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 (FEV1) 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>
<thead>
<tr>
<th>Phenotype</th>
<th>Natural history</th>
<th>Clinical and physiological features</th>
<th>Pathobiology and biomarkers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Early-onset allergic</td>
<td>Early onset; mild to severe</td>
<td>Allergic symptoms and other diseases</td>
<td>Eosinophils; specific IgE; TH2 cytokines; thick subepithelial basement membrane</td>
</tr>
<tr>
<td>Late-onset</td>
<td>Adult onset; often severe</td>
<td>Sinusitis; less allergic</td>
<td>Corticosteroid-refractory eosinophilia; IL-5</td>
</tr>
<tr>
<td>Exercise-induced</td>
<td></td>
<td>Mild; intermittent with exercise</td>
<td>Mast-cell activation; TH2 cytokines; cysteinyl leukotrienes</td>
</tr>
<tr>
<td>Obesity-related</td>
<td>Adult onset</td>
<td>Women are primarily affected; very symptomatic; airway hyperresponsiveness less clear</td>
<td>Lack of TH2 biomarkers; oxidative stress</td>
</tr>
<tr>
<td>Smoking-related</td>
<td></td>
<td>Low FEV1; more air trapping</td>
<td>Sputum neutrophilia; TH17 pathways; IL-8</td>
</tr>
</tbody>
</table>

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-IL-5 [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.


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].
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 \[51\].

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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.
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 TRanscriptiONal 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
Chapter 1

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.
References


