58.4 ± 9.1 [c]
	BMI (kg/m²)	25.2 ± 4.2	26.4 ± 3.5	24.7 ± 3.2	25.6 ± 4.4
	Pack years (years)	46.7 ± 19.8 [b]	45.6 ± 27.7	29.0 ± 11.6 [b c]	NA [c]
PFT	PC_{20} methacholin (mg/ml) [#]	0.9 [0.2 – 2.3]	0.3 [0.1 – 1.4]	NA	NA
PFT	FEV_1 % predicted (%)	63.8 ± 7.8 [b]	60.9 ± 10.5	104.0 ± 11.2 [b]	108.7 ± 13.9
PFT	FEV_1/IVC ratio (%)	45.2 [40.7 – 53.5] [b]	45.1 [37.8 – 52.2]	73.5 [69.8 – 76.7] [b]	73.4 [70.9 – 76.1]
PFT	RV/TLC ratio (%)	49.3 ± 7.9 [b]	45.6 ± 7.7	30.1 ± 2.7 [b]	31.2 ± 5.4
PFT	FeNO (ppb)	4.8 [3.9 – 8.4] [a]	14.9 [9.3 – 19.6] [a]	NA	NA
Blood	Total IgE (IU/L)	131.1 ± 265.1	164.7 ± 272.8	NA	NA
Blood	Periostin (ng/ml) [#]	51.8 [48.4 – 59.8] [b]	54.8 [47.8 – 62.2]	44.6 [39.8 – 51.2] [b]	49.7 [41.8 – 54.7]
Blood	≥75th %ile periostin (≥55.4 ng/ml) (%)	17 (37.8%) [b]	12 (48.0%)	1 (4.5%) [b]	5 (21.7%)
Blood	Eosinophils (%) [#]	2.2 [1.3 – 3.4]	2.8 [1.4 – 3.9]	2.2 [1.7 – 3.1]	2.2 [1.5 – 3.6]
Blood	Basophils (%) [#]	0.5 [0.3 – 0.7]	0.5 [0.3 – 0.8]	0.4 [0.2 – 0.6] [c]	0.6 [0.4 – 1.0] [c]
Blood	Neutrophils (%)	58.8 ± 7.2	57.5 ± 12.7	57.7 ± 8.8	54.9 ± 6.4
Blood	Monocytes (%)	8.8 ± 2.4	9.1 ± 2.6	8.2 ± 1.9	7.4 ± 1.6
Blood	Lymphocytes (%)	29.2 ± 7.0	29.9 ± 11.3	31.2 ± 7.7	34.3 ± 5.6
Sputum	Eosinophils (%)	1.0 [0.3 – 2.2]	1.3 [0.3 – 2.5]	0.4 [0.2 – 0.9] [c]	0.0 [0.0 – 0.3] [c]
Sputum	Basophils (%)	0.0 [0.0 - 0.0]	0.0 [0.0 – 0.0]	0.0 [0.0 - 0.0]	0.0 [0.0 – 0.0]
Sputum	Neutrophils (%)	66.2 [49.6 – 73.1] [a b]	73.2 [64.1 – 75.4] [a]	50.0 [41.7 – 69.7] [b]	45.7 [34.1 – 60.9]
Sputum	Macrophages (%)	28.2 [21.3 – 39.0] [a b]	22.0 [18.1 – 28.8] [a]	44.3 [26.9 – 55.5] [b]	47.0 [34.6 – 62.0]
Sputum	Lymphocytes (%)	1.7 [1.2 – 2.2] [a b]	2.3 [1.9 – 4.0] [a]	0.4 [0.0 – 0.8] [b]	0.7 [0.3 – 0.9]
Biopsy	Eosinophils (count / 0.1mm²)	1.0 [0.5 – 4.0] [b]	2.0 [0.5 – 5.5]	0.8 [0.0 – 1.5] [b]	0.8 [0.0 – 2.3]
Biopsy	Neutrophils (count / 0.1mm²) [#]	4.0 [1.5 – 7.5]	5.0 [2.0 – 8.8]	1.7 [0.7 – 5.0] [c]	7.1 [3.5 – 11.2] [c]
Biopsy	Macrophages (count / 0.1mm²) [#]	8.5 [4.5 – 12.0]	10.5 [5.3 – 13.3]	4.9 [1.5 – 14.3]	7.1 [2.8 – 12.3]
Biopsy	Lymphocytes (count / 0.1mm²)	109.0 [61.8 – 167.8] [b]	169.5 [79.8 – 220.8]	21.1 [12.7 – 37.5] [b]	30.9 [17.0 – 41.7]

*Data is presented as mean ± standard deviation, median [interquartile range] or dichotomous (%), #: log2 transformed and presented in geometric mean [original IQR], **a**: statistical significance (P <.05) between smoking COPD and ex-smoking COPD group, **b**: statistical significance (P <.05) between smoking COPD and healthy smoker group, **c**: statistical significance (P <.05) between healthy smoker and healthy never-smoker group, **ICS**: inhaled corticosteroids, **BMI**: Body Mass Index, **NA**: not available.*

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Chapter 11

Bronchial gene expression clustering in COPD identifies a subgroup with higher level of bronchial T-cells and accelerated lung function decline

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Submitted

Abstract

Chronic Obstructive Pulmonary Disease (COPD) is usually diagnosed, staged, and monitored by clinical features, while it is known that these parameters poorly reflect underlying pathology. More insight to distinguish pathophysiological mechanisms, so-called endotyping, is needed to predict disease progression in COPD and improve treatment. Linking genome-wide gene expression profiling to disease pathology has the potential to contribute to this endotyping.

The aim of the study was to relate gene expression-based clusters of COPD patients to physiological and histopathological parameters in COPD to identify new endotypes.

An existing COPD-associated gene signature was applied on our bronchial biopsy RNA-sequencing dataset derived from mild-moderate COPD patients, to perform an unsupervised clustering analysis using ConsensusClusterPlus. The gene expression-based clusters of COPD patients were related to cross-sectional clinical and histopathological features, as well as longitudinal lung function decline over 7 years.

We identified two clusters of COPD patients: COPD-associated Airway Gene Expressed 1 (CAGE1)-cluster (n = 39) and CAGE2-cluster (n = 17). CAGE2 was characterized by higher baseline sputum lymphocyte percentage, higher biopsy CD4+ and CD8+ T-cell counts, more rapid lung function decline over 7 years and inhaled corticosteroid unresponsiveness compared to CAGE1. The CAGE2 gene signature was associated with more severe COPD in the validation cohort.

Unsupervised clustering analysis based on an existing COPD gene expression signature enabled the identification of new COPD endotypes with significant differences in bronchial T-cell count and acceleration of lung function decline.

Introduction

Chronic Obstructive Pulmonary Disease (COPD) is primarily diagnosed, staged, and monitored by pulmonary function tests and symptom severity [1], even though these parameters poorly reflect the underlying pathology [2]. Several COPD studies have focussed on identification of new biomarkers, such as blood neutrophils, $CD8^+$ T-cells, IL-6 and genetics [3,4]. However, with the exception of α-1-antitrypsin deficiency and the newly identified rare dominant mutation in protein tyrosine phosphatase non-receptor type 6 (PTPN6) [5], most biomarkers have fallen short of predicting long-term outcome in COPD. More insight in the pathologic changes and identification of so-called endotypes of COPD, i.e. subgroups with distinct pathologic features, is highly needed.

Combining genome-wide gene expression profiling with clinical outcomes and pathological features represents a promising contribution for endotyping of obstructive pulmonary diseases [6]. Several unsupervised gene expression cluster analyses have been performed on sputum samples [7,8] and bronchial biopsies [9,10]. In asthmatics, Baines *et al.* distinguished three different clusters based on gene expression differences in asthma linked to distinct clinical features [8]. Additionally, their sputum-derived gene signature could predict the clinical response to inhaled corticosteroids (ICS). Unsupervised gene expression clustering in COPD patients has been studied less; Chang *et al.* performed unsupervised clustering in 141 COPD patients and 88 smoker controls using blood microarray gene expression data and identified four distinct endotypes [11]. One of the endotypes was associated with severe lung function impairment, respiratory symptoms and CT-scan characterised emphysema, whereas another endotype was characterized by a more preserved lung function and less emphysema. These studies suggest that combining genome-wide gene expression profiling with clinical outcomes and pathological features enables us to identify key COPD endotypes that may help in determining prognosis, treatment response and in the end novel treatments.

Previously we have shown that a 98-gene signature from airway epithelial brushes can distinguish COPD patients from healthy controls [12]. This gene expression-signature was found to be enriched for genes involved in a variety of pathogenic categories, e.g. glycoproteins, proteins involved in acute inflammation (both up-regulated) and

epidermal growth factor-like domains (down-regulated), suggesting an association with inflammatory response and altered epithelial restoration [12].

In the current study, we performed unsupervised clustering using this COPD-associated gene-signature in RNA-seq data obtained from bronchial biopsies of well-characterized COPD patients from the Groningen and Leiden Universities Study of Corticosteroids in Obstructive Lung Disease (GLUCOLD) study. We related these clusters to physiological and histopathological parameters to identify clinically relevant COPD endotypes.

Methods

Patient inclusion

We included subjects who participated in the GLUCOLD study [13]. Briefly, patients were 45 – 75 years old, had an FEV_1/FVC ratio <70%, ≥10 packyears of smoking and no history of asthma. COPD patients were characterised with the following tests, i.e. spirometry, peripheral blood samples (for cell differential), sputum induction (for inflammatory cell percentages) and bronchoscopy with bronchial biopsies, which were immunostained for inflammatory cells (i.e. $CD3^+$, $CD4^+$, CD8+, $CD68^+$, neutrophil elastase, tryptase, $EG2^+$). GLUCOLD was a randomized, double-blind, placebo-controlled study that consisted of four treatment regimens for 30 months, i.e. fluticasone, fluticasone/salmeterol, placebo, or fluticasone the first 6 months followed by placebo for the remaining 24 months of the study. After the end of this 30-month double-blind treatment period, spirometry was repeated once yearly for an additional 5 years (7.5 years in total) [14]. After the 30 month trial, ICS treatment was considered ongoing when a subject used ICS for >50% of the total follow-up period [15]. The local ethics committee approved the study protocol and all subjects gave written informed consent.

Cluster algorithm and statistical analysis

Methods for RNA isolation from bronchial biopsies and RNA-seq were described previously [16]. Processing of RNA libraries and RNA sequencing methods were outlined previously as well [17]. The 98 gene-signature associated with COPD was selected from a previous study [12]. Of the 98 genes in this signature, 93 genes were detected in our baseline RNA-seq dataset. Genes with an expression value of less than one fragment per million reads in all samples were excluded.

The R package ConsensusClusterPlus was used to identify clusters based on the 93 gene-signature list and its algorithm is described by Wilkerson *et al.* [18]. In brief, using this algorithm, a consensus value was calculated per number of clusters, resulting in Cumulative Distribution Functions (CDF). The number of clusters with the lowest CDF value (i.e. the least gene expressional overlap between the clusters) was selected for further analysis. Subsequently, baseline and longitudinal clinical and histopathological features were compared between the clusters, using SPSS 23.0.03 software (SPSS Inc., Chicago, IL, USA). When data was non-normally distributed, a log2 transformation was performed. If these variables remained non-normally distributed, we used the original values. Differences in baseline and longitudinal clinical features between clusters were compared using independent sample T-tests in case of normal distribution, Mann-Whitney U test for non-normally distributed data and chi-square tests for categorical variables. Subsequently, a logistic regression was used to correct for smoking status, age, and sex.

A linear mixed effects model was used to pool treatment arms and analyse annual FEV_1 decline (in millilitres per year). In order to use all subjects for a linear mixed effects model and expel treatment changes during the study as much as possible, the six months' time point was used as baseline, as described previously [15]. This way, a linear mixed effects model was applied between six months and 7.5 years, correcting for smoking status, ICS treatment (i.e. >50% ICS use between 30 months – 7.5 years) and inflammatory cell count as possible confounders.

Differential expression and pathway analysis

To identify genes differentially expressed between the identified clusters whole genome differential gene expression analysis was performed using a linear model correcting for smoking status, gender, and age (R-package Limma version 3.40.6), using a False Discovery Rate (FDR) of <0.05. G-Profiler and Gene Set Enrichment Analysis (GSEA) were used for pathway analysis of genes associated with the different clusters, as described previously [19]. For GSEA, genes were ranked based on their T-value statistic, comparing the number of clusters.

Validation of clusters associated with severity of COPD

To determine if the identified clusters could be replicated, we examined the signature of the differentially expressed genes in a dataset previously published by Steiling *et al.*, consisting of bronchial brushings from ever-smokers with and without COPD enrolled in the British Columbia Lung Health Study (BCLHS), using gene set variation analysis (GSVA) between the clusters in a subset of this dataset [12]. We used gene symbols to match the genes identified by sequencing and affymatrix micro-array. Separate GSVA analyses were performed for the genes that were up- or down-regulated in the comparison between CAGE1 and CAGE2 (t < or > 0), resulting in separate GSVA scores. These two GSVA scores were then projected into the validation dataset and linear models were used to test whether the GSVA scores were associated with baseline and longitudinal lung function tests, correcting for sex, age, packyears, and smoking status.

Results

Of the original 114 randomised COPD patients in the GLUCOLD study at baseline, 89 frozen biopsies were available, and 56 had RNA of sufficient quality for RNA sequencing. All subjects had longitudinal pulmonary function measurements until 30 months of follow-up and 31 individuals had measurements until 7.5 years. The mean duration of the follow-up was 5.7 ±2.6 years.

Based on the existing COPD gene signature, CDF clustering analysis showed that a model with two clusters resulted in the lowest inter-consensus value and therefore these two clusters were selected for further analysis (figure 1). One cluster, termed the COPD-associated Airway Gene Expressed 1 (CAGE1), consisted of 39 COPD patients. The second cluster, termed the COPD-associated Airway Gene Expressed 2 (CAGE2) cluster, was comprised of 17 COPD patients.

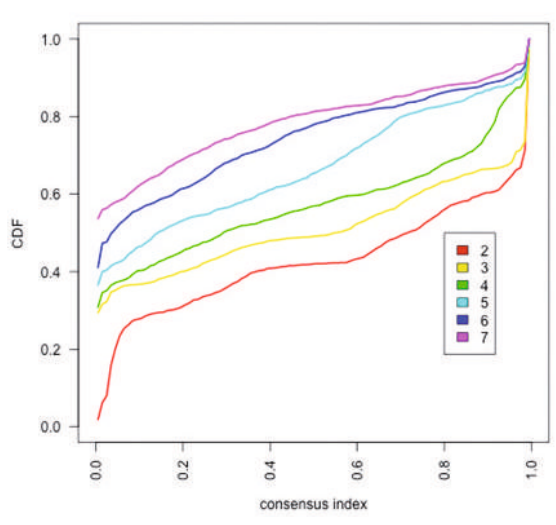


Figure 1: cluster quantity and least inter-cluster consensus. The red line represents the cluster quantity (red is two clusters, yellow is three, green is four, etc.) with the least inter-cluster consensus, which is based on Cumulative Distribution Functions (CDF on y-axis).

CAGE2 is associated with higher lymphocyte levels

Baseline demographics, clinical and histopathological characteristics for CAGE1 and CAGE2 are presented in table 1. There was no significant difference in gender, age, smoking status, pack years and baseline pulmonary function tests between the CAGE1

and CAGE2 clusters. Sputum lymphocyte percentage was higher in CAGE2 compared to CAGE1 (2.2% (IQR 1.8 – 3.5) vs. 1.8% (IQR 1.2 – 2.2), P=0.045, figure 2A), as well as $CD3^+$, $CD4^+$ and $CD8^+$ T-cell counts in biopsies ($CD3^+$: 153.8/0.1 mm^2 (IQR 107.8 – 211.3) vs. 103.4/0.1 mm^2 (IQR 64.0 – 169.5), P=0.01, $CD4^+$: 68/0.1 mm^2 (IQR 57 – 97) vs. 44/0.1 mm^2 (IQR 25 – 76), P=0.02, $CD8^+$: 30/0.1 mm^2 (IQR 20 – 37) vs. 17 /0.1 mm^2 (IQR 8 – 28), P=0.006, figure 2B-D). After correcting for either smoking status or age, both bronchial $CD3^+$ and $CD8^+$ T-cell count remained significantly associated with CAGE2 (P=0.035 and P=0.044, respectively), but significance was lost after correcting for both. Bronchial $CD4^+$ T-cell count was not significantly different between CAGE1 and CAGE2 when correcting for smoking or age.

Table 1: baseline characteristics of COPD patients in CAGE1 and CAGE2

	CAGE1 n = 39	CAGE2 n = 17	p	p^S	p^A	p^{SSA}
Sex, male (n, %)	35 (89.7%)	15 (88.2%)	1.0 #	-	-	-
Mean age in years	61.5 (±7.1)	60.3 (±9.1)	.59 ¶	-	-	-
Mean BMI in kg/m^2	25.2 (±3.1)	25.5 (±4.6)	.83 ¶	-	-	-
Current smoking (n, %)	29 (74.4%)	9 (52.9%)	.13 #	-	-	-
Median pack years	42.0 (31.9 – 55.6)	36.5 (31.5 – 51.9)	.45 §	-	-	-
Reversibility (≥12% and >200ml improvement)	8 (20.5%)	4 (23.5%)	1.0 #	-	-	-
GOLD II to III Classification ratio (n, %)	36 (92.3%)	15 (88.2%)	.63 #	-	-	-
Mean FEV_1 % predicted Post-BD	61.5 (±8.7)	64.9 (±9.0)	.46 ¶	-	-	-
Mean FEV_1/IVC ratio (%)	45.3 (±9.0)	47.9 (±8.9)	.32 ¶	-	-	-
Mean RV/TLC ratio (%)	48.8 (±8.3)	46.9 (±6.6)	.42 ¶	-	-	-
Mean TLCO % predicted	63.9 (±22.6)	63.9 (±14.5)	1.0 ¶	-	-	-
PC_{20} methacholine mg/ml $	0.7 (0.2 – 2.8)	0.5 (0.1 – 1.6)	.46 ¶	-	-	-
PC_{20} methacholine ≤8 mg/ml (n, %)	36 (94.7%)	16 (94.1%)	1.0 #	-	-	-
Median CCQ, total score	1.2 (0.8 – 1.8)	1.4 (0.9 – 1.8)	.75 §	-	-	-
Mean SGRQ, symptom score	31 (±14)	29 (±15)	.68 ¶	-	-	-
Treatment arm: placebo (n, %)	8 (20.5%)	6 (35.3%)				
Treatment arm: fluticasone/salmeterol (n, %)	10 (25.6%)	6 (35.3%)				
Treatment arm: fluticasone ≥0 months (n, %)	9 (23.1%)	4 (23.5%)	.21#	-	-	-
Treatment arm: fluticasone ≥6 months (n, %)	12 (30.8%)	1 (5.9%)				
Treatment arm: placebo (n, %)	8 (20.5%)	6 (35.3%)				

Table 1: (continued)

	CAGE1 n = 39	CAGE2 n = 17	p	p^{S}	p^{A}	p^{SSA}
ICS use until 30 months (n, %)	31 (79.5%)	11 (64.7%)	.32 #	-	-	-
>50% ICS use of the period 2.5-7.5 years (n, %)	9 (29.0%)	2 (15.4%)	.46 #	-	-	-
Mean % eosinophils $	1.9 (1.3 – 3.2)	2.4 (1.2 – 4.4)	.24 ¶	-	-	-
Mean % basophils $	0.5 (0.3 – 0.7)	0.4 (0.3 – 1.1)	.49 ¶	-	-	-
Mean % neutrophils	60.2 (±9.3)	57.7 (±10.1)	.38 ¶	-	-	-
Median % monocytes	8.8 (7.5 – 9.5)	8.3 (7.4 – 10.6)	.99 §	-	-	-
Mean % lymphocytes	28.3 (±7.6)	29.8 (±10.6)	.55 ¶	-	-	-
Mean % eosinophils $	1.1 (0.3 – 2.0)	0.9 (0.3 – 2.5)	.96 ¶	-	-	-
Median % neutrophils	70.0 (59.7 – 81.5)	72.0 (64.0 – 75.0)	.98 §	-	-	-
Median % macrophages	25.2 (15.0 – 34.5)	21.7 (18.5 – 25.7)	.68 §	-	-	-
Median % lymphocytes	1.8 (1.2 – 2.2)	2.2 (1.8 – 3.5)	**.045** §	.176	**.027**	.086
CD3^{+} T-cells, Count / 0.1mm^{2} $	103.4 (64.0 – 169.5)	153.8 (107.8 – 211.3)	**.013** ¶	**.037**	**.022**	.051
CD4^{+} T-cells, Count / 0.1mm^{2} $	44.2 (25.0 – 75.5)	67.4 (56.5 – 96.5)	**.022** ¶	.094	.063	.144
CD8^{+} T-cells, Count / 0.1mm^{2} $	17.0 (8.0 – 28.0)	29.7 (20.3 – 36.5)	**.006** ¶	**.042**	**.040**	.071
NE^{+} neutrophils, Count / 0.1mm^{2} $	3.9 (2.5 – 8.0)	4.6 (3.5 – 6.5)	.55 ¶	-	-	
Tryptase^{+} mast cells, Count / 0.1mm^{2} $	27.2 (20.5 – 37.0)	26.1 (23.0 – 32.8)	.78 ¶	-	-	
CD68^{+} macrophages, Count / 0.1mm^{2} $	8.4 (5.0 – 13.5)	9.8 (9.0 – 13.5)	.49 ¶	-	-	
EG2^{+} eosinophils, Count / 0.1mm^{2}	0.4 (0.3 – 0.7)	0.2 (0.5 – 0.8)	.94 §	-	-	

BMI: body mass index, ICS: inhaled corticosteroids, BD: bronchodilator (salbutamol 400mcg), PC_{20}: substance dosage needed for a 20% FEV_1 drop, CCQ: COPD Control Questionnaire, SGRQ: St. George Respiratory Questionnaire, NE: neutrophil elastase, # : Fisher's Exact Test, ¶ : Independent T-test, § : Mann-Whitney U Test. $: log2 transformed and presented in geometric mean (IQR). S: logistic regression corrected for smoking status, A: logistic regression corrected for age, SSA: logistic regression corrected for sex, smoking status, and age.

CAGE2 is associated with faster lung function decline and ICS unresponsiveness

To investigate the association between CAGE status and the longitudinal decline of lung function, we analysed the change in FEV_1 over 7 years in both groups. Between 6 months and 7.5 year follow-up, patients in the CAGE2 cluster showed a faster decline of FEV_1 compared to patients in CAGE1 (-69.9 ml/year (95% CI -55.7 to -84.0 ml) versus -44.0 ml/year (95% CI -30.3 to -57.7 ml/year), P=0.002), after correcting for smoking status, ICS treatment and biopsy CD4^{+} and CD8^{+} T-cell counts (figure 2E). In addition, CAGE2-patients responded less after 30 months of ICS treatment compared to CAGE1-patients as reflected by the change in $_{11}$ (-29.1ml/year (95% CI -89.2 – 30.9ml) versus 24.4ml/year (95% CI -30.9 – 79.8ml) (P=0.048, corrected for smoking status and, biopsy CD8^{+} T-cell count; P=0.146 when additionally corrected for CD4^{+} T-cell count, figure 2F). In addition, we investigated whether patients with high or low CD4^{+} or CD8^{+} T-cell counts at baseline numbers (i.e. high >50th percentile versus low <50th percentile) showed differences in FEV_1 decline, but found no significant difference (P=0.94 and P=0.92 respectively), corrected for smoking status, supplementary figure 1A). No correlations were found between baseline FEV_1 % predicted with either biopsy CD4^{+} or CD8^{+} T-cell count (Pearson's R=-0.036, P=0.79 and R=0.13, P=0.33 respectively). Finally, since the CD4^{+} and CD8^{+} T-cells were different between clusters, which could be a possible confounder for findings related to lung function decline, we also analysed their direct association, which was not significant (CD4^{+} Spearman's R=0.139, P=0.34 and CD8^{+} R=0.062, P=0.67).

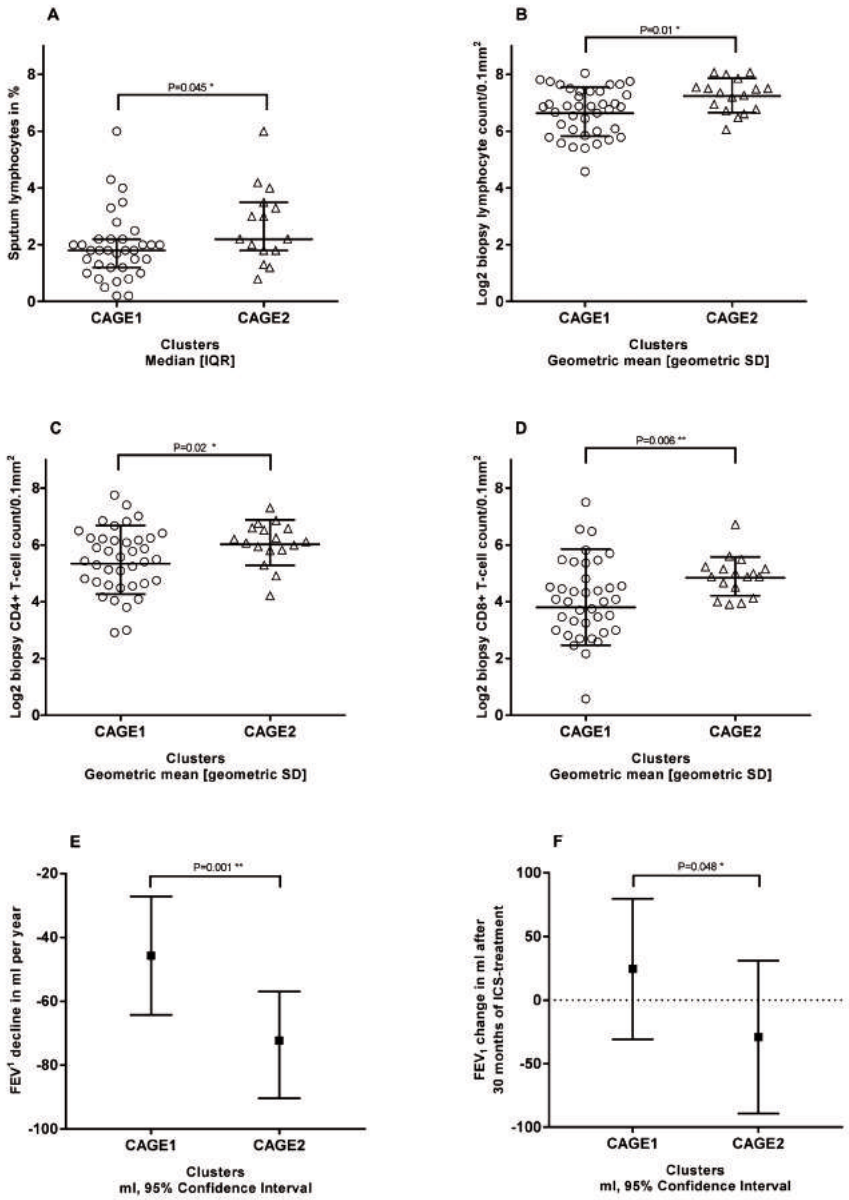


Figure 2: inflammatory level parameters in CAGE1 and CAGE2. **2A:** sputum lymphocyte percentage of total amount of cells per cluster, presented in median with IQR, **2B:** log2-transformed biopsy $CD3^+$ T-cell count in 0.1mm² per cluster, presented in geometric mean and SD, **2C:** log2-transformed baseline $CD4^+$ T-cell count count in 0.1mm² per cluster, presented in geometric mean and SD, **2D:** log2-transformed baseline biopsy $CD8^+$ T-cell count in 0.1mm² per cluster, presented in geometric mean and SD, **2E:** annualized FEV_1 change (ml per year, 95% CI) between 6 months and 7.5 years of follow-up per cluster, corrected for smoking status, ICS treatment and log2-transformed $CD4^+$ and $CD8^+$ T-cell biopsy count variance from geometric mean, **2F:** FEV_1 change in ml (and 95% CI) between 0 and 30 months of ICS treatment per cluster, corrected for smoking status and log2-transformed $CD8^+$ T-cell biopsy count variance from geometric mean.

Gene expression differences between CAGE1 & CAGE2 and pathway analyses

Given that the CAGE1 and CAGE2 patients clusters differed in COPD-related phenotypic variables such as T-cell counts, and lung function decline, we next examined differences in genome-wide gene expression profiles between the 17 patients in CAGE1 and 39 patients in CAGE2. We identified 200 genes that were differentially expressed between the two groups with fold change FC>|2| and FDR<0.05, with 186 higher expressed in CAGE2 as compared to CAGE1, and 14 genes whose expression levels were lower in CAGE2 as compared to CAGE1 (table 2). Pathway analysis using G-profiler on the 186 higher expressed genes showed an enrichment in pro-inflammatory pathways (e.g. antigen binding, humoral immune response mediated by circulating immunoglobin and B-cell mediated immunity, table 3). No pathways were associated with the 14 lower expressed genes.

Table 2: top 20 genes differentially expressed in CAGE2 compared to CAGE1

	Increased	FDR	Decreased	FDR
1.	NXPE2	2.05E-24	SCEL	2.57E-05
2.	LYZ	7.58E-21	CLCA4	8.80E-05
3.	LTF	8.37E-18	CAPN14	2.51E-04
4.	AZGP1	8.81E-22	TGM3	3.24E-04
5.	DMBT1	5.26E-16	NMRAL2P	1.07E-04
6.	ZG16B	5.07E-19	SERPINB2	4.12E-04
7.	MGAM2	8.00E-17	A2ML1	4.24E-04
8.	PPP1R1B	1.19E-18	MUC21	3.88E-04
9.	CRISP3	2.91E-15	HS6ST2	1.46E-06
10.	CA2	5.75E-18	CYP3A5	4.59E-04
11.	PRR4	1.09E-17	SLC7A11	3.27E-04
12.	PIP	7.90E-15	FGFBP1	1.02E-03
13.	CCDC129	2.68E-15	KLK13	1.46E-03
14.	GP2	3.20E-14	LYPD3	1.44E-03
15.	TTYH1	1.27E-14	-	-
16.	LINC02009	1.09E-13	-	-
17.	C6orf58	3.26E-13	-	-
18.	MYCN	8.00E-17	-	-
19.	LPO	9.81E-13	-	-
20.	NPY1R	4.08E-15	-	-

Table 3: clusters gene-expression pathways (G-profiler)

Rank	Term name	Term ID	Term genes (N)	Query genes (N)	Common genes (N)	Corrected P
1.	Antigen binding	Go:0003823	189	164	19	6.07×10^{-12}
2.	Complement activation, classical pathway	Go:0006958	133	164	15	1.08×10^{-9}
3.	Immune response-activating signal transduction	Go:0002757	576	164	27	1.10×10^{-9}
4.	Activation of immune response	Go:0002253	638	164	28	$1,91 \times 10^{-9}$
5.	Immune response-regulating signalling pathway	Go:0002764	605	164	27	3.48×10^{-9}
6.	Humoral immune response mediated by circulating immunoglobin	Go:0002455	144	164	15	3.50×10^{-9}
7.	Immunoglobin mediated immune response	Go:0016064	205	164	17	4.51×10^{-9}
8.	B cell mediated immunity	Go:0019724	207	164	17	5.28×10^{-9}
9.	Positive regulation of immune response	Go:0050778	775	164	30	6.41×10^{-9}
10.	Adaptive immune response	Go:0002250	488	164	24	9.10×10^{-9}

To provide complementary functional assessment of the gene expression signature, GSEA was used to compare the cluster's pathways. Figure 3A illustrates a pathway analysis on the higher expressed genes, including antigen processing and presentation (e.g. antigen processing and presentation, antigen presentation folding assembly and peptide loading of class I MHC) and T-cell pathways (e.g. immunoregulatory interactions between lymphoid and non-lymphoid cells, TCR signalling, phosphorylation of $CD3^{+}$ and TCR ζ chains, interferon γ signalling). Figure 3B presents a pathway analysis of the lower expressed genes, e.g. cell senescence (meiosis, packaging of telomere ends and telomere maintenance) and mitochondrial pathways (citric acid and respiratory electron transport, mitochondrial transcription).

Figure 3: GSEA pathway analysis of increased and decreased genes. **3A:** GSEA pathway analysis of increased and decreased genes, **3B:** GSEA pathway analysis of increased and decreased genes.

Replication of CAGE2 using an independent cohort

Analysis of the BCLHS data was used for replication of CAGE2 in an independent cohort [12]. Baseline demographics of this study are presented in supplementary table 1. In brief, the validation cohort consisted of 87 COPD patients. Thirty subjects were current-, and 57 were former-smokers. The mean age was 65 ±6 years and the mean amount of packyears was 51 ±25. The mean baseline FEV_1 % predicted was 60.3% ±13.8 and follow-up period was four years.

Of the 186 higher and 14 lower expressed genes between the clusters, 143 were expressed in the validation cohort. Of these 143 genes, 140 were higher expressed and 13 were lower expressed in CAGE2 compared to CAGE1. In the COPD patients, we found that a higher expression of CAGE2 associated genes were associated with more severe COPD, i.e. a trend for a lower baseline FEV_1 % predicted (T=-1.70, P=0.09) and lower FEV_1/FVC ratio (T=-2.74, P=7.44 x 10^{-3}) (table 4). GSVA for genes decreased with CAGE2 were not associated with severity of COPD. In addition, GSVA for genes increased and decreased with CAGE2 were not associated with lung function decline in COPD.

Table 4: validation of CAGE2 signature in British Columbia Lung Health Study

	T GSVA of up-regulated genes in CAGE2 vs. CAGE1	P-value	T GSVA of down-regulated genes in CAGE2 vs. CAGE1	P-value
COPD only				
ΔFEV_1 COPD only (ml per year)	-0.35	0.73	0.15	0.88
Baseline FEV_1 % predicted (%)	-1.70	0.09	-1.42	0.16
FEV_1/FVC ratio (%)	**-2.74**	***7.44 x 10^{-3}***	-0.04	0.97

Corrected for sex, age, pack years, and smoking status.

Discussion

In the current study, we identified two COPD subgroups based on bronchial biopsy gene expression data, i.e. CAGE1 and CAGE2. The CAGE2 endotype is characterized by a higher sputum lymphocyte percentage, and higher biopsy $CD4^+$ T-cell and $CD8^+$ T-cell counts, but most importantly, associated with more rapid lung function decline, independent of smoking, ICS treatment and baseline CD8+ T-cell counts when compared to CAGE1. Pathway analysis confirmed that genes who's expression is associated within CAGE2 are involved in T-cell immune responses supporting the known relationship between chronic inflammation and lung function decline in COPD patients; these signatures now allow the discrimination of slow (CAGE1) and rapid (CAGE2) decline in lung function.

We found CAGE2 to be associated with higher $CD4^+$ and $CD8^+$ T-cell numbers in the airway wall compared to CAGE1. Several studies found that bronchial $CD8^+$ T-cells are associated with lung function impairment in COPD [20–22]. Using surgically resected lung tissue, Hogg et al. showed that the percentage of airways containing $CD8^+$ T-cells was higher in the higher COPD stages [22]. $CD8^+$ T-cells play a dominant role in airway inflammation [23–25] and have been associated with increased epithelial apoptosis as is often observed in COPD [26]. Whereas CAGE2 when compared to CAGE1 shows differences in T-cell profile, it is important to note that the association between CAGE2 COPD and more rapid loss of lung function remained significant after adjusting for baseline bronchial $CD8^+$ and $CD4^+$ T-cell counts. Therefore, other pathways defined by the CAGE2 signature are likely to be involved in decline of FEV_1. The latter is supported by the finding that bronchial $CD8^+$ and $CD4^+$ T-cells themselves were not associated with either FEV_1 impairment or decline between 6 months and 7.5 years follow-up.

The CAGE2 endotype may be useful as a biomarker for the following reasons. First, it is associated with greater lung function decline, and could potentially be used to identify COPD patients who are at risk for rapid lung function deterioration. Second, we found that CAGE2 patients show less improvement in FEV_1 after ICS treatment than patients with CAGE1 COPD. Thus, these clusters potentially distinguish between patients who are responsive and unresponsive to ICS therapy, and perhaps other (future) treatments. So far, a limited number of studies have been performed that applied unsupervised

clustering on COPD patients based on gene expression signatures. Chang et al. analysed genome-wide blood gene expression from 229 former smokers in the ECLIPSE study [11], and identified four distinct clinical subtypes of COPD, which were successfully reproduced in an independent sample. The four groups were well differentiated by baseline FEV_1/FVC and FEV_1, and had significant differences in emphysema and symptom severity. Blood sampling would be easier in terms of clinical application and be more patient friendly compared to taking bronchial biopsies. Nevertheless, our current analyses of parameters tested indicate that the baseline identified CAGE2 gene expression cluster is linked to prospective lung function decline rather than associated with existing lung damage, thus yielding more prognostic value. For a biomarker that can predict future risk of rapid lung function loss, a more invasive diagnostic method such as bronchoscopy might be justified as this also is a better representation of the activity of all the cells involved in the disease process, whereas analysis of white blood cells is limited to (off-site) inflammatory cells only.

Amongst the 20 most significantly higher and lower expressed genes in CAGE2 compared to CAGE1, the following were previously found to be associated with obstructive pulmonary disease: lactotransferrin (LTF) and Chromosome 6 Open Reading Frame 58 (C6orf58) were higher, while Cytochrome P450 Family 3 Subfamily A Member 5 (CYP3A5) and Serpin Family B Member 2 (SERPINB2) were lower expressed.

The LTF gene encodes lactotransferrin (also called lactoferrin), a globular glycoprotein which is widely present in secretory body fluids, has both direct and indirect antimicrobial effects [27], and is found to be elevated in non-typeable Haemophilus influenzae and seasonal influenza A virus infections [28]. In virus-exposed human bronchial epithelial cells, pre-treated with concentrations of budesonide, levels of LTF expression were significantly higher [28]. $CD8^+$ T-cell subsets in influenza A infected mice express LTF as well [29].

The function of the C6orf58 gene, apart from being involved in liver development in zebra fish, is unknown [30,31]. Nevertheless, C6orf58 was identified as a differentially expressed protein in sputum supernatant of COPD patients compared to asymptomatic smokers [31].

The CYP3A5 protein is a well-known member of the cytochrome P450 superfamily of enzymes, which metabolize (inhaled) toxicants, such as tobacco smoke, resulting activation or inactivation [32,33]. Therefore, altered expression of CYP3A5 may contribute to the risk of developing lung diseases [32]. While a CYP3A5 gene polymorphism was associated with a faster FEV_1 and FVC decline in current smokers [33], Hukkanen et al. found a decreased CYP3A5 expression level in alveolar macrophages from current-smoking patients with respiratory diseases [32]. Our data suggest that lower expression of the CYP3A5 gene in CAGE2 might be linked to a more rapid lung function decline in COPD.

The SERPINB2 gene enables transcription of the plasminogen activator inhibitor-2, a coagulation factor that is present in most cells, especially in monocytes and macrophages. A negative correlation was found between SERPINB2 expression by PCR in respiratory epithelial cells and FEV_1/FVC ratio, FEV_1 % predicted, and disease severity in asthmatic adults [34]. The high expression of this gene is also included in a signature that enabled identification of the TH2-high, ICS responsive asthma endotype [35]. In line with this, CAGE2 subjects, who have a lower expression of the SERPINB2 gene compared to CAGE1, were unresponsive to ICS treatment.

GSEA pathway analysis of the genes down-regulated in CAGE2 showed enrichment for genes associated with packaging of telomere ends and telomere maintenance, including a number of histone related genes including many members of the HIST1H2 family. There is evidence that COPD is a disease of accelerated lung aging [36]; the progression of the disease and lung function decline is associated to the failure of the lung to repair DNA damage by oxidative stress and from telomere shortening, caused by tobacco smoke [37]. In agreement with this, factors linked to telomere shortening may play a role in the accelerated lung function decline in the CAGE2 subjects.

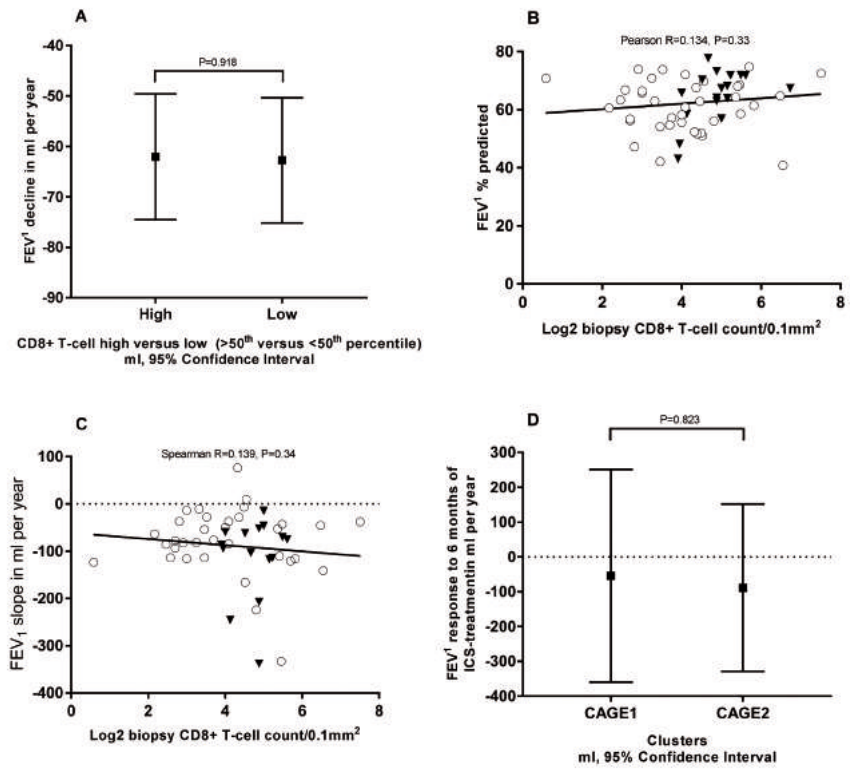
In the validation cohort, we saw a trend of more severe COPD in CAGE2 GSVA compared to CAGE1. However, GSVA for genes increased and decreased with CAGE2 were not associated with lung function decline in COPD. The latter may be due the fact that we used a bronchial biopsy gene signature including a wider variety of (inflammatory) cells and replicated this on a gene set that contains predominantly epithelial cells. Another possible explanation could be that COPD patients with less severe obstruction have

more room to deteriorate and experience more rapid decline expressed in ml per year, as described in previous studies [39,40].

This is the first study to apply an unsupervised clustering method on gene expression data derived from bronchial biopsies in COPD patients. The validation was limited as it is difficult to replicate our findings as there is a lack of studies investigating bronchial gene expression profiles associated with severity of COPD, let alone analysing longitudinal outcomes. We investigated a composite score of genes up- and down-regulated in our CAGE2 signature in the British Columbia Lung Health Study, which were derived from epithelial brushings. Not all genes were present on the array of expressed genes that was used for the validation study, which could be due to the different cell populations represented in bronchial brushings versus biopsies.

Conclusion

In conclusion, our gene expression profile based clustering approach we identified a new COPD endotype, i.e. CAGE2, that is characterized by a higher bronchial $CD8^+$ and $CD4^+$ T-cell level, and most notably, a more rapid lung function decline, compared to the CAGE1 endotype. The identity of genes linked to the expression signatures of the CAGE2 and CAGE1 endotypes confirm underlying immune responses driving COPD, but as such do not explain the difference in lung function decline. These results show that unsupervised clustering analysis based on an existing COPD gene expression signature enables the identification of a new COPD endotype, which in turn could be relevant for diagnosis, staging and treatment of this complex and heterogeneous disease.



Supplementary figure 1: S1A: effect of $CD8^+$ T-cell high/low level (i.e. cut-off at median) on FEV_1 decline per year (95% CI) between 6 months and 7.5 years of follow-up, corrected for smoking status, **S1B:** correlation between baseline FEV_1 % predicted and baseline log2-transformed biopsy $CD8^+$ T-cell count (white circle is CAGE1 and black triangle is CAGE2), **S1C:** correlation between individual FEV_1 ml slope per year between 6 and 7.5 years and baseline log2-transformed biopsy $CD8^+$ T-cell count (white circle is CAGE1 and black triangle is CAGE2), **S1D:** FEV_1 ml change per year (95% CI) between 0 and 6 months of ICS treatment per cluster, corrected for smoking status and log2-transformed $CD8^+$ T-cell biopsy count variance from geometric mean.

Supplementary table 1: baseline demographics of the validation cohort

	BCLHS	GLUCOLD
N	87	56
Mean age	65 ±6	61 ±8
Mean pack years	51 ±25	45 ±18
Current/former smoking ratio (n, %)	30 (34.5%)	38 (67.9%)
Male ratio (n, %)	35 (40.2%)	50 (89.3%)
Inhaled medications (n, %)	23 (26.4%)	Placebo: 14 (25.0%) Fluticasone/salmeterol: 16 (28.6%) Fluticasone 6 months: 13 (23.2%) Fluticasone 30 months: 13 (23.2%)
Baseline FEV_1 % predicted	60.3% ±13.8	62.5% ±8.9
ΔFEV_1 (ml/year)	-40.0 ±50.0	-66.1 [-59.6 - -72.7]
Follow up time (years)	4	5.7 ±2.6

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Chapter 12

Summary

In this thesis, we provide an overview of the pathophysiology of asthma remission and the asthma-obesity syndrome, and present novel techniques for endotyping asthma, pheno- and endotyping remission of asthma, endotyping COPD, and analyzing airway remodeling. The main results of the chapters are summarized below.

In **chapter 2**, an overview of what is known about clinical and complete asthma remission is presented. Despite the fact that the definition of asthma remission is a complex issue and varies greatly between studies, some clinical features have been reproducibly observed to be associated with asthma remission: younger age of onset, mild asthma at onset, male sex, higher baseline lung function, less bronchial hyperresponsiveness at baseline, lower blood eosinophils and IgE at baseline, lower number of allergens with a positive skin prick test, absence of allergic environmental factors (e.g. pets in household), absence of specific comorbidities (i.e. nasal polyps, eczema, atopy or rhinitis), lack of/absence of a history of pneumonia, a negative family history of asthma and atopy, and cessation of smoking. In subjects with asthma remission, the levels of inflammatory markers were lower, especially in complete asthma remission. However, Broekema *et al.* stated that both clinical and complete asthma remission subjects still had a degree of airway remodeling. The most important consideration of this chapter is that the pathophysiological state of complete asthma remission yields more scientific interest than that of clinical remission, since this strict phenotype has higher potential to elucidate biological pathways of the molecular and cellular mechanisms that hold the potential for future therapeutic intervention aimed a inducing asthma remission in patients with persistent asthma.

In **chapter 3** we further elaborate on remission of asthma. By following a cohort of asthmatic children, we were able to calculate the prevalence of clinical and complete asthma remission at age 25 and 49. We showed that this long-time, persistent remission of asthma does occur, but the prevalence was only 11% of the individuals who had childhood-onset asthma. Moreover, persistent asthma remission was more likely in subjects with complete compared to clinical asthma remission at age 25.

In **chapter 4,** we analyzed the differences in amounts of exhaled particles in asthmatics, subjects with clinical- or complete remission, and healthy controls from the *exploring Asthma ReMission by Single-cell TRansciptiONal sequencinG* (ARMSTRONG) study, using the

Particles of Exhaled Air (PExA) device. We hypothesized that the number of exhaled particles is reduced in asthmatics and subjects with clinical asthma remission due to the ongoing small airways dysfunction. Indeed, the mass of exhaled particles in asthmatics proved to be significantly lower compared to healthy controls (P=0.009) and subjects with complete asthma remission (P=0.028). Additionally, subjects with clinical remission had significantly lower exhaled particle mass than healthy individuals (P=0.018). We also correlated the mass of PExA particles in nanogram per liter (ng/L) in exhaled air by the participants with both small- and large airways disease parameters. PExA mass was significantly associated with a variety of airway parameters, such as small airways-associated lung function parameters (i.e. $FEF_{25-75\%}$), lung function reversibility to salbutamol, bronchial hyperresponsiveness (i.e. PC_{20} methacholine and adeno-5-monophosphate slope), small airways resistance (i.e. impulse oscillometry R_5-R_{20} resistance and AX reactance), hyperinflation (i.e. body plethysmography RV % predicted and RV/TLC ratio), and conductive airway nitrogen clearance (i.e. multiple breath nitrogen wash-out S_{cond}). Stepwise multiple regression analysis showed that the small airways disease-associated parameter S_{cond} was the only independent factor associated with PExA mass. These results suggest that better large and small airways function is linked to higher PExA mass.

A comparison of small airways function and inflammatory cell count in healthy subjects, subjects in clinical- and complete asthma remission, and asthmatics is presented in **Chapter 5**. We found evidence that individuals with clinical asthma remission still have a degree of small airways disease and inflammatory markers, while complete asthma remission subjects are clinically similar to healthy controls. Again, we propose that in order to elucidate the pathogenesis of true asthma remission, studying complete asthma remission is the most promising approach. To explore this in detail, further work is needed to establish histological differences such as other remodeling and bronchial inflammatory parameters, and provide a cellular landscape of human lung tissue of clinical and complete asthma remission subjects at the single-cell level.

In **chapter 6,** we evaluate a recently published model predicting more than 80% of the asthma remission cases in young adulthood, by testing its performance in our Dutch asthma remission cohorts. The model from the *Childhood Asthma Management Program* (CAMP) study for predicting asthma remission later in life consisted of the

following characteristics: asthmatic children having no pulmonary obstruction as reflected by FEV_1/FVC% ≥85%, no severe bronchial hyperresponsiveness defined as PC_{20} methacholine ≥1mg/ml, and low blood eosinophils (<500 cells/μL). In concordance with the findings of the CAMP authors, children in our cohorts had a significantly higher FEV_1, FEV_1/IVC%, and PC_{20} threshold and significantly lower blood eosinophils in the asthma remission group compared with the persistent asthma group. Even though the cohorts had similar characteristics, the clinical asthma remission rates of our subjects and CAMP cohort were 10.0% versus 26.1%, while the model predicted future development of asthma remission in 40.0% versus 82.6% respectively. The FEV_1/FVC% measured in the CAMP trial was overall higher than in ours, probably contributing to the discordance. We showed that the proposed model has predictive value in the CAMP cohort, but would be insufficient in our cohort. Therefore, we propose that biomarkers that are associated with pathways inducing complete asthma remission should be incorporated in such models to achieve a better predictive power.

In **Chapter 7** we review the relation between asthma and obesity. There is evidence that obesity increases the risk for asthma development and enhances the difficulty of gaining control of asthma symptoms. Even though the mechanisms underlying the link between asthma and obesity are not well elucidated, we point out several pheno- and endotypical features: first, obesity reduces the expiratory reserve volume (ERV) and slightly increases bronchial hyperresponsiveness. Second, asthma with obesity is associated with a higher neutrophil count than asthma in non-obese patients. Third, obesity is linked to various comorbidities that could aggravate asthma symptoms and which could lead to their persistence. And finally, corticosteroid therapy is less effective in obese asthmatics, which would consequently contribute to the worsening of asthma control. To conclude, this review addressed the relevance and complexity of the asthma-obesity phenotype combination, including current therapeutic strategies to treat patients affected by this syndrome. Future studies should focus on exploring the pathogenic relation of asthma and obesity, in order to find novel treatment options.

In **Chapter 8** we present our exploration of the cellular landscape of the healthy lung, and of the asthmatic airways by single-cell RNA-sequencing (scRNA-Seq). We describe the transcriptomic profile of lung-resident structural and inflammatory cells and their interaction in healthy lung tissues from several tissue sources: nasal brush, bronchial

biopsy and brush, lung resection specimens, and transplant donor lungs. In addition, we profile airway wall biopsies from six patients with asthma and six healthy controls from the ARMSTRONG-study. We identified a tissue-resident $CD4^+$ T-cell subset with transcriptomic features of both (circulating) central memory T cells and tissue-resident memory T cells. Second, by comparing the cellular composition of the airway wall between asthmatics and healthy volunteers, we identify novel epithelial cell states, asthma-associated changes in the composition of the airway wall and predict large changes in cell-cell communication in the airway wall in asthma. Summarized, **chapter 8** generates novel insights into the epithelial cell changes and transcriptomic-defined communication patterns between immune- and structural cells of the airways that underlie asthmatic airway inflammation and remodeling.

In **Chapter 9** we focus on detecting and quantifying airway remodeling using OCT imaging. We paired histological and immunohistochemical stainings on sections of 36 tissue samples from the airways with ex-vivo obtained OCT images of the exact same physical location, derived from five lobectomy specimens. The histological sections were stained with Picosirius Red (total collagen), Masson's Trichrome (blue color: total collagen and bone), anti-collagen A1 antibody (collagen type 1 A1), Verhoeff's (elastin) and anti-fibronectin antibody (fibronectin) to quantify the ECM component area and intensity in the airway wall. All of the ECM component areas were positively correlated with the paired OCT areas, while total collagen, Masson's Trichrome blue (marking e.g. total collagen), and collagen A1 mean intensity correlated with OCT intensity as well.

Chapter 10 assessed the potential of serum periostin, a promising biomarker in the asthma field, to predict disease severity and inhaled corticosteroid (ICS) responsiveness in patients with COPD. Baseline serum periostin was associated with cross-sectional and longitudinal features, including levels of inflammatory cells in three compartments (i.e. blood, sputum and bronchial biopsies) and properties of bronchial extracellular matrix (ECM) components in COPD patients from the *Groningen and Leiden Universities Corticosteroids in Obstructive Lung Disease* (GLUCOLD) study. Smoking and former-smoking COPD patients had significantly higher serum periostin values compared to healthy smoking controls. Nevertheless, the periostin levels did not predict ICS responsiveness in COPD: there was no correlation with improvement in lung function (FEV_1% predicted), decrease in hyperinflation (RV), or COPD Control Questionnaire score after either 6 or

30 months of ICS therapy. Finally, no correlation was found between serum periostin and change in ECM component area or density after 30 months of ICS or placebo treated COPD patients. Overall, we show that measuring serum periostin has little clinical relevance in COPD.

In **Chapter 11**, we demonstrated the use of transcriptional profile clustering of bronchial biopsies to predict long-term clinical outcome of COPD patients, which was originally introduced in asthma research in 2014. COPD- and asthma phenotypes have been frequently associated with specific gene expression signatures. The other way around, associating transcriptional clusters from relevant tissue samples with asthma phenotypes, has been studied less extensively. By using a COPD-associated gene signature from an American bronchial brush cohort, we performed unsupervised clustering of our own COPD cohort. The number of bronchial RNA clusters was determined by an algorithm that calculated the least transcriptional consensus between these clusters. Consequently, the GLUCOLD-enrolled COPD patients were divided into two clusters: COPD Associated Gene Expression #1 (CAGE1) and CAGE2 subjects. The latter group had significantly higher lymphocyte percentage in sputum and T-cells in bronchial biopsies, compared to CAGE1 subjects. But most importantly, CAGE2 subjects had more rapid lung function decline between 0.5 and 7.5 years compared to the CAGE1 cluster. We concluded that this gene expression signature enables us to identify a COPD phenotype with a more rapid lung function decline.

Chapter 13

Discussion and
future perspectives

The discussion and future perspectives of this thesis are subdivided in five themes: asthma remission, small airways dysfunction, the asthma-obesity relationship, airway wall remodeling, and endotyping of COPD. Suggestions for future studies are delineated at the end of the paragraphs. Finally, the discussion and future perspectives are summarized in the conclusion.

13.1 Asthma remission

This thesis summarizes what is known about clinical and complete asthma remission. Apart from additional phenotyping of these phenomena, various chapters emphasize the need for further endotyping. As an example, in **chapter 6** it is shown that the *Childhood Asthma Management Program* (CAMP) prediction model lacks biomarkers that are associated with cellular or molecular pathways linked to the induction of asthma remission, which might contribute to the low predictive power to determine asthma remission in the Dutch cohorts.

To predict and determine persistent remission of asthma, further endotyping is needed. Endotyping of asthma remission can be achieved via multiple strategies: defining endotypes based on structural features, or on molecular features that are collectively referred to as –omics, such as transcriptomics, metabolomics, and proteomics [1,2]. Future approaches to characterize complete asthma remission through various -omic modalities (genomics and microbiomics are highlighted in **chapter 2**), will be discussed below.

Structural endotyping

Exploration of the disease activity by visualizing structure and location of airways affected by asthma can be performed by quantifying high-resolution computed tomography (HR-CT) parameters [3,4], by assessing airway remodeling using optical coherence tomography (OCT) of the airway walls [5], or by analyzing the architecture of airway wall histology sections [6]. Current HR-CT analyses are able to quantify the degree of airtrapping, airway thickness and lumen diameter in asthma patients [7]. One increasingly used method for quantifying HR-CT images is parametric response mapping (PRM) [3,4], which we also tested in **chapter 4 & 5** (PRM-defined small airways disease is discussed in *paragraph 13.2*). In the *exploring Asthma ReMission by Single-cell TRansciptiONal sequencinG* (ARMSTRONG) study, we observed a significantly lower PRM-defined interstitial lung density in subjects with complete asthma remission than in persistent asthmatics. Additionally, these HR-CT analyses revealed a ventilation-shift towards the upper lobe in asthmatics, which was not seen in individuals in complete asthma remission and healthy individuals. Ultimately, automated HR-CT programs could be easily incorporated to quantify asthma activity, absence, or treatment response. The evaluation of OCT imaging linked to airway remodeling is discussed in *paragraph 13.4*.

Transcriptomic endotyping

Gene expression profiling [8–11] allows to create gene signatures that we can subsequently link to a phenotype, as demonstrated in **chapter 11**. Various studies have aimed to define endotypes of asthma by transcriptomics in blood [12], sputum [8,11], bronchial epithelium [10], and bronchial biopsies [13]. However, studies focusing on the genetic background of complete asthma remission are scarce [14,15], and no study to date has reported transcriptomic profiling of lung tissue samples in complete remission patients. Unfortunately, complete asthma remission is a unique phenotype representing only a small proportion of all asthmatics [16,17], thereby limiting the possibilities for recruiting the numbers of participants needed for large transcriptomic studies required to realize this kind of endotyping.

In **chapter 8**, we demonstrate a novel technique in transcriptomic endotyping, so-called single-cell RNA sequencing (scRNA-seq) [18,19]. scRNA-Seq has a major advantage compared to previously described genome wide transcriptomic profiling on complex tissue samples such as airway wall biopsies: it enables identification of transcriptionally divergent cell subsets within a certain cell type. We index-sorted blood samples and bronchial biopsies of persistent asthmatics and healthy controls to obtain a 200 to 300 $CD4^+$ T-cells per donor, and performed single-cell transcriptomic profiling by SmartSeq2 on these cells [20]. The second method, the *10X Genomics Chromium* microfluidics platform, can be used to process larger numbers of cells, allowing us to analyze 2,000 to 5,000 single cells from a suspension of all cells contained in the bronchial biopsy [18]. In contrast to the FACS-sorted $CD4^+$ T cells analyzed by SmartSeq2 scRNA-Seq, the cell types in 10X Genomics scRNA-Seq datasets are primarily categorized by their transcriptomic profile. With both SmartSeq2 and 10X, we had the ability to define cell

types through unsupervised clustering based on transcriptome similarity [21,22]. In the near future, we will implement these and novel scRNA-seq methods on blood samples, bronchial and nasal brushes, and bronchial biopsies of subjects with clinical or complete asthma remission in an attempt to characterize the biology of the airway wall structural cells in asthma remission patients.

Several issues need to be resolved in order to make scRNA-Seq more accessible for clinical practice. First, due to the high dimensionality of transcriptional data of so many cells, it is very difficult to conveniently visualize molecularly and clinically relevant cell types [21]. Future research should focus on lifting "the curse of dimensionality"[23], by introducing new analysis methods that integrate biologically linked –omics. Second, scRNA-Seq needs to become cheaper than the current €3,600 to €8,900 per sample [24]. By becoming less expensive, more institutes will invest in scRNA-Seq projects, and more samples can be processed to increase statistical power and scientific impact. And last, a challenge for scRNA-Seq studies is generating a high-quality single-cell suspension, which needs to be from fresh tissue free from both RNA degradation and transcriptional stress responses [25]. A promising technique to tackle these technical demands, is to analyze the samples via single-nucleus instead of single-cell RNA-sequencing. For future studies, we could perform single-nucleus RNA-sequencing on our extensively characterized ARMSTRONG dataset and extend the subjects numbers by adding frozen biopsy samples of older asthma datasets as well. To realize the full potential of these novel technologies also in a clinical setting, the international *Human Cell Atlas* consortium aims to establish optimized workflows and analysis pipelines, share best practices and establish a reference dataset describing all cell types, states and their interactions in all organs of the healthy human body [26].

Epigenomic endotyping

Epigenetic factors (e.g. microRNAs and DNA methylation) are expected to have a significant impact on the cellular and molecular interactions driving asthma [27]. Epigenetic signatures from tissue samples integrate information from genetic makeup, cell type composition and programming due to environmental factors accumulated during the life course, thereby holding great promises as potential biomarkers for disease progression or therapy response. In concordance with this, epigenetic factors may very well be relevant for understanding complete asthma remission. Boudewijn *et al.* searched for underlying mechanisms of asthma remission by investigating bronchial microRNA expression [28], and found that complete asthma remission had a distinct bronchial microRNA expression profile compared to persistent asthmatics and healthy participants. They found differential expression of microRNAs such as *mir-320d*, which is associated with anti-inflammatory effects. By understanding how microRNAs like *mir-320d* interact with protein-coding RNAs that are responsible for these anti-inflammatory effects, it would bring us one step closer to revealing the pathways driving the induction of asthma remission.

Proteomic endotyping

Analysis of the proteins which mediate the pathogenesis of asthma, can be applied on various samples, such as sputum [29,30], bronchoalveolar lavage fluid [31–33], bronchial biopsies [34], but also particles in exhaled air [35]. Analyses in exhaled air have shown that albumin, and surfactant proteins can be collected by the (Particles of Exhaled Air) PExA device and analyzed using ELISA [35–37]. Proteins like surfactant, an immunomodulator involved in innate immune recognition and regulation of surface tension [33], could be produced insufficiently or in an altered form thereby affecting asthma severity [33]. For instance, Soares *et al.* demonstrated that topological data analysis of albumin and surfactant was able to non-invasively identify a small airways disease phenotype in asthma [35]. One important limitation that needs to be addressed when implementing this approach to endotype complete asthma remission is the difference between the number of exhaled particles in diseased and healthy, which is thought to be elicited by blockage of the smaller airways in the asthmatics (see *paragraph 13.2*). Future studies should focus on the discovery of exhaled, aberrant proteins linked to asthma, absence of which could be associated with complete asthma remission.

Metabolomic endotyping

Analyzing biochemical molecules derived from metabolic processes in a disease [1,38], is a strategic approach called metabolic endotyping. Profiling of exhaled biochemical molecules is a concept that is applied in the electronic nose, called "breathprints" [39,40]. Since the electronic nose is able to differentiate healthy controls from asthmatics [41], it is plausible that the exhaled breathprints of complete asthma remission subjects are different from persistent asthmatics and clinical asthma remission subjects as well. Future studies should explore this approach, since it is non-invasive, cheap, and

has potential to work as an early biomarker, for instance of those subjects that could discontinue medication.

13.2 Small airways dysfunction

Abnormalities in small airways, defined as the airways with an internal diameter of less than 2 mm, contribute to the clinical expression of asthma [42]: their dysfunction is associated with reduced asthma control, higher number of exacerbations, more severe bronchial hyperresponsiveness, and exercise-induced asthma [42,43]. Two research questions are addressed in this thesis with regard to small airways: one, whether abnormalities in the mass of exhaled particles reflects small airways function. Two, whether there is persistent small airway dysfunction in clinical- and complete asthma remission subjects.

Various tests are available to assess small airways disease [44,45], and we propose measuring exhaled particles with the PExA device as a novel technique. In **chapter 4**, we correlated the amount of PExA particles in nanogram per liter, exhaled by the participants with both small- and large airways disease parameters. Apart from Soares *et al.*, who found a positive correlation between the PExA mass per exhalation and R_5-R_{20} airway resistance (R=0.257, P<0.05) [35], no other studies investigated associations between PExA mass and small airways dysfunction. We find… All in all, our data suggest that the total exhaled particle mass could potentially be used as a tool to assess small airways dysfunction. However, there are some limitations to this technique. First, the PExA device does not distinguish the origin of exhaled particles, and does not reveal the location of airway blockage that causes the observed decrease in PExA mass. Figure 2 illustrates the hypothesis of the origin of PExA particles [46]. Basically, the concept is that the exhaled particles are generated whenever the airway re-opens an area of aerosol-producing airway tract. In line herewith, it is plausible that the number of small, but also that of large airways is negatively correlated with the exhaled particle mass, since both would result in a smaller lining tract area. Even though age, length and gender were not associated with exhaled PExA mass, we cannot rule out that obstruction of the larger airway areas has more effect on the measured PExA particles that of the smaller airways. Other factors could have effect on the exhaled particle mass, such as season

[47], airway wall remodeling (including airway wall elasticity), comorbidity, and many other factors.

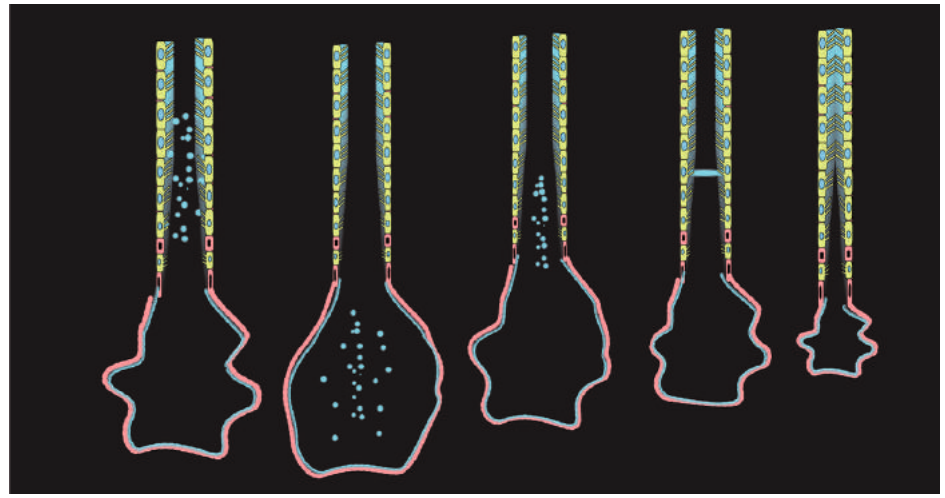

Figure 2: Schematic illustration of the airway reopening concept. When airways close, opposing airway walls get in contact creating a plug of respiratory tract lining fluid. As the airway walls distend during inspiration, forming a meniscus that finally breaks and generate particles. Figure of Bake B, et al. Respir Res. 2019 Jan 11;20(1):8 is distributed under the terms of the Creative Commons Attribution 4.0 International License.

Second, differences in breathing manoeuvres may affect the mass of exhaled particles. Morawski *et al.* studied exhaled particle concentrations and size distributions in healthy volunteers, while performing different types of breathing manoeuvres, such as whispering, voiced expiration, and coughing [48]. In agreement with a previous study [49], their data confirmed that air velocity and vibration of vocal cords affect the mass of exhaled particles. Hence, the individual airflow limitation of each patient or the instructions provided to the patient during measurement could alter the mass of PExA particles.

Even when taking these limitations into account, it is likely that measuring the exhaled PExA mass relates to both large and small airways function. As already highlighted in *paragraph 13.1*, future PExA studies should focus on proteomics and – if possible – transcriptomics in exhaled air.

Our main findings in **chapter 5** were that subjects with complete asthma remission had significantly lower inflammatory markers, and better large and small airways function. Moreover, complete asthma remission and healthy controls were indistinguishable on these parameters. In our opinion, these findings demonstrate that complete asthma remission is the closest to the healthy condition, meaning that in a sense these patients were spontaneously cured, while clinical asthma remission is similar to the definition "sub-clinical asthma". Steinbacher *et al.* investigated the multiple breath nitrogen washout (MBNW) parameters, that are thought to reflect small airway function, in children who were into asthma remission for more than one year, and either normoresponsive or hyperresponsive to cold air challenge [50]. In the hyperresponsive children, there was a significant increase and subsequent decrease in the MBNW parameters after cold air challenge, and salbutamol inhalation, respectively. This was not the case in normoresponsive children. The MBNW response illustrates the presence of small airways dysfunction in subjects with asthma remission who still appear to react on exogenous triggers. This is in concordance to our findings, since clinical asthma remission subjects, practically all having a positive provocation test (i.e. exogenous trigger), had higher MBNW S_{cond} and S_{cond} and worse small airways function.

There is one important limitation in the assessment of small airways dysfunction in clinical and complete asthma remission: it is challenging to evaluate the history of asthma severity, let alone the history of small airways disease. All individuals with asthma remission described in **chapter 4 & 5**, had a diagnosis of asthma, confirmed by documented spirometry and provocation tests before the age of 20. Again, remission subjects had early-onset asthma, and were 40 – 65 year at enrollment for the ARMSTRONG study. It is a delicate process to find the diagnoses in old charts, because these documents are frequently stored remotely or even destroyed due to rules and regulations. The limited availability of medical tests and statuses hindered the estimation of asthma severity at onset. Additionally, tests to assess small airways function were not performed at the time of diagnosis of these asthma patients. Therefore, we can only assume that individuals that are into complete asthma remission nowadays had a significant degree of small airways disease at asthma-onset.

As already put forward, there is no gold standard to determine small airway dysfunction. Future studies will need to introduce novel or improve current methods to do so, e.g.

by visualizing airtrapping, calibrating localized airway resistance, or measuring small airways-specific metabolites and gases. An example of an upcoming technique is the quantification of functional small airways disease reflected by HR-CT PRM (PRM ^{fsad}) [4] (figure 3).

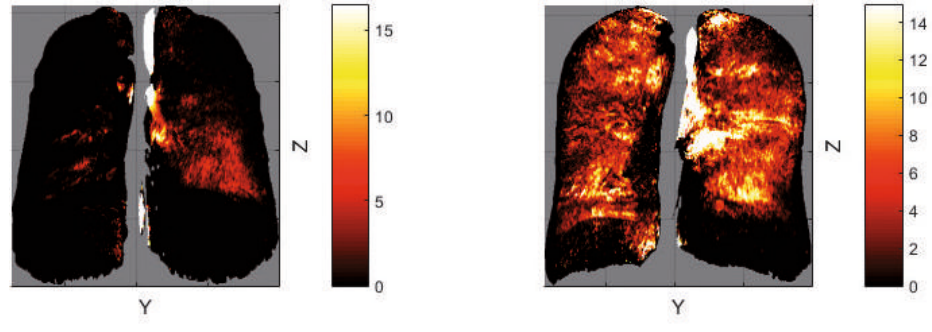

Figure 3: examples of parametric response mapping functional small airways disease (PRM ^{fsad}) quantified by inspiration-expiration HR-CT. Left pair of lungs are from a subject with complete asthma remission, right pair from an asthmatic. Degree of color intensity resembles percentage of PRM ^{fsad}.

Unlike our study, a larger study found a significantly higher PRM ^{fsad} in asthmatics than healthy controls [51]. Within asthma, a higher PRM ^{fsad} was associated with an eosinophil high (>300 cells per µl) group, while a lower PRM ^{fsad} was associated with low eosinophil counts (<150 cells) [52]. In our own cohort of healthy never- and former-smokers, more PRM ^{fsad} was found to be associated with higher RV/TLC- and lower $FEF_{25-75\%}$/FVC ratios [53], indicating early pulmonary alterations in lungs without subjects having symptoms. Applying the PRM algorithms to HR-CTs should be easily feasible.

In contrast to HR-CT, thoracic magnetic resonance imaging (MRI) has historically played a minor part in respiratory medicine, because of inferior spatial and temporal resolution of lung tissue compared to HR-CT [54,55]. HR-CT excels in determining lung morphology but relies on indirect signs in images of obstructive pulmonary disease [55]. Therefore, functional MRI combining with inhaled hyperpolarized noble gases such as helium-3 and xenon-129 has been put forward as a potential method to integrate lung morphology with small airways function and gas transfer within the lungs [54–57]. It would be of interest to associate hyperpolarized MRI parameters with asthma disease severity levels, and correlate it with small airways parameters in healthy and asthmatics.

13.3 Asthma-obesity relationship

The asthma-obesity relationship is a poorly understood, and the phenotype is difficult-to-treat. A major issue in obese asthmatics is the over-use of corticosteroids [58]. A distinct endotypical process presumably determines this phenotype, making it less responsive to ICS. Patients are therefore more likely to receiveimmunosuppressants. To fight the combined chronic condition, one intervention attracts attention as an effective treatment: bariatric surgery [59–61]. In addition to improvement of asthma control, bariatric surgery has been shown to improve small and large airway function and bronchial hyperresponsiveness. Although bariatric surgery is considered to be a potent intervention to treat obesity (and related comorbidities) and reduce mortality [62], post-operative complications are well known and common [63]. The overall complications rate is around 17% with a re-operation rate of 7% [64]. The 30-day rate of death among patients who underwent gastric bypass is around 0.3% [65]. But when successful, it has major beneficial consequences for the patient [62]. Thus, future improvements of bariatric surgery, decreasing the complication rate, would be of interest for patients with severe asthma and morbid obesity that are eligible for this type of intervention.

It is well-known that the early- and late-onset asthma phenotypes have distinct clinical and genetic features [66]. In line with this, it is conceivable that this also applies specifically to obese astmatics [59,67]. For instance, childhood-onset obesity with late-onset asthma might well have a distinct pathogenesis from late-onset obesity with childhood-onset asthma. The findings of the *Severe Asthma Research Program* are in accordance with this idea, since the authors found that obese subjects with early-onset asthma had more airway obstruction, bronchial hyperresponsiveness, and more oral steroid use per year, or intensive care unit admissions due to asthma, compared to their obese peers with late-onset asthma [68]. These results highlight the need to understand the interaction between both chronic conditions, in order to develop tailored treatment regimens. As a speculation, patients with early-onset asthma and late-onset obesity might be more responsive to weight reduction, while subjects with asthma and obesity since childhood might be less susceptible, due to their chronic exposition to the metabolic syndrome or oxidative stress.

Either way, treating overweight in asthma is relevant to control the latter condition; the metabolic syndrome is thought to up-regulate pathways contributing to asthma [69], via inflammatory cytokines such as adipokines [70,71]. Future therapy to resolve the asthma-obesity syndrome will presumably focus on pathways decreasing leptin [72], increasing adiponectin [73], and on further development of bariatric surgery.

13.4 Airway wall remodeling

Obstructive pulmonary diseases are characterized by structural airway remodeling, including alterations in the extracellular matrix (ECM) [6,74]. To date, two diagnostic tools are used to assess airway remodeling: HR-CT of the chest, and immunohistochemistry in bronchial biopsies [5,75], both having their drawbacks. A novel technique, OCT, generates infrared-based cross-sectional images of the airway wall [76], with potential to visualize airway remodeling. Some studies investigating OCT imaging have quantified collagen deposition, a parameter of airway wall remodeling, in skin [77], ovarian tissue [78], and primary lung tumors [5]. However, no study has linked OCT images to other ECM components within a healthy airway wall. Exploring the potential of OCT to analyze airway remodeling is of interest for at least two reasons: first, by using remodeling parameters (e.g. localized or overall airway wall infrared intensity area), early stages of pulmonary obstructive conditions could be detected [79]. And second, airway wall remodeling could be correlated more directly to effects of treatment [80].

In **chapter 9,** we demonstrated the use of OCT imaging to analyze airway wall remodeling. To our knowledge, this is the first study demonstrating that OCT imaging enables us to detect and quantify ECM collagen deposition in the airway wall, without the need of extracting and processing bronchial biopsies. By measuring the thickness [5,79], but also the ECM content of the airway wall, we hope to further explore the potential of OCT to analyze airway remodeling in asthma and COPD. Between 2016 and 2019, OCT imaging has been performed in several airway branches of never-smoking and former-smoking healthy controls, persistent asthmatics, past-smoking GOLD I or II COPD patients, subjects with clinical-, and complete asthma remission. Together with OCT airway wall images of severe asthmatics, collected by colleagues from the departments of Respiratory Medicine and Biomedical Engineering & Physics,

Amsterdam University Medical Center, we hope to compare the OCT images of asthma- and COPD severity groups with healthy controls. With this data, we can analyze whether there is a degree of airway remodeling in individuals with asthma, clinical- and complete asthma remission, and whether remodeling has ceased in complete asthma remission subjects. Additionally, we can relate the OCT-defined airway remodeling parameters with the degree of fixed airflow obstruction in asthmatics, and with scRNA-seq inflammatory cell types and proportions. These future projects would increase our knowledge on the molecular mechanisms behind airway wall remodeling, which would in turn give insight on how to reverse it.

13.5 Endotyping COPD

There is consensus that asthma and COPD share features [81], such as epidemiological and clinical characteristics. The theory of pathophysiological overlap, is known as the "Dutch hypothesis", proposed by Orie and colleagues in 1961 [82]. In line with this, it is of interest to test biomarkers and transcriptional profiles that are used in asthma research, on COPD patients and explore their clinical relevance.

We tested the clinical relevance of serum periostin in COPD patients in **chapter 10**, yet this biomarker did not reflect TH2-driven inflammation, airway remodeling, longitudinal FEV_1 decline, and inhaled corticosteroid (ICS) treatment responsiveness. We found two other studies focusing on the use/application of periostin in COPD patients and its predictive value for treatment response [83,84]. Even though these studies linked serum periostin to clinical outcome, their design or outcome restricted their persuasiveness: Park *et al.* studied 130 COPD patients before and after three months of ICS/long-acting β agonist treatment and found that a combination of high periostin level and blood eosinophil count was able to predict FEV_1 improvement. However, patients with high periostin and blood eosinophils who received this therapy, already had a higher bronchodilator response at baseline [84]. Therefore, the FEV_1 improvement might have been due to long-acting β agonists in patients with documented reversibility alone. The second study measured serum periostin in COPD patients admitted for a COPD exacerbation, as well as differences in death during follow-up, exacerbation-rate after discharge, or hospitalizations between low and high periostin (i.e. <25 ng/ml and

≥25 ng/ml respectively) [83]. Based on the current knowledge, it is unlikely that serum periostin will be integrated in future COPD clinical decision-making.

In **chapter 11,** we demonstrated that transcriptional profile (i.e. COPD Associated Gene Expression #1 (CAGE1) and CAGE2) was able to identify clusters of COPD patients that are distinguished on clinical features. Patients categorized into the CAGE2 cluster had significantly higher lymphocyte percentage in sputum and T-cells in bronchial biopsies, less ICS responsiveness, and more rapid lung function decline compared to CAGE1 COPD patients. Generating and implementing a gene signature such as CAGE2, may be useful as a biomarker for lung function decline and response to ICS. Blood or sputum sampling would be more convenient and more patient friendly compared to extracting bronchial biopsies. Nevertheless, if the CAGE2 signature truly predicts rapid lung function decline, a more invasive diagnostic method such as bronchoscopy could be justified when externally validated in a prospective study. Ultimately, harmonizing the multiple levels of –omics is key towards the development of biomarkers for personalized prognosis and treatment response.

13.6 Conclusion

The chapters presented in this thesis provide an overview of what is known about asthma remission, further characterize of complete asthma remission, elaborate on the asthma-obesity complexity, apply cutting edge techniques to endotype asthma and COPD as well as novel devices to analyze airway remodeling and small airways dysfunction.

Various conclusions have been made:

I. In order to elucidate the pathophysiological state of asthma remission, future studies should focus on complete asthma remission, since this phenomenon is likely to yield superior prognostic and scientific impact. This is of interest, since elucidation of the pathophysiology of asthma remission could potentially lead to new treatment options for asthma.
II. To clearly predict asthma remission later in life, we need to integrate biomarkers with clinical features at asthma-onset.
III. Measuring particles of exhaled air correlates with large, and indirectly, small airways parameters, in asthmatics, clinical-, complete asthma remission subjects, and healthy controls.
IV. Transcriptomic bronchial cell typing (e.g. single-cell RNA-sequencing) characterizes the landscape of lung-resident structural and inflammatory cells and their interactions, enabling us to identify differences in proportions and transcriptional output of cells between asthmatics and healthy.
V. Optical coherence tomography enables us to quantify extracellular matrix components in the airway wall, such as collagen. This now allows for future studies 'in vivo' to explore the clinical characteristics and the underlying pathobiology related to airway remodeling in asthma and asthma remission.
VI. The asthma-obesity syndrome is a common combination of diseases with its own distinct pathophysiological processes.
VII. There will presumably be no room for serum periostin in COPD clinical decision-making.
VIII. Transcriptomic profiling can be implemented as a biomarker for COPD patient prognosis.

Unavoidably, aforementioned conclusions lead to new questions and recommendations for future studies. These recommendations include:

I. To expand our knowledge on asthma remission by implementing single-cell RNA-sequencing on blood samples, bronchial- and nasal brushes, and bronchial biopsies of subjects with clinical and complete asthma remission, while comparing to asthmatics and healthy controls.
II. To test single-nucleus RNA-sequencing in the *ARMSTRONG* study. This method enables sequencing of frozen biopsy samples of former datasets, consequently extending the number of subjects.
III. To analyze exhaled, aberrant proteins linked to asthma, instead of merely counting exhaled particles.
IV. To compare metabolomic breathprints of various asthma severities.
V. To introduce novel methods in small airways disease-phenotypes in asthma, which enable visualization of airtrapping and gas exchange, such as functional MRI.
VI. To study the effects of leptin and adiponectin in the asthma-obesity syndrome. Specifying the eligibility for bariatric surgery in patients with severe asthma and morbid obesity, in order to treat this phenotype more safely.
VII. To analyze the presence of airway remodeling – defined by optical coherence tomography and histological parameters - in complete and clinical asthma remission, compared to asthmatics and healthy controls.
VIII. To correlate optical coherence tomography-defined airway wall remodeling parameters with both fixed airway obstruction and single-cell RNA sequencing inflammatory cell types or proportions.

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Chapter 14

Nederlandse samenvatting

14.1 Astma

Astma is een veel voorkomende, chronische longziekte. In Nederland zijn ruim 640.000 mensen die astma hebben. Van deze groep zijn ongeveer 100.000 kinderen. Astma kenmerkt zich door klachten van kortademigheid, hoesten en piepen, welke kunnen fluctueren in ernst en duur. Bij astmatici zijn de longen overgevoelig voor externe prikkels, zoals rook, parfum, koude lucht, huisstofmijt, huisdieren of iets anders. De gevoeligheid uit zich op verschillende manieren: de slijmvliezen kunnen meer vocht en slijm gaan produceren, de spieren rondom te luchtwegen kunnen meer verkrampen en samentrekken, en ontstekingsvocht kan in de luchtwegwand ophopen. Al deze factoren maken de luchtwegen nauwer, wat er voor zorgt dat een astmapatiënt minder lucht kan verplaatsen bij het ademhalen en benauwd wordt. Als dit plotseling zeer heftig opkomt, noemt men dat een astma-aanval.

Omdat de klachten van astma vaak wisselen van ernst, is het stellen van de diagnose niet altijd gemakkelijk. De arts redeneert op basis van het klachtenpatroon of de klachten bij astma kunnen passen, waarna een longfunctietest wordt gepland. Middels deze blaastest wordt bepaald of de desbetreffende persoon, ten opzichte van zijn of haar lengte-, geslacht-, en leeftijdgenoten, voldoende liters lucht uitblaast. De blaastest is in goede periodes (met weinig klachten) vaak normaal en dus niet altijd conclusief waardoor er vaak herhaalde metingen nodig zijn. Ook kunnen andere aanvullende onderzoeken helpen bij het stellen van de diagnose, zoals bloedonderzoeken, allergie- en provocatietests.

Er wordt onderscheid gemaakt tussen verschillende soorten astma, zoals allergisch astma, niet-allergisch astma, inspanningsastma, astma met obesitas, en ernstig astma. Onderzoekers en artsen hebben deze astmasoorten zo gelabeld zodat ze astmapatiënten beter kunnen behandelen; de ene soort astma reageert bijvoorbeeld makkelijker op een luchtwegverruimer, dan de andere.

De oorzaak van astma is niet altijd te achterhalen. Onderzoek heeft uitgewezen dat astma vaak erfelijk is, d.w.z. dat kinderen van astmatische ouders een grotere kans hebben op het ontwikkelen van astma. Daarnaast is er ook een grotere kans op het verkrijgen van de diagnose astma als iemand allergieën of eczeem heeft. Andere oorzaken voor het

ontwikkelen van astma zijn: het hebben gehad van een rokende moeder gedurende de zwangerschap, vroeg geboren zijn, of veel longontstekingen hebben gehad als kind, of het werken met bepaalde stoffen die schadelijk zijn voor de longen.

De ernst van astma wordt bepaald door hoe vaak iemand zijn of haar medicijnen moet nemen, hoe vaak iemand piept of 's nachts wakker wordt vanwege de astmaklachten, in welke mate men beperkt wordt door de ziekte in zijn of haar dagelijkse activiteiten, en hoe vaak iemand vanwege astma naar de spoedeisende hulp moet of zelfs in het ziekenhuis opgenomen moet worden. De longfunctietest wordt meegenomen bij het inschatten van de ernst, maar een goede of slechte longfunctie betekent niet per se dat een astmaticus respectievelijk weinig of veel klachten hoeft te hebben.

Er is een breed scala aan behandelingen voor astma. De belangrijkste medicijnen zijn inhalatiecorticosteroïden. Deze puffers - ook wel inhalatoren genoemd - remmen de voor astma karakteristieke ontsteking van de luchtwegen, wat leidt tot een afname van kortademigheid, astma-aanvallen, en gevoeligheid voor externe prikkels, en tot een verbeterde kwaliteit van leven. Daarnaast bestaan er ook luchtwegverwijders die de spieren rondom de luchtwegen tijdelijk verslappen, waardoor de luchtwegen weer wijder worden. Vaak worden de genoemde inhalatiecorticosteroïden en luchtwegverwijders gecombineerd in één puffer. Naast puffers bestaan er ook allergiepillen, ontstekingsremmers in tabletvorm, en verschillende ingrepen die de astmaklachten kunnen verminderen, afhankelijk van welk type astma de patiënt heeft.

14.2 Astma remissie

Astma is tot op heden niet te genezen; de huidige behandelingen verminderen de ernst van symptomen of verlagen de kans op een astma-aanval. Er bestaat echter wel een kleine kans dat men "over astma heen groeit". Dit fenomeen heet astma remissie. Patiënten met astma remissie hebben geen luchtwegklachten meer, en hoeven ook geen inhalatiemedicijnen meer te gebruiken. Astma remissie kan worden opgedeeld in twee groepen: ten eerste is er klinische astma remissie, waarbij het individu geen astmaklachten, geen astmamedicijnen, maar nog wel longfunctieafwijkingen heeft. Daarnaast is er een groep ex-patiënten met complete astma remissie, waarbij zij ook geen longfunctieafwijkingen meer hebben. Simpel gezegd is in de laatste groep de

diagnose astma niet meer te stellen met de gebruikelijke testen, terwijl deze in het verleden wel is gesteld. Het is interessant om te onderzoeken welke biologische factoren leiden tot het ontstaan van astma remissie, omdat dit in de toekomst kan leiden tot betere voorspelling van het beloop van de ziekte en tot het ontwikkelen van nieuwe astmamedicijnen.

Hoofdstuk 2 is een overzicht van wat er bekend is over klinische en complete astma remissie. Vele studies hebben onderzoek gedaan naar factoren die geassocieerd zijn met astma remissie. Er worden in de diverse studies echter verschillende definities gebruikt, en er doen maar weinig instituten onderzoek naar complete astma remissie. Ondanks de variatie aan definities, zijn de kenmerken van de patiënten die geassocieerd zijn met astma remissie vrij consistent. Verschillende onderzoeken wijzen uit dat astmapatiënten een grotere kans op astma remissie hebben wanneer zij op jongere leeftijd astma hebben gekregen, milde klachten of minder gevoelige longen hadden rond het ontstaan van de ziekte, wanneer zij man zijn, geen ernstige longontstekingen hebben gehad, en geen vader en moeder hebben met astma of allergie. De studies die ook nog een onderscheid maakten tussen klinische en complete astma remissie zagen dat patiënten met complete astma remissie lagere ontstekingswaarden hadden (op het moment dat de diagnose astma werd gesteld) ten opzichte van personen met klinische astma remissie of met astma. Ook wijzen sommige studies erop dat aanleg een rol speelt bij complete astma remissie.

In **hoofdstuk 3** hebben we onderzoek gedaan naar patiënten die op kinderleeftijd astma kregen. Op hun 25e en 49e hebben we gekeken of er sprake was van klinische of complete astma remissie. Daarnaast hebben we onderzocht of verschillende kenmerken op kinderleeftijd voorspellend waren voor klinische of complete remissie op latere leeftijd. Uit deze studie bleek dat slechts 11% van de toenmalige kinderen in astma remissie was op 49-jarige leeftijd. De grootste groep van deze personen had reeds complete astma remissie op 25-jarige leeftijd. Verschillende factoren op kinderleeftijd waren voorspellend voor astma remissie in de volwassen jaren: het krijgen van meer dan zes maanden borstvoeding van de moeder, het hebben van een niet-astmatische of niet-allergische moeder, en intrigerend genoeg, het hebben van een familielid die gediagnosticeerd was met leukemie. De link tussen leukemie en astma is eerder beschreven in de literatuur. Maar wat de precieze relatie is, is tot op heden onbekend.

In **hoofdstuk 6** gebruiken we een astma remissie voorspellingsalgoritme dat gepubliceerd was door een andere onderzoeksgroep in november 2018. Dit algoritme of model hebben we toegepast op twee Nederlandse studies bestaande uit kinderen met astma die gevolgd zijn tot jongvolwassen leeftijd, om te achterhalen wie wel of niet over het astma heen zijn gegroeid. Ondanks het feit dat het model enige voorspellende waarde in onze resultaten bleek te hebben (40% juist inschatten van astma remissie op latere leeftijd), laat dit hoofdstuk zien dat het gebruik van enkel klinische kenmerken tekortschiet voor het opstellen van zo'n voorspellend model. Derhalve illustreert dit hoofdstuk een tekortkoming van de kennis over het ontstaan van astma remissie. In toekomstig onderzoek willen we op zoek gaan naar een biomarker voor astma remissie. Een bio(-logische) marker is een meetbare indicator van een biologische toestand of conditie. Als voorbeeld; prostaatspecifiek antigeen (PSA) is een biomarker voor de prostaatgrootte en snelle veranderingen in deze biomarker kunnen wijzen op prostaatkanker. Zo hopen we in de toekomst de klinische kenmerken en biomarkers van het astmatische kind te integreren in één model, waarmee we met (meer) zekerheid uitspraken kunnen doen over het verloop van de ziekte en het hebben van astmaklachten in de toekomst.

Samenvattend, complete astma remissie is op prognostisch en wetenschappelijk vlak relevanter dan klinische astma remissie, en iemand met complete remissie lijkt wat betreft de klinische beschrijving erg op iemand die nooit astma heeft gehad. Om te achterhalen waardoor mensen over astma heen kunnen groeien, moet men fundamenteel onderzoek doen naar personen met complete astma remissie. Zulk onderzoek is het meest waardevol wanneer het de beschrijving van de klinische kenmerken ontstijgt, zoals in studies naar genetische en moleculaire kenmerken van complete astma remissie. Wellicht leidt het daaruit voortvloeiende biologisch inzicht tot een nieuwe behandeling van astma gericht op genezing van de ziekte.

14.3 Remodeling van de luchtwegen

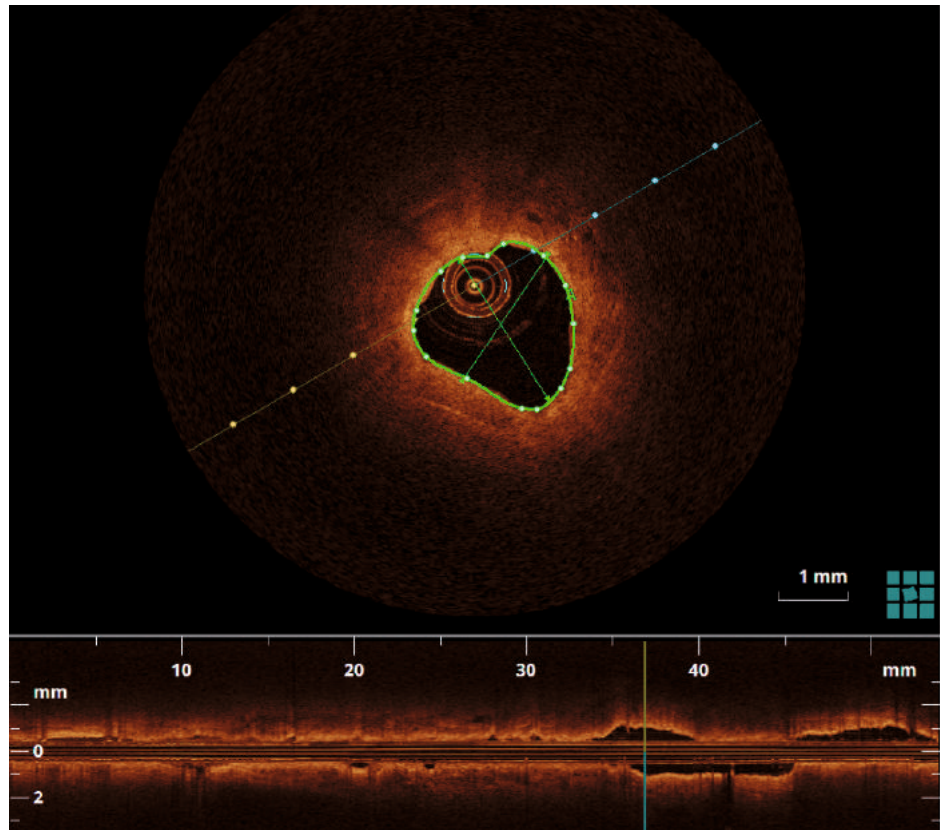
Bij mensen met astma of COPD kan de chronische ontsteking blijvende, structurele veranderingen in de longen veroorzaken. Deze ontstekingen hebben de nodige gevolgen voor de luchtwegen; chronische invasie van witte bloedcellen in de luchtwegwand, verdikking van de luchtwegwand, al of niet met bindweefselvorming, nieuwe

vaatvorming, veranderingen van bedekkend en onderliggend steunweefsel, en toename en groei van het gladde spierweefsel. Een term die al deze structurele veranderingen omvat is “remodelering van de luchtwegen”. Het afweersysteem speelt een belangrijke rol in het ontstaan en instandhouding van deze remodelering, maar het is nog grotendeels onduidelijk hoe dat precies in zijn werk gaat, en of en hoe dit is terug te draaien. Puffers met ontstekingsremmers hebben enig effect op de remodeling, maar hebben geen curerend effect.

Er is momenteel maar één zekere manier om remodelering vast te stellen: middels het beoordelen van een biopt uit de luchtwegen, welke verkregen wordt via een bronchoscopie. Een bronchoscopie is een kijkonderzoek in de longen, waarmee kleine hapjes van het longweefsel (van 1 à 2 mm) kunnen worden afgenomen. Om de mate van remodelering te bepalen, moeten deze biopten vervolgens verwerkt, gekleurd en beoordeeld worden door een patholoog. De nadelen van biopten zijn de volgende: het is een invasief onderzoek, het beoordelingsproces kost veel tijd, en de uitslag van een klein biopt wordt gegeneraliseerd als geldend voor de hele long. Er is dus behoefte aan betere manieren om remodelring van de luchtwegen te meten. Een techniek die gebruikt wordt om aderverkalking in het hart vast te stellen, kan een oplossing bieden: OCT (Optical Coherence Tomography) maakt gebruik van een kleine infrarood sensor, die ingebracht kan worden in de luchtwegen via de bronchoscoop, om vervolgens beelden te maken van de luchtwegen. Het grote voordeel van OCT is dat dwars- en lengtedoorsnede (tot 5cm) van de luchtwegen kunnen worden gemaakt, die bovdendien binnen 10 seconden na het verwerven van de beelden al kunnen worden geanalyseerd.

De interpretatie van OCT-beelden van de luchtwegen is op dit moment nog niet goed ontwikkeld, waardoor men nog niet met zekerheid kan stellen welke structurele veranderingen in de luchtwegwand gevisualiseerd wordt door de infraroodbeelden. Eerder onderzoek met OCT op de huid toonde aan dat een toename van structuurweefsel zorgde voor een toename van infrarood signaal. Onze hypothese is dat dit ook voor de longen zal gelden. In **hoofdstuk 9** legden we ons toe op het testen van bovenstaande hypothese. Hier hebben wij samengewerkt aan een project van het Amsterdam UMC waar OCT beelden op precies dezelfde plek zijn gemaakt als de plaats waar vervolgens weefsel is afgenomen voor het verkrijgen van een dwarsdoorsnede van de luchtwegwand.

De afgenomen weefselblokjes zijn vervolgens gekleurd om verschillende structuren in de luchtwegwand te kunnen onderscheiden, zoals het bindweefseleiwit collageen.



Figuur 1: voorbeeld van een dwars- (boven) en lengtedoorsnede (onder) OCT beeld. Gele kleur staat gelijk aan infrarood intensiteit. Doorsnee beeld is een coupe van een luchtwegwand in de rechter onderkwab.

In zo’n kleuring wordt collageen rood, waardoor dit luchtwegwandbestandsdeel te kwantificeren is in oppervlakte en intensiteit. Met een computerprogramma werden ook de waarden bepaald voor de wandoppervlakte en -intensiteit van de OCT beelden. Door deze getallen te koppelen, zagen we dat een toename van weefseloppervlakte en -intensiteit van het collageen eiwit, overeenkwam met een toename van de wandoppervlakte en –intensiteit op de OCT. Met andere woorden, de OCT beelden leken inderdaad collageen te meten, wat een van de eiwitten is betrokken bij remodelering van de luchtwegwand. In de toekomst willen wij de OCT beelden van astmapatiënten,

astmapatienten in remissie, en gezonde deelnemers analyseren, om zo te achterhalen of er enige remodelering plaatsvindt bij personen die over astma heen zijn gegroeid.

14.4 Kleine luchtwegziekte

Lucht die mensen inademen gaat via de luchtpijp naar de longen, en via de grote luchtwegen die steeds verder vertakken, naar de kleinere luchtwegen, en komt zo uiteindelijk in de longblaasjes terecht. In de longblaasjes vindt gaswisseling van zuurstof en afvalstoffen plaats via een netwerk van kleine bloedvaatjes die om de longblaasjes heen liggen. Als je alle longblaasjes in de longen van een gezond persoon zou uitklappen en naast elkaar zou leggen, zou de totale oppervlakte zo'n 70 tot 100m^2 beslaan. Zoals in de vorige paragraaf is besproken kan bij astma en COPD remodelering van de luchtwegen plaatsvinden, waardoor het transport van de ingeademde lucht in de luchtweg afneemt. Dit heeft in het bijzonder consequenties voor de kleine luchtwegen (met een diameter van minder dan 2mm), omdat deze dan geblokkeerd raken en worden afgesloten voor verdere gaswisseling. Dit wordt kleine luchtwegziekte of –dysfunctie genoemd, en komt voor bij alle stadia van astma en COPD. Er wordt zelfs gesuggereerd dat kleine luchtwegziekte de belangrijkste oorzaak is van kortademigheidsklachten, omdat het de effectieve oppervlakte van longblaasjes drastisch verlaagt. Het is echter zeer moeilijk om kleine luchtwegziekte te onderzoeken; de conventionele longfunctietests zeggen iets over de algehele longfunctie en kunnen onvoldoende onderscheid maken tussen het functioneren van de grote en kleine luchtwegen. Op dit moment zijn er verschillende gespecialiseerde longfunctietests die de mate van kleine luchtwegziekte wel kunnen meten, maar er is nog geen gouden standaard (diagnostische methode die de grootste zekerheid heeft over het al dan niet aanwezig zijn van een aandoening). Derhalve wordt er veel onderzoek gedaan naar verschillende tests die de kleine luchtwegen in kaart zouden kunnen brengen. In **hoofdstuk 4** stellen we een nieuwe methode voor, en in **hoofdstuk 5** passen we reeds bestaande kleine luchtwegdiagnostiek toe op een groep patiënten met astma, gezonde vrijwilligers, en deelnemers met klinische- of complete astma remissie.

In **hoofdstuk 4** maken wij gebruik van een nieuw experimenteel apparaat, de PExA (Particles of Exhaled Air). Dit instrument meet de hoeveelheid uitgeademde partikels (deeltjes) en kan deze ook opvangen voor analyses. Wij waren geïnteresseerd of

de hoeveelheid van uitgeademde partikels een maat zou kunnen zijn voor kleine luchtwegziekte. De theorie is dat als de kleine luchtwegen zijn afgesloten vanwege luchtweg remodelering, de desbetreffende persoon ook minder partikels uitademt. Onze resultaten toonde aan dat een lagere hoeveelheid van uitgeademde partikels overeenkwam met een slechtere functie van zowel de grote als de kleine luchtwegen. Een beperking van het PExA apparaat is dat het bij een afname van de uitgeademde partikels niet duidelijk is, wáár de blokkade de luchtwegen heeft plaatsgevonden. Verder onderzoek zal zich moeten toeleggen op analyse van de verschillende soorten uitgeademde partikels en hun precieze relatie tot astma en astma remissie.

In **hoofdstuk 5** zetten we de klinische kenmerken, waaronder kleine luchtwegfuncties van astmapatiënten, gezonde proefpersonen en deelnemers met klinisch-, en complete astma remissie uiteen. De belangrijkste uitkomst van deze studie was dat personen met complete astma remissie een vergelijkbare kleine luchtwegfunctie (bepaald door middel van stifstofuitwas-, stikstofoxide-, luchtwegweerstand- en CT-scantests) hadden als gezonde deelnemers.

14.5 De astma-obesitas relatie

In **hoofdstuk 7** wordt aandacht geschonken aan de relatie tussen astma en obesitas (overgewicht). Astma met obesitas onderscheidt zich van andere types astma door specifieke veranderingen in de longfunctie, een ander ontstekingsbeeld en een moeilijker te behandelen ziekteprofiel. Het gaat vaak gepaard met andere obesitas-gerelateerde aandoeningen die ook weer effect op astma kunnen hebben. Tot op heden bestaat er nog geen goede behandeling die specifiek ingrijpt op astma met obesitas. Vaak krijgt deze patiëntengroep een hogere dosering (inhalatie) ontstekingsremmers voorgeschreven, hetgeen veel bijwerkingen geeft. Wat echter wel goed helpt tegen obesitas (en indirect tegen astma), is een maagverkleining. Maar deze ingreep heeft een grote impact en geeft een kans op complicaties. Toekomstig onderzoek gericht op astma met obesitas zal zich moeten focussen op het verder ontwikkelen van maagverkleiningen, maar ook op het vinden van factoren die beide chronische aandoeningen in stand houden.

14.6 De cel-atlas van de mens

The Human Cell Atlas is een groot internationaal onderzoeksproject dat is opgezet om alle cellen van het menselijke lichaam in kaart te brengen. Dit moet leiden tot een soort referentiekaart waarop per orgaan alle aanwezige celtypes – en hun onderlinge verhoudingen - zijn afgebeeld. Dit project is ontstaan toen onderzoekers in staat waren om met nieuwe, geavanceerde moleculaire technieken, weefsels cel voor cel te bestuderen aan de hand van hun genexpressie. Genexpressie is het proces waarbij de genetische informatie die besloten ligt in een gen 'tot uiting (expressie) komt', doordat het DNA afgelezen wordt en er een RNA kopie van wordt gemaakt. Dit RNA molecuul wordt vervolgens vertaald tot een eiwit, wat vervolgens een specifieke functie in een cel gaat uitvoeren. De samenstelling van het RNA van een weefselmonster bevat dus informatie over de functies die in dat weefsel worden aangezet. Deze weefsels kunnen bloed, sputum, en andere lichaamsvloeistoffen zijn of kleine stukjes van een orgaan zoals de long. In **hoofdstuk 8** maken we gebruik van deze zogenoemde *single-cell RNA sequencing* (scRNA-seq) techniek om het genexpressieprofiel van elke cel in het longweefsel in kaart te brengen. Zo vergeleken we de cellen uit de longen van gezonde proefpersonen met die van patiënten met astma. Met behulp van scRNA-seq hebben we nieuwe celtypes ontdekt, en unieke veranderingen in bepaalde celtypes die alleen in astma optreden, zoals slijmproducerende trilhaarcellen, T-cellen in de transitie van weefsel-residente naar een migrerend celtype, maar ook de recent beschreven ionocyten.

14.7 Van astma naar COPD

Chronic Obstructive Pulmonary Disease (COPD) is net zoals astma, een obstructieve longziekte. De aandoening kenmerkt zich door progressieve kortademigheid en verslechterende inspanningstolerantie. Op dit moment is COPD de derde doodsoorzaak ter wereld. De belangrijkste oorzaak van COPD is roken, maar deze longziekte kan ook veroorzaakt worden door het inademen van andere schadelijke stoffen zoals rook van open (haard)vuur of luchtverontreiniging. Dit leidt tot ontsteking van de luchtwegen, vernauwing van de kleine luchtwegen, en verlies van longblaasjes, ook wel emfyseem genoemd. Ook COPD kent geen genezing. De huidige therapie van COPD is met name gericht op stoppen met roken, luchtwegverwijding middels puffers, conditieverbetering en ontstekingsremming. Patiënten met ernstig COPD kunnen in aanmerking

komen voor longvolume-reductie therapie, waarbij bv met ventielen de meest zwaar beschadigde, emfysemateuze longkwabben worden afgesloten, waardoor de resterende longkwabben meer ruimte krijgen voor het ademen. In de uiterste gevallen kan een longtransplantatie uitkomst bieden.

Klassieke verschillen tussen patiënten met COPD en astma zijn dat de eerste vaak ouder zijn dan 40 jaar, vele jaren gerookt hebben, minder vaak ergens allergisch voor zijn, een stabiele of langzaam verslechterende longfunctie hebben, en een gedeeltelijk andere ontstekingsreactie in hun longweefsel hebben. Echter, in de praktijk is het onderscheid tussen COPD en astma moeilijker te maken, want er bestaan ook astmatici die roken, patiënten met COPD die allergisch zijn, patiënten met astma waarbij de longfunctie langzaamaan achteruitgaat. Dit idee is niet nieuw in Nederland, want vroeger vielen astma en COPD nog onder de parapluterm CARA, wat chronisch aspecifieke respiratoire aandoening betekent. Het gedachtegoed dat astma en COPD vele kenmerken delen, wordt ook wel 'de Nederlandse hypothese' genoemd, hetgeen professor Orie en collegae uit Groningen in 1961 hebben opgeschreven. Vanwege de nodige overeenkomsten is het gebruikelijk dat technieken en tests die gebruikt worden voor astma-, ook worden toegepast op COPD-onderzoek. **Hoofdstuk 10 & 11** geven daar voorbeelden van.

In **hoofdstuk 10** staat de klinische relevantie van periostin, gemeten in het bloed van COPD patiënten centraal. Periostin is in astmaonderzoek als biomarker voor eosinofiele ontsteking genoemd. Eosinofielen zijn een bepaald type witte bloedcellen, die vele afweerstoffen aanmaken en een rol spelen bij de afweer tegen bepaalde infecties zoals bij parasieten. Eosinofielen kunnen in aantal toenemen bij zulke infecties, maar ook bij allergische ziekten zoals astma, hooikoorts en eczeem. Om deze eosinofiele ontsteking in astma te onderdrukken worden inhalatiecorticosteroïden voorgeschreven. In COPD is het gebruik van inhalatiecorticosteroïden niet zo effectief in het onderdrukken van de luchtwegontsteking als in astma, behalve wanneer er veel eosinofielen aanwezig zijn, bijvoorbeeld in het bloed of in opgehoest slijm (sputum). Het dilemma is dat het voorspellen van een goede respons op inhalatiecorticosteroïden in COPD door het meten van eosinofielen niet waterdicht is. Daarom is men op zoek naar betere biomarkers voor de respons op inhalatiesteroïden in COPD, zoals de reeds genoemde periostin. Helaas blijkt uit ons onderzoek dat periostin in het bloed niet geassocieerd is met

klinische kenmerken van COPD, of met de respons op inhalatiecorticosteroïden bij COPD patiënten.

In **hoofdstuk 11** onderzoeken we de mogelijkheid om COPD types te scheiden op basis van automatisch gegenereerde genexpressieprofielen uit longbiopten. In dit geval is er geen genexpressieprofiel gemaakt per cel zoals in **hoofdstuk 8**, maar van het gehele longbiopt. Deze techniek bestaat al wat langer, is veel goedkoper, maar ook minder specifiek dan de single-cell RNA-seq. Op basis van een COPD genexpressieprofiel uit een studie uit Amerika, hebben wij, zonder gebruik van klinische gegevens van de patiënten, COPD patiënten geclusterd in twee groepen. Eén van deze twee clusters was geassocieerd met een hoger aantal witte bloedcellen ($CD4^+$ en $CD8^+$ T-cellen) in het weefsel, een slechtere respons op inhalatiecorticosteroïden en een snellere longfunctieachteruitgang. Dit onderzoek toont aan dat automatisch onderscheidende genexpressieprofielen als biomarker kunnen dienen voor het typeren van longfunctieachteruitgang in COPD patiënten.

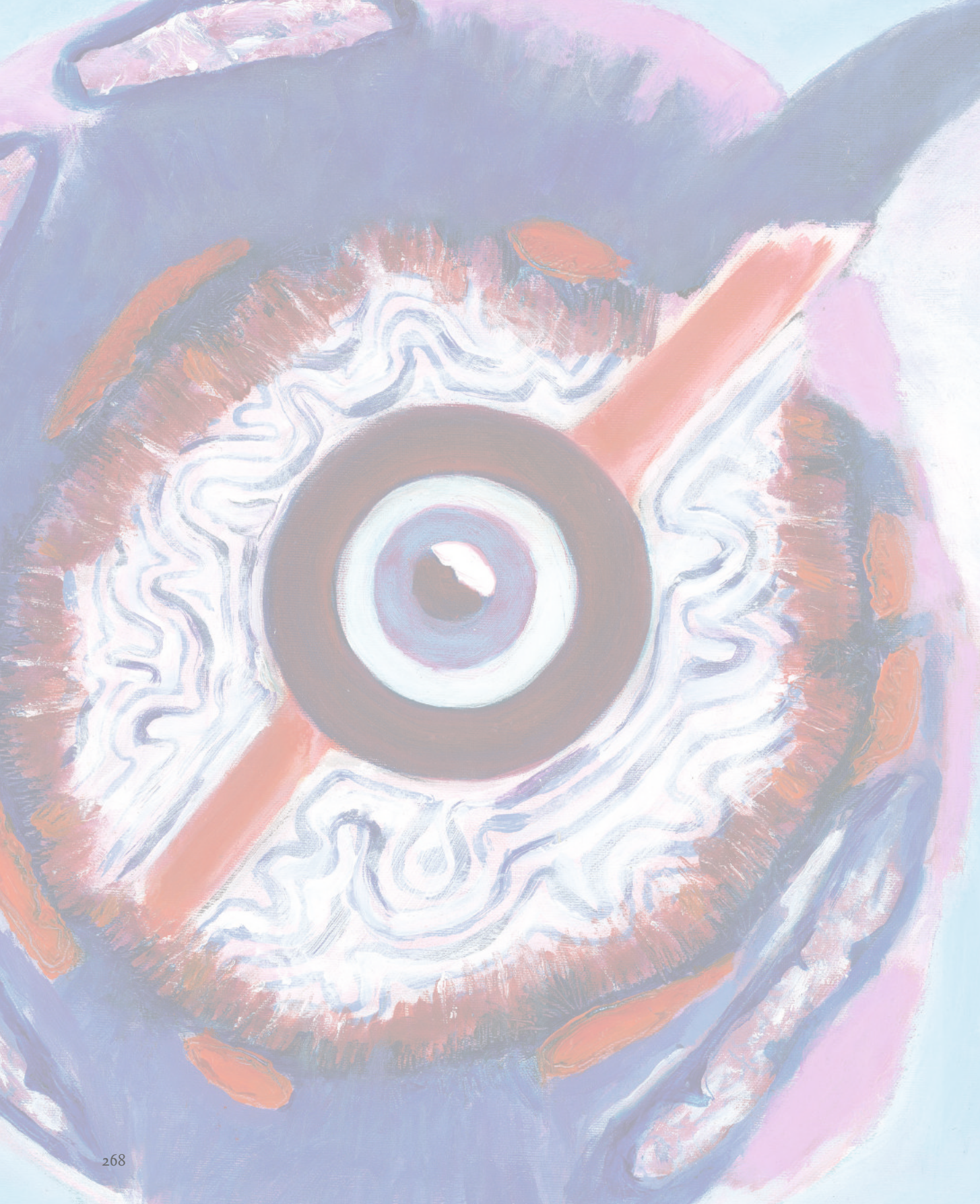
14.8 Conclusie

De verschillende hoofdstukken in dit proefschrift beslaan verschillende onderwerpen, zoals astma remissie, biomarkers voor patiënten met COPD, luchtweg remodelering en kleine luchtwegziekten. Hoewel dit als een relatief onsamenhangend palet van studies kan aanvoelen, hebben alle hoofdstukken ook een overeenkomst: het zijn allemaal onderwerpen die proberen mensen met astma of COPD nader te typeren, om beter de oorzaak van ontstaan, remissie of verergering te begrijpen, en om uit al die zaken te streven naar preciezer gerichte of nieuwe therapieën.

Verschillende conclusies zijn te trekken uit dit proefschrift. Eén, complete astma remissie is een fenomeen dat de meeste prognostische en wetenschappelijke interesse verdient als het gaat om het ontrafelen waarom mensen “over astma groeien”. En astma remissie onderzoeken is boeiend, omdat daaruit voortvloeiende biologisch inzichten kunnen leiden tot een nieuwe behandeling van astma, welke mogelijk gericht is op genezing van de ziekte. Twee, we weten welke factoren op kinderleeftijd geassocieerd zijn met astma remissie op latere leeftijd, maar met de meest recente modellen kunnen we astma remissie nog niet echt goed voorspellen. Drie, metingen van uitgeademde

luchtwegpartikels correleren met kleine en grote luchtwegparameters in individuen met astma en astma remissie. Vier, de OCT lijkt een bruikbare techniek om luchtweg remodeling in luchtwegziekten aan te tonen. Vijf, periostin gemeten in het bloed is niet geassocieerd met klinische kenmerken of respons op ontstekingsremmende puffers bij COPD patiënten. Zes, met genexpressieprofilering is het mogelijk om COPD patiënten in te delen. En zeven, nieuwe methoden die genexpressieprofilering van cel per cel mogelijk maken, stellen ons in staat om verschillende soorten en subsoorten van celtypen te ontdekken, maar ook fundamentele verschillen tussen astma en gezond te vinden.

Alle hoofdstukken roepen logischerwijs om een vervolg: verder onderzoek is hard nodig om weer de volgende stap te kunnen zetten. Toekomstig onderzoek zal zich focussen op het integreren van onderzoeksmethodes, bijvoorbeeld door klinische kenmerken (waaronder kleine luchtwegtests), bloeduitslagen, luchtweg remodeling parameters, genetisch onderzoek, CT-scanuitslagen, en nog meer te combineren. Het doel moet zijn dat dit zal leiden tot een betere, individuele behandeling. Deze onderzoeksmethoden zijn nog niet uitgediept in astma en COPD, laat staan in het fenomeen dat astma remissie heet. De reeds lopende *Astma Remissie Studie* zal hier verandering in brengen.



Curriculum Vitae

Orestes Alexander Carpaij was born on November 22nd 1988 in Nijmegen, the Netherlands. The majority of his youth, he lived on the Oosterhoutsedijk in Lent with his mother Christina van Lokven, father Marcel Carpaij, stepmother Tilly Spakman, and half-brother Solon Carpaij. After graduating from the Nijmeegse Scholengemeenschap Groenewoud, Orestes moved to Utrecht to study Medicine at the University of Utrecht. In his first year of college, he joined the Utrecht Rowing Club Triton, where he met the predominance of his friends and boosted his interest in endurance sports. Orestes started working as an internal medicine intern at the Diakonessenhuis in Utrecht, where he discovered his excitement for pulmonology. Subsequently, chest physician dr. F. Brijker helped Orestes to get in touch with prof. dr. D.S. Postma. This led him to PhD trajectory in the University Medical Center Groningen (UMCG), under supervision of dr. M.C Nawijn, dr. M. van den Berge, and prof. dr. H.A.M. Kerstjens. During his research, Orestes became a member of the GVAV Triathlon Club and completed a full Ironman. After finishing his PhD thesis, Orestes started his internal medicine residency in the Onze Lieve Vrouwen Gasthuis in Amsterdam, and will finish his pulmonogy residency in University Medical Center Groningen.

Dankwoord

Zoals Wouter P. Kluijfhout ooit in zijn proefschrift heeft geschreven, “welkom bij het meest gelezen onderdeel van mijn proefschrift” [1], het dankwoord. Logischerwijs wil ik ten eerste alle proefpersonen bedanken die mee hebben gedaan aan de Astma Remissie Studie. Ik ben hen zeer dankbaar dat zij viermaal wilden langskomen voor verschillende tests, waaronder een bronchoscopie. Ondanks het feit dat deze onder een roes plaatsvond, was dit niet altijd een pretje.

Huib, mijn promotor, ik kijk enorm tegen je op. De manier hoe jij betrokken bent met de afdeling, met GRIAC, met mijn onderzoek, is buitengewoon en inspirerend. Dit besefte ik verschillende keren gedurende het jaar: tijdens de kerstborreltoespraak, wanneer ik jou opbelde voor overleg tijdens mijn dienst in de revalidatiekliniek, en tijdens de internationale congressen. Je had het net zo goed niet hoeven doen, maar jij loopt wel even langs ieders poster en praatje, en ik heb wel jou aan de telefoon tijdens de diensten. Verder zag ik ons maandelijks overleg vaak als een verademing; dan kwam ik gedurende dit uurtje er weer achter dat het traject best prima verliep, en ik onnodig aan het piekeren was. Huib, ik spreek vol lof over jou.

Maarten, UHD van den Berge, ik heb een aantal maanden na het beginnen aan promotietraject gevraagd of ik niet te amicaal met je omging. Toen je vervolgens zei dat je het ervoer als een prettige omgang, was het hek van de dam. Onno Akkerman sprak zelfs van decorumverlies, en misschien heeft hij daar ook wel een beetje gelijk in; met alle (sportieve) activiteiten buiten het werk om vind ik het moeilijk om een lijn te trekken tussen baas en vriend. Je bent echt een fantastische pragmaticus, en ik heb daar enorm veel van geleerd. Jij rijdt met jouw BMW over de beren heen. Misschien verklaren die beren de deuken in jouw oude wagen. Als ik ergens twijfels over had, haalde jij jouw schouders op en je keek me aan alsof mijn aarzelingen compleet overbodig waren. Dat heeft mij veel vertrouwen gegeven in mijzelf. Maarten, ik hoop dat wij, als de twee overgebleven leden van het *Broncho Dream Team*, nog veel zullen koersen, honderd meters zwemmen, en wetenschap (lees: meer studienamen verzinnen zoals ARMSTRONG, INDURAIN, PANTANI, CIPOLLINI) bedrijven.

Martijn, Mar-10X, the Big Easy, jij ademt wetenschap; ik hoef er maar een muntje in te gooien en een scala aan wetenschappelijke mogelijkheden en ideeën worden voorgelegd. Lesgeven

en presenteren doe je met verve. Al is het voor mij geen nieuws meer, ik blijf genieten van jouw *ik-weet-niet-wat-groen-of-rood-is-op-deze-PowerPoint-slide-want-ik-ben-kleurenblind* catchphrase. Ik had eigenlijk een video-opname willen hebben van jouw praatje tijdens *Human Lung Cell Atlas: beyond single cell RNA-sequencing* in Dallas. Jij bent ook kritisch; ik heb nog nooit zo’n levendig en direct commentaar gekregen als van jou. Mijn narratieve review wordt als narrige review teruggestuurd. De paragraaf waarin ik probeer de immunologische link tussen astma remissie en leukemie te verklaren, werd door jou becommentarieerd met het volgende: “Serieus? Is acute leukemie een auto-immuunziekte? Waar heb jij jouw coschappen gelopen? Begrijp ik goed dat jij redeneert dat als familieleden dendritische cellen in elkaars gezicht spugen ze dan wel leukemie dan wel astma remissie kunnen ontwikkelen?” Ik heb een ogenblik sip naar het manuscript gekeken, maar heb dit commentaar zeer weten te waarderen. Ik hoop dat wij in de toekomst nog vaak onnodig verhitte, doch plezante discussies zullen voeren. Misschien leer ik er iets van.

The reading committee, professor Ian Pavord, professor Peter Sterk, and professor Reinoud Gosens, thank you for accepting to be a member of the reading comittee and reading my thesis critically.

Dear sponsors, thank you financially supporting the printing costs for this thesis.

Mijn cyclonimfen Karin & Dirk-Jan, jullie zijn een *Strava*-begrip. Ik kwam tijdens het feest in ’t Feithhuis, dat jullie gaven ter eren van de *New England Journal of Medicine* publicatie, erachter dat jullie fietsfanaat waren. Vervolgens zijn we nooit gestopt met praten over wielrennen, onderzoek, maar ook persoonlijke kwesties. Ik wil jullie danken voor alle ritten, maar met name voor de onvoorwaardelijke steun. En aan iedereen die met ons mee heeft gefietst; Dirk-Jan dan wel Karin begon met harder fietsen. Ik zweer het!

The Long Distance Team Onno, Maarten, Tji, Maaike, Bram, Alen, Mathieu, Anne-Greet, Rob, Wim en Birgitta, I’m very grateful that Alen tought me how to swim freestyle. Otherwise, I would not have had the experience to enter the Groningen Swim Challenge, three times already. It feels like you are swimming faster every year, while I’m not. Whatever the training intensity, in the next years, I would have a swim-buddy for every speed level, ranging from Onno for the Michael Phelps-like, to Maarten for the nearly drowning. It has been a privilege.

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Sharon, (hé) buddy, ik kijk met veel plezier terug naar de eerste jaren van ons Astma Remissie Studie-avontuur. Ik moet nog steeds glimlachen als ik me herinner hoe "leuk" jij het vond als Maarten en ik langskwamen om te vragen hoeveel $CD4^+$ T-cellen er door de FACS waren getrokken. De sfeer tijdens de bronchoscopieën waren altijd feestelijk, zeker als we opereerden met het "Dream Team".

Laura Hesselhoff, de Hessias, Martina Nawijn, ik krijg enorm veel energie van jouw aanwezigheid. Misschien komt het door al het snoep in jouw omgeving, misschien omdat we allebei vrij druk zijn. Ik wens jou veel geluk en succes toe met het afronden van jouw proefschrift.

Medewerkers van het endoscopiecentrum, ik ga die bronchoscopieën wel echt missen! Ik denk dat velen zullen beamen dat de atmosfeer in kamer #4 energiek en enthousiasmerend was. Hulde voor jullie hulp; het waren veel brushes.

Tji, Jellie en Nick, ik hoop dat jullie de research bronchoscopieën net zo fantastisch vonden als ik. In het begin vond ik het vrij onwennig om jullie tijdens de ingreep te dirigeren. Ik heb geleerd dat elke bronchoscopist de ingreep anders uitvoert, wat in mijn ogen dit vak nog mooier maakt.

Medewerkers van de longfunctieafdeling, de basis van alle wetenschappelijke cohorten. Ik weet dat de visites van de Astma Remissie Studie – met name visite 2 - logistieke en arbeidsintensieve exercities waren. Maar dankzij jullie hebben wij een groot deel van de klinische karakteristieken van de deelnemers. Als blijk van erkentelijkheid zal ik in de toekomst de oude, koude koffie uit de kan drinken, als een soort gifbeker.

Monique en Marian, al staan er geen resultaten van de SHERLOCk studie in dit proefschrift, ik wil graag benadrukken dat samenwerken met jullie vrijwel vlekkeloos verliep, zeker

vanwege jullie toewijding. Ik heb veel respect voor jullie. En, die data van Sherlock worden nog gepubliceerd hoor!

Professor Janette Burgess, our paths crossed later during my PhD years. This is unfortunate, since I really see you as a mentor, but also because I like your unique field of expertise. I am very grateful for what you thought me, and for believing in my capabilities. Your students are very lucky.

Professor Wim Timens, vader GRIAC, dank voor het secuur lezen en becommentariëren van een aantal van deze manuscripten. Jij ziet de addertjes onder het gras en verbeterde een scala aan foutjes.

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Felipe, Gozde, and Sarah, thank you for helping us with the single-cell RNA-sequencing and subsequent analyses, which were also performed by our own *Marijn*. Then again, you are mainly responsible for my imposter syndrome [2]; you, including Marijn, raise the bar of what I think is expected from a researcher.

Team GSK, Antoon and Karen, although our contact gradually reduced over the years, I appreciated your input, trust, and informal meetings during the international conferences. I am indebted to you for giving me this opportunity.

Alex, Aleksa, professor Salman Siddiqui and professor Craig Galban, thank you for the collaboration and rapid processing of the Parametric Response Mapping analyses. Your services were outstanding.

Members of the GRIAC, undoubtedly the most diverse and beautiful institute of the UMCG, keep it going! And whenever the clinical implication is unclear, please keep asking. Kudos to the current presidents and role models Gerard and Reinoud.

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Harde kern van pubquiz team "Lekker Long Lekker" Marieke en Jorrit, al hebben we verscheidene keren gewonnen in café Oblomov (éénmaal valsspel wegens samenwerking met Jan Zijlstra), wat mij het meeste bijblijft zijn de ontdekkingen van *Indian Pale Ales*, minutenlange tunnelvisies, extreme blunders, de 25% van Marieke, en de waardeloze muziekkennis van Jorrit. Moeten we zeker weer oppakken als ik terugkom!

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(Oud) research collega's Ben, Menno, Corneel, Ilse, Claire, Kai, Dianne, Nynke, Helma, Niels, Tim, Judith, Grietje en Floor, ik heb in vele formaties in verschillende kamers met jullie samengezeten. De ene kamer werd gesloopt terwijl we er nog in werkten, de andere was drie koprollen van het chique personeelsrestaurant vandaan. Deze herschikkingen hebben voor verscheidene groepsdynamieken gezorgd, iets wat ik achteraf ben gaan waarderen. Met z'n zessen in één hok gepropt te worden was stiekem best wel gezellig. Ik wil jullie bedanken voor alle hulp, en mijn excuses aanbieden voor mijn verscheidene monologen en tirades. Ilse, jouw proefschrift was een heerlijke template!

De heilige drie-eenheid Sietske, Trudy en Inez, ik hoop eigenlijk dat jullie samenstelling eeuwig is. Als de afdeling *long*ziekten een *hart* zou hebben, dan waren jullie dat. Ik prijs jullie emotionele en bureaucratische steun.

Mijn paranympo's Mart & Jens, Smartie & Jenzel Smashington, No Emotions Marty & Jay Infinity, wat hebben wij de afgelopen jaren veel samen ondernomen. De vakanties, de uitdagingen, het aantal uren wielrennen, zwemmen, en hardlopen; Ik denk dat ik niet zo ver kan tellen. En wat heb ik misbruik gemaakt van het feit dat jullie een bed hadden in Utrecht. De reden dat ik jullie heb gevraagd heb naast me te staan moge duidelijk zijn; jullie zijn mijn sociale steun en toeverlaat geweest tijdens mijn promotietraject. Ik denk dat jullie het even logisch vinden als ik dat jullie mijn paranimfen zijn.

Poly, Wouter en Rob, ik heb nog steeds de foto's van onze eerste keer gala en canyoning in mijn kamer hangen. Op een paar grijze haren na, is er weinig veranderd; even ijverig, even luidruchtig, en nog even onvolwassen. Ik hoop dat we zo blijven. *Piem*, spijtig dat wij de switcheroo Groningen - Amsterdam verkeerd gepland hebben. Laatste jaar opleiding samendoen?

ABS, als ergens aanvoelde als thuis (voorheen Oosterhoutsedijk), dan was het Abstederdijk. Dank voor jullie gastvrijheid de afgelopen jaren. Ik heb nu al zin in aankomende kerstdiner, hopelijk met "the original Six".

Oud-Triton Pro-Continental & Endurance Hogan, met jullie heb ik op de mooiste en gekste plekken van Europa en eveneens 'down in Africa' gefietst. Mij blijven de adembenemende Gorge du Dades, 260km lange woestijnrit naar Tafraoute, en snoeiharde windetappe naar Zagora het meest bij, al was de in 2019 gereden Pico Veleta ook verschrikkelijk mooi. Laten we dit blijven doen, totdat we niet meer kunnen. And *Alan*, thanks for reminding me to stay slim.

Breaking Zürich & the Loveboat, 2018 was mede dankzij jullie een van de mooiste jaren van mijn leven. We blijven aanmoedigen, dineren en hurken.

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mogen gebruiken als kaft. Ook al kan je het nooit laten om hier en daar een meerkoet of gans in te verstoppen.

Referenties

1 Kluijfhout W.P. Thesis: Parathyroid Imaging. Dankwoord. 2017.

2 Kolligian J & Sternberg RJ. Perceived fraudulence in young adults: is there an “imposter syndrome”? J Pers Assess. 1991 Apr;56(2):308-26.