Future Research Directions in Asthma
An NHLBI Working Group Report

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Abstract

Asthma is a common chronic disease without cure. Our understanding of asthma onset, pathobiology, classification, and management has evolved substantially over the past decade; however, significant asthma-related morbidity and excess healthcare use and costs persist. To address this important clinical condition, the NHLBI convened a group of extramural investigators for an Asthma Research Strategic Planning workshop on September 18–19, 2014, to accelerate discoveries and their translation to patients. The workshop focused on (*1*) *in utero* and early-life origins of asthma, (*2*) the use of phenotypes and endotypes to classify disease, (*3*) defining disease modification, (*4*) disease management, and (*5*) implementation research. This report summarizes the workshop and produces recommendations to guide future research in asthma.

Keywords: asthma; prevention; phenotype; disease modification; implementation

At a Glance

Scientific Knowledge on the Subject: This report summarizes the Workshop participants' discussions, recommendations, and priorities for future research in asthma.

What This Study Adds to the Field: The report represents a collective body of scientific expert opinion conveyed to the NHLBI for use in strategic planning. The recommendations will be of interest to the scientific, professional, and patient communities because they constitute a summary of the directions asthma research may take in the near future.

Since the NHLBI last convened an asthma research planning workshop in 2003 (1), the NHLBI and other organizations have made a number of efforts to summarize available evidence that reflects the advances and gaps in our understanding of the onset of asthma, and its pathobiology, classification, management, and research (2, 3). For example, research has identified prenatal and early childhood environmental, genetic, and immune risk factors for the development of asthma that

(Received in original form May 18, 2015; accepted in final form August 24, 2015)

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Supported by the NHLBI, National Institutes of Health.

Author Contributions: Conception and design: B.D.L., S.C.E., C.O., P.G.W., S.N.G., N.N.J., M.M.C., M.M.F., and P.J.N. Writing and contributing important intellectual content: all authors.

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Am J Respir Crit Care Med Vol 192, Iss 11, pp 1366–1372, Dec 1, 2015

Originally Published in Press as DOI: 10.1164/rccm.201505-0963WS on August 25, 2015
Internet address: www.atsjournals.org

act in concert, and perhaps sequentially, at critical developmental time points to determine an individual's risk for the development of disease. Nevertheless, we remain uncertain how to prevent the onset of asthma, while recognizing that primary prevention of asthma is one ultimate goal (4). Ideally, preventing or perhaps encouraging specific environmental exposures at critical time points in developmental pathways would allow us to prevent the onset of asthma (Figure 1). Once a diagnosis is established, patients were traditionally classified as having mild, moderate, or severe asthma. We now refine the classification to reflect disease heterogeneity and the biological basis of such heterogeneity using clinical and/or molecular phenotypes. The current approach may allow us to make more informed treatment decisions that will modify or reverse the disease (i.e., disease-modifying asthma therapies) (Figure 1), rather than simply relieving symptoms temporarily. Until we can prevent asthma or modify existing disease, patient care will remain challenging because of our inability to predict or prevent exacerbations and the persistence of substantial barriers to the translation of scientific evidence into the everyday lives of patients. On September 18–19, 2014, the Division of Lung Diseases of NHLBI convened a group of extramural investigators to discuss their recommendations for the direction(s) of future asthma research and to identify opportunities for scientific advancement.

Workshop participants were divided into working groups that focused on five areas of asthma research: asthma origins and primary prevention; phenotypes and endotypes; disease modification; personalized asthma control strategies and management; and implementation research. Each working group identified barriers to progress and made recommendations for future research. During the workshop, several cross-cutting themes and associated research questions emerged from the working groups. This report provides a summary of the workshop participants' discussion and cross-cutting themes, as well as their recommendations and priorities for future research in asthma

Asthma Origins and Primary Prevention

As a multifactorial disease, asthma onset, severity, and natural history vary, which reflects the combined effects of development-specific exposures (e.g., *in utero* vs. early-life vs. childhood exposures) and host responses to those exposures (4–6). Despite this heterogeneity, most asthma manifests in the preschool years; therefore, asthma origins and primary prevention research have focused on childhood onset of the disease. Although many epidemiological, genetic, environmental, and immune risk factors for asthma are known, distinguishing which are causal, and how these factors interact and lead to overt disease remains poorly understood. In clinical investigations, specific questions to be addressed include identifying which risk factors are causal, the mechanisms through which these factors initiate asthma, and how combinations of exposures interact to ultimately initiate disease. Studies that address both the relevance of specific developmental windows and the importance of coincident exposures are also necessary to understand the etiology of asthma. To do so, metrics for the exposome, originally defined as "encompasses(ing) life-course environmental exposures (including lifestyle factors), from the prenatal period onwards," (7) will be needed. Incorporating comprehensive longitudinal exposure data (with relevant exposure specificity, time, dose, and frequency) collected from the prenatal period through the onset of asthma into rigorous analyses in a meaningful way remains a challenge. Furthermore, investigation into the role of extrapulmonary organ systems on the development of asthma through unique or shared mechanisms could provide insights into important developmental pathways.

Current barriers to our understanding of the origins of asthma include the limitations on clinical research that are permissible during gestation and the perinatal period, inadequacies of animal models in recapitulating the onset of human disease, and differences between human and experimental animal developmental stages. For example, although mouse allergen challenge models recapitulate many features of sensitization and allergic lung responses, acute exposures in mice differ substantially from the chronic/intermittent and diverse exposures antecedent to the onset of asthma in humans. Therefore, the utility of animal

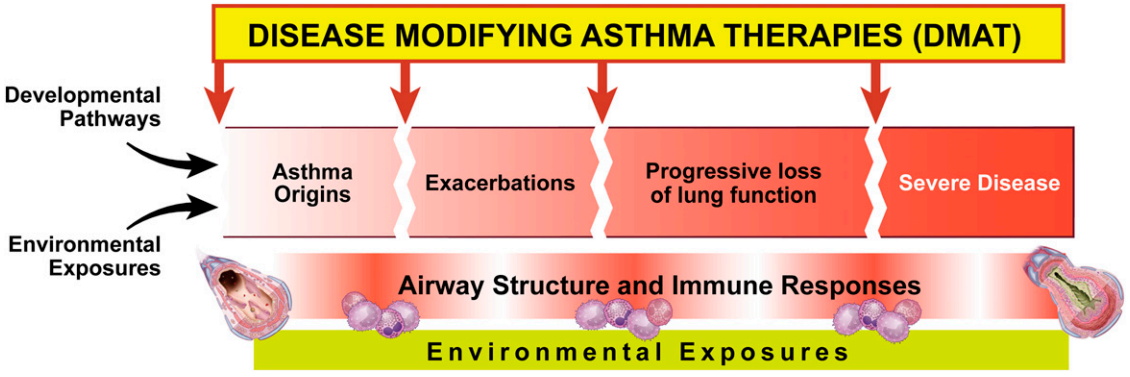

Figure 1. Preventive strategies and disease-modifying therapies for asthma. The immune system and lung structure pathobiology in asthma arise from developmental pathways and exposures. Asthma exacerbations and progressive decline of lung function are morbid outcomes for asthma. Standard treatment is focused on reversing bronchoconstriction with β-adrenergic agonists (relievers) and treating inflammation with corticosteroids (long-term controllers) to alleviate the downstream signs and symptoms of asthma. Disease-modifying asthma therapies target upstream effectors and mechanisms underlying asthma pathobiology. Preventive strategies require understanding of the critical windows of environmental exposures over the life-course and the personal biological predispositions that put individuals at risk for development of asthma or its progression.

models to elucidate the origins of asthma is limited, particularly when assessing potential interventions for the primary prevention of asthma.

To address these issues, the following recommendations were made:

1. Develop methods to assess the developing human infant immune system and lung.
2. Define the critical windows in human development when environmental exposures are most likely to increase the risk of asthma or its onset, specifying the relevance of interactions between specific risk factors and the timing (critical window) of the exposure.
3. Elucidate the critical windows in human development when non-environmental risk factors cause or influence the onset of asthma, specifying the relevance of interactions between specific risk factors and the timing (critical window) of the exposure.
4. Test *in utero* or early-life strategies targeted to mitigate potential determinants of asthma onset (primary prevention).
5. Generate and evaluate methods to measure and analyze the asthma-relevant "exposome" at different stages of human development (e.g., determine how peripubertal changes in the exposome differ by sex and the associated differences in immune response, lung development, and the onset of asthma).

Asthma Heterogeneity: Phenotypes and Endotypes

Several approaches have been taken to classify the disease heterogeneity observed in patients with asthma (8–12). For example, cluster analyses, which define sets of patient characteristics based on mathematical modeling to create clusters, have been used within specific cohorts. However, in clinical practice, prospective application of cluster analyses to individual patients has limited utility because of the granularity of phenotyping and analytical approaches used to aggregate candidate variables into a cluster. In addition, the natural history of phenotypes over the life span and the longitudinal course of phenotypes with treatment and among racially or ethnically diverse populations remain unclear. The potential integration of technologically advanced data (e.g., molecular or "omics" data or other biomarkers) to refine phenotypes into endotypic clusters further complicates the classification of disease. For example, patients may be characterized as having "type 2–high" asthma on the basis of elevated gene expression of markers of type 2 inflammation in airway epithelial cells or on the basis of type 2 cytokine expression in bronchial biopsies (13). Nonetheless, type 2–high patients may be similar to type 2–low patients (as defined by gene or cytokine expression) if they are compared on the basis of lung function or hyperresponsiveness. Moreover, the analytical approach to classification may be modified based on the purpose (e.g., predicting patient response to therapy) or disease status (e.g., patient on immunomodulatory therapy or having an exacerbation) (13–15).

Endotyping and phenotyping research requires well-characterized cohorts with appropriate representation of the spectrum of disease and modifying factors, as well as bioinformatics and computational biology expertise. The complexity of managing large data sets, including access to electronic medical record data and lack of data harmonization among large cohort studies create significant barriers to progress.

To address these issues, the following recommendations were made:

1. Sustain basic and translational research into the mechanisms underlying endotypes and phenotypes of asthma, including, but not limited to, the molecular phenotypes that underlie type 2–low asthma.
2. Develop new models for molecular phenotyping, including organotypic cultures of cells from patients.
3. Foster public–private scientific collaborations between academia and industry to uncover endotypes in human asthma as revealed by the increasing use of newly developed type 2–specific antagonists.

Disease Modification

Although asthma therapeutics have been traditionally identified as either "controllers" or "relievers," this dichotomous classification does not address the therapeutic potential to modify the underlying disease. Ideally, disease-modifying asthma therapies (DMATs) should target fundamental pathobiological mechanisms involved in asthma and/or pathologic alterations in lung structure. Evidence suggests that the mucosal immune system (16), and specifically airway epithelial-derived signals, play a pivotal role in asthma (17). In addition, dysregulation of innate antiviral defenses (17) and/or the role of type 2 inflammation are important features of disease pathobiology in many individuals with asthma.

To achieve disease modification, disruption of the critical mechanisms of disease is likely to be necessary, and the timing of such disruption may also be important. Mouse models suggest that innate lymphoid cells (ILCs), which respond to epithelial-derived signals to mediate allergic airway inflammation (18, 19), may be useful to target the mucosal immune system. Although ILCs have been identified in the healthy human lung (20) and among patients with asthma (21), more research is needed to understand the role of ILCs in asthma, to quantify the degree of dysregulation in mucosal immunity, and to assess epithelial integrity (22) in human subjects. Moreover, the relationship between critical mechanisms of disease and clinically meaningful disease manifestations or outcomes requires further exploration. For example, airway remodeling can be an early event in the natural history of asthma, but it is often dissociated from inflammation or airflow limitations (23, 24). Finally, as described in the Asthma Heterogeneity: Phenotypes and Endotypes section, although the type 2–high phenotype (characterized by elevated gene or cytokine expression of markers of type 2 inflammation in airway epithelial cells or bronchial biopsies, respectively) has been targeted to modify disease, our understanding of the type 2–low phenotype and mechanisms of disease that are independent of type 2 inflammation continues to be a challenge.

To address these issues, the following recommendations were made:

1. Promote investigation of asthma by multidisciplinary groups of scientists and clinicians with diverse expertise, and the ability to use new tools and animal models to explore the heterogeneity of disease mechanisms,

identify the critical pathobiological mechanisms of disease, and understand the dynamic interactions between such processes.
2. Evaluate the relationship between pathobiological pathways and clinical manifestations of disease to enable the identification of appropriate targets for DMATs.
3. Assess critical windows for disease modification and clarify when end-effector mechanisms (e.g., smooth muscle structure or function, mucous production, or airway fibrosis) are reversible or modifiable.

Personalized Asthma Control Strategies and Management

As indicated by the asthma phenotypes and endotypes working group, there is substantial heterogeneity in "asthma," and this heterogeneity is reflected by a broad range of therapeutic responses in clinical trials (8, 25–27). Such heterogeneity makes it challenging to manage asthma, particularly in the absence of prognostic indicators. Moreover, diagnostic biomarkers that could be of great value for young children and for determining the earliest origins of disease (28) are also not yet available. Individualized, evidence-based approaches to managing asthma on the basis of disease endotypes are lacking (29–33), as are approaches to management in diverse healthcare settings. In addition to cross-sectional approaches to defining an individual's disease characteristics, there is a paucity of longitudinal research linking molecular mechanisms and environmental exposures with clinical manifestations of disease (such as lung function changes or exacerbations) that might improve our understanding of the heterogeneity of responses to treatment.

For precision in asthma management, more mechanistic approaches to care are needed. These new approaches would be based on an integrated understanding of the individual patient's biological mechanisms, including the interplay among the exposome, genetics, epigenetics, immune response, psychosocial support and lung physiology. To address these issues, the following recommendations were made:

1. Design nontraditional clinical trials to identify the causes of disease heterogeneity and to facilitate the discovery of diagnostic and prognostic biomarkers.
2. Evaluate alternative methods to assess risk factors, mechanisms, and predictors of heterogeneity in the response to treatment, including medication-related adverse effects and morbidity.
3. Identify effective longitudinal asthma management models that integrate essential components of care (e.g., diagnosis, monitoring, education, exposures, medications, adherence) to prevent asthma, improve asthma control, or mitigate disease progression while improving efficiency and adaptation over time.
4. Asthma control strategies and management models should address the needs and behaviors of patients of all ages (soliciting patient feedback to do so), with different social, economic, and cultural backgrounds in a variety of healthcare settings.

Implementation Research

Implementation research investigates the processes by which evidence results in the modification of patient care (34). In asthma, implementation research integrates the relevant context in which care occurs, the needs and concerns of patients, the most appropriate provider and location for care to occur, and the organizations and communities in which patients live and care providers work. Current examples of advances made through implementation research include interventions to achieve evidence-based practices in some communities (35) and use of school programs (36, 37) for children with asthma. Other examples have defined modifiable barriers that may be addressed to improve asthma care (38).

Despite significant advances in our understanding of the management of asthma over the past decade, there are numerous barriers to translating the efficacy measured in randomized clinical trials into the everyday lives of patients. One key issue is that advances in therapy have not been equally distributed among different racial and ethnic groups. In particular, recent improvements in asthma outcomes are larger among non-Hispanic white Americans than among African Americans (39). Systematic reviews have shown that many healthcare providers do not adhere to clinical practice guidelines, and efforts to improve providers' compliance have had limited success (40). In addition, many interventions are now available for broad-scale dissemination, but these interventions lack an efficient mechanism for dissemination. The establishment of standard definitions of asthma outcomes based on collaboration among investigators, NHLBI, and National Institute of Allergy and Infectious Disease (41) may facilitate comparative effectiveness research particularly when interventions are disseminated across different communities. Despite previously weak incentives that have impeded implementation, current secular influences, most notably the Affordable Care Act (42) and Patient-Centered Outcomes Research Institute (PCORI) have created opportunities to which the asthma research community should respond.

To address these issues, the following recommendations were made:

1. Create a continuous learning/training collaborative of implementation researchers with the following goals:
   a. Develop new study designs and methodologies that enable the integration of evidence into clinical practice efficiently while assessing implementation outcomes to meet the dynamic needs of patients with asthma. This will require patient engagement in the research process.
   b. Investigate best practices for the dissemination of scientific discoveries and evidence-based practices in a variety of clinical care settings.
   c. Study how novel models of health care and its delivery promote implementation and dissemination in vulnerable and underserved populations that have a disproportionate burden of asthma, as well as in novel contexts, including nonmedical facilities (e.g., school, community center) and alternative healthcare providers (e.g., patient navigator, community health worker).
2. Develop a standard training curriculum within the implementation research collaborative for junior investigators to learn how to overcome challenges and

attain success in implementation research.
3. Establish a comparative cost-effectiveness core center within the collaborative that uses a systematic approach to compare various interventions for asthma.

Cross-Cutting Themes to Direct Future Research

Throughout the workshop and in the discussions of all of the working groups, several common recommendations emerged that merit further discussion because of the potential for synergistic advances in asthma research.

Informatics

The importance of advancing the use of informatics tools to enable asthma research was emphasized. One goal was to allow the creation, harmonization, and merging of large datasets, including prospective patient populations and existing administrative or electronic health record data. This would enable the identification of phenotypes on a population level using existing data. Enrollment of homogenous patient populations in clinical trials could be facilitated by such phentoypes. Ideally, these large datasets would also integrate existing clinical and "omics" data (e.g., genomic, proteomic, lipidomic, and microbiomic). Of critical importance, such datasets would require the use of a harmonized set of terms to define patient characteristics and outcomes, and provide links to available standardized protocols and repositories for biospecimen collection in current and future asthma cohorts. Such rigorously collected and integrated data would be useful to further clarify the heterogeneity of disease to allow development of predictive indexes to target populations at risk for disease development, DMATs, and new approaches to asthma management that account for the healthcare setting. Similarly, a registry of data from the electronic health records of patients with asthma would provide insight into the current state of asthma care/practices across the nation and promote planning of how to scale and efficiently implement discoveries to the population level.

Longitudinal Cohorts

The need for longitudinal cohorts of patients with asthma was also recommended for several purposes. First is to better understand the origins of the disease and the interdependence of both disease-promoting and disease-suppressing exposures and developmental changes over the lifespan. Such cohorts would also allow investigation of the durability and natural history of clinical phenotypes and molecular endotypes from infancy to adulthood, and help predict individuals at high risk for severe disease. Longitudinal collection of biospecimens would also allow for identification of predisease biomarkers and DMATs at different stages of life for patients with a variety of phenotypes.

Translational Research

The promotion of translational research incorporating multidisciplinary teams was also championed. For example, clinical phenotypes and molecular endotypes could be prospectively validated using evidence-based clinical management strategies to improve asthma care across the severity spectrum. Such teams would require the promotion of training to fill knowledge gaps, such as information technology skills to create user-friendly large datasets. Investigators with expertise in molecular phenotypes would need to engage in research planning with the informatics, genetics, clinical, and drug development experts. Such efforts may be useful to identify novel approaches to defining biomarkers for predicting the response to treatment or the disease course.

Mechanisms of Disease

Although much has been learned in the last decade regarding the underlying mechanisms of asthma, the principal focus has been on type 2–high asthma. With the remarkable development by industry of several new therapeutics that target molecular mechanisms for type 2 immune responses, their translation to clinical practice will provide a virtual learning laboratory for the importance of type 2 immunity in asthma. Renewed efforts for basic and translational research are needed for type 2–independent mechanisms in asthma, including immune, neural, and structural mechanisms, as well as developmental and regenerative pathways. These and other precision interventions and tools will allow for the clarification of type 2–dependent and –independent pathways in the pathobiology of asthma exacerbations, underlying disease, and progression.

Prevention, Disease Modification, and Cure

Asthma remains without primary prevention or cure. Multidisciplinary teams should be encouraged to investigate early-life events, including environmental exposures, to identify high-risk patients and populations to target for primary prevention. In the near term, new mechanistic-based therapies offer promise for a new era of disease modification with reductions in asthma morbidity, including improved quality of life, lung function, and reduced risk of exacerbations even for those with severe, persistent disease. Implementation research on the delivery of current asthma medications and new DMATs will also be needed to realize the full potential of existing and emerging therapies. Finally, long-term approaches to disease modification will enable the ultimate goal of asthma secondary prevention and cure.

Conclusions

The emerging themes from the workshop are intended to be a challenge for investigators to connect technological advances to the understanding of asthma pathobiology for disease management and its eventual dissemination to the general population. The ultimate goals are to develop effective strategies for the primary prevention of asthma and to discover or create precision interventions for asthma patients that optimize airway function and the health of individuals and their communities. These priority topics will be used to create a strategic approach for asthma research to achieve these goals, thereby improving patient outcomes and reducing the public health burden of asthma. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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REVIEW ARTICLE

Jeffrey M. Drazen, Editor, *M.D.*

Early-Life Origins of Chronic Obstructive Pulmonary Disease

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N Engl J Med 2016;375:871-8.
DOI: 10.1056/NEJMra1603287

In the United States, more people die from chronic obstructive pulmonary disease (COPD) than from any other condition except cancer and cardiovascular illnesses.[1] There is currently no curative therapy for COPD, and treatment is mostly palliative. Given the degree of airway or parenchymal damage (or both) in most advanced cases of the disease, a strategy aimed at reversing the condition once it is established is fraught with challenging obstacles. Thus, the approach with the highest likelihood of success is one that addresses the predisposing factors of this condition. By far the most common cause of COPD is smoking (and exposure to biomass smoke in poor countries[2]), and smoking cessation continues to be the potentially most successful prevention strategy.

Until recently, the prevailing idea was that during development (i.e., from birth to approximately 25 years of age), all people — those destined to have no lung disease and those destined to have COPD — reached the same plateau for lung function as measured by the forced expiratory volume in 1 second (FEV_1). What determined whether COPD developed was the rate of subsequent decline in the FEV_1 level. Was this rate at the physiologic level (approximately 25 ml per year) and thus not associated with lung disease, or was it much faster owing to the continuous and progressive noxious effects of cigarette or biomass smoke on small airways and surrounding parenchyma,[3] leading to COPD (Fig. 1)? This "classic" form of COPD is indeed a very frequent clinical presentation of the disease[4]; it is associated with chronic bronchitis, emphysema, or a combination thereof and is characterized by neutrophilic and CD8-mediated inflammation. The complex biologic mechanisms of this form of COPD have been studied extensively.[5]

Emerging evidence, however, has radically challenged the concept of a single natural history for COPD, indicating that the spectrum of patients presenting with chronic respiratory symptoms and irreversible airway obstruction (as assessed by an abnormally low FEV_1) is much more heterogeneous than previously thought. Results from longitudinal cohort studies have shown that in a considerable proportion of patients with COPD the decline in the FEV_1 was not steeper than that in healthy adults.[6] It has also been shown that some people with COPD who do not show excessive lung-function decline reach a lower FEV_1 level early in adult life than those with future rapid decline and normal populations[7] (Fig. 1). These findings thus identify an entirely different pathway leading to the diagnosis of COPD from the rapid-decline form, one in which smoking can certainly play a role, especially in the clinical expression of the disease, but in which the central derangement is already present early in adult life. What emerges is a fundamentally new concept of COPD, in which the factors that determine the maximal (or "plateau") FEV_1 level attained during the third decade of life become major elements in the pathogenesis of the disease.

In this review, three major questions are addressed. First, what causes certain persons to enter into adult life with a diminished FEV_1 level? Second, which diverse biologic mechanisms are involved in determining this lower plateau? Third, could early-life events influence the rate of FEV_1 decline that is observed in classic COPD?

THE ROLE OF GENETICS IN LUNG FUNCTION, STARTING AT BIRTH

It is now well established that trajectories of lung function during childhood are already at least partially established at birth. Levels of maximal expiratory flow that are measured shortly after birth with the use of a chest-compression technique are significantly correlated with the ratio of FEV_1 to forced vital capacity (FVC) into adult life.[8] Infants in the lowest quartile of maximal expiratory flow at birth as measured by this technique had FEV_1:FVC ratios in early adult life that were significantly lower than those of infants in the highest quartile (0.82 vs. 0.88, $P=0.001$ for trend) (Fig. 2). These results thus point to genetic and prenatal influences as major contributing factors in the level reached at the plateau of lung function.

Lung function, as measured by the FEV_1 or FVC, has a large heritable component. Studies that use pedigree data and those that are based on single-nucleotide polymorphism (SNP) data have reached similar conclusions: genetic factors explain 50% of the phenotypic variance for the FEV_1 and up to two thirds of that for the FEV_1:FVC ratio.[9] Although there are several genomewide association studies assessing the role of SNPs as determinants of FEV_1, most of such studies enrolled adults and included smokers, thus making it difficult to identify bona fide genetic determinants of baseline FEV_1, as opposed to genes influencing the decline in the FEV_1 associated with smoking.

Nevertheless, the largest published genomewide association study, involving almost 100,000 white participants, suggested that 26 loci and more than 100 variants could collectively explain 7.5% of the additive polygenic variance for the FEV_1:FVC ratio and 3.4% of the variance for the FEV_1 values, independent of smoking.[10] Some of the SNPs that were identified were present in genes with potentially plausible roles in lung growth and development, including genes involved in the structure of cilia, elastin-associated microfibrils, and retinoic acid receptor beta, an important factor in lung growth.[11]

The only study that spanned the whole age spectrum from infancy to adulthood showed an association between lung function and a variant in the gene encoding vascular endothelial growth factor, also a critical growth factor for airway development,[12] which was not captured in the larger genomewide association study.[13] Of interest was the discovery that there are genetic variants associated with the FEV_1 that are also associated with myocardial infarction, cancer, and height. These and other studies that combined

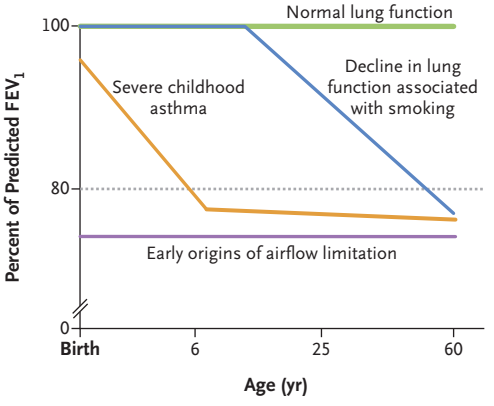

Figure 1. Three Major Lung-Function Pathways That Lead to Stage 2 Chronic Obstructive Pulmonary Disease (COPD).

Stage 2 COPD is defined as a forced expiratory volume in 1 second (FEV_1) that is less than 80% of the predicted value plus a ratio of FEV_1 to forced vital capacity (FVC) of less than 0.70. The green line represents a normal lung-function trajectory. The blue line (classic COPD) represents a decline in lung function associated with cigarette smoking or chronic exposure to biomass smoke; illnesses due to respiratory syncytial virus and parental smoking may increase susceptibility to these exposures. The orange line represents the three-phase lung-function trajectory associated with severe childhood asthma. Lung function is lower than normal at birth, shows a major decline during the preschool years, and may continue to decline at a slower pace thereafter. Lower respiratory tract illnesses (especially pneumonia before 3 years of age) and air pollution may similarly alter lung-function growth. The purple line represents the early origins of airflow limitation. Genetic and prenatal factors influence lung function at birth, and deficits persist into adult life.

genetic with gene-expression analyses[14] suggest that the FEV_1 level is controlled by a very large number of biologic pathways, most of which remain to be identified, with genetic variation in each pathway having small effects on phenotypic expression.

PRENATAL INFLUENCES

Exposure to noxious stimuli in utero may have long-term effects on lung health and influence the maximally attained FEV_1 level. The most widely studied among such exposures is maternal smoking, which has been consistently found to be associated with small but significant reductions of approximately 1.5% in the FEV_1 and 5% in the maximal expiratory flow in older children and young adults.[15] In animal models, exposure of the fetus to nicotine showed similar effects to those observed in fetuses whose mothers were exposed to cigarette smoke.[16,17] Specifically, prenatal nicotine exposure led to a decreased maximal expiratory flow by stimulating epithelial-cell growth and lung branching, resulting in longer and more torturous airways, which led to greater resistance to airflow.[18] If most of the effects of maternal smoking during pregnancy are caused by nicotine, e-cigarettes are likely to be as harmful to the fetal lung as standard cigarettes.

Approximately 10% of all births in the United States occur prematurely (i.e., before 37 weeks of gestation), and preterm birth has been shown to have profound effects on long-term lung function. This is especially true for the 0.3% of all newborns who are born very prematurely (before 27 weeks of gestation)[19] and who require oxygen supplementation for more than 28 days after birth, the standard definition of bronchopulmonary dysplasia. A recent meta-analysis[20] of studies involving children and young adults born prematurely concluded that major deficits in the FEV_1 level among those who had bronchopulmonary dysplasia occurred in 16.2% of those requiring oxygen for more than 28 days and in 18.9% of those requiring oxygen for more than 36 weeks. These results identify bronchopulmonary dysplasia as a major risk factor for major deficits in maximally attained FEV_1 in early adult life.

The results of a recent clinical trial strongly suggest that a potentially critical biologic mechanism for persistent airway obstruction in bronchopulmonary dysplasia is airway inflammation resulting from mechanical ventilation and oxygen therapy. Very-low-birth-weight children with severe respiratory distress syndrome who were given budesonide, an inhaled glucocorticoid, together with surfactant intratracheally had a significantly lower incidence of bronchopulmonary dysplasia or death than those receiving surfactant only (55 of 131 children [42.0%] vs. 89 of 134 [66.4%], P<0.001). They also had a lower concentration of inflammatory mediators in tracheal fluids.[21]

Although the most severe consequences are observed in newborns with bronchopulmonary dysplasia, premature infants without the disorder also have FEV_1 levels later in life that are 7.2% lower than those of children born at term.[20] Small deficits in adult FEV_1 have also been reported in children with intrauterine growth retardation.[22]

In chronically undernourished populations, maternal micronutrient deficiency may also affect maximally attained FEV_1 and FVC in the offspring. Nepalese children of women whose diet had been supplemented with vitamin A before, during, and after pregnancy had higher levels of

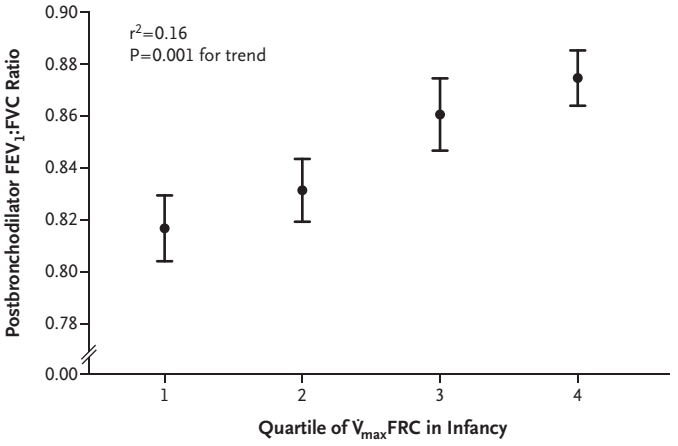

Figure 2. Relationship between the Quartile of Lung Function in Early Life and the FEV_1:FVC Ratio at 26 to 32 Years of Age.

Maximal flows at functional residual capacity ($\dot{V}_{max}FRC$) were measured shortly after birth in 67 infants and were adjusted for infant length and sex; spirometry was performed in the same participants in adult life. An estimated 16% of the variance in the FEV_1:FVC ratio was explained by the level of lung function in infancy. I bars indicate standard errors. Data are from the Tucson Children's Respiratory Study (unpublished data).

FEV_1 and FVC at 9 to 13 years of age than those whose mothers received placebo.[23]

RESPIRATORY ILLNESSES IN EARLY LIFE

Several longitudinal studies have shown that children who have lower respiratory tract illnesses in early life are at increased risk for subsequent chronic respiratory symptoms and FEV_1 deficits, which often persist into adult life.[24] The largest deficits have been observed in adults who had radiologically ascertained pneumonia before 3 years of age, who had an FEV_1:FVC ratio that was significantly lower than those with no early-life respiratory illnesses (0.76 vs. 0.80, P<0.001), whereas those with lower respiratory tract illnesses but no pneumonia had less severe impairment in the FEV_1:FVC ratio.[25] Both in developed[26,27] and developing[28] countries, the most frequent agents currently associated with pneumonia in early life are viruses, especially respiratory syncytial virus.

From the available data, it is impossible to determine whether the association between pneumonia and impaired lung function is due to airway damage caused by the etiologic agent triggering the episode of pneumonia or to preexisting deficits in lung function in young children in whom pneumonia develops. However, some young children with pneumonia that is caused by adenovirus serotypes 3, 7, and 21 have severe long-term sequelae, including bronchiolitis obliterans and bronchiectasis,[29] and others have moderate subsequent deficits in the FEV_1 level.[30] It is thus plausible to surmise that the deficits in the FEV_1 observed in adults with a history of early childhood pneumonia may be due in part to abnormalities present before the episode occurred but also, as has been shown for adenoviral pneumonia, to airway damage caused by the episode itself.

AIR POLLUTION

There is now convincing evidence that exposure to airborne contaminants is associated with reduced growth in lung function during adolescence and lower maximally attained FEV_1 levels. In a comprehensive study in the Los Angeles area,[31] mean deficits in the growth of FEV_1 between 10 and 18 years of age for participants living in the most polluted communities, as compared with those living in the least polluted communities, within the area were 105.8 ml for acid vapor (P=0.004), 101.4 ml for nitrogen dioxide (P=0.005), 87.9 ml for elemental carbon (P=0.007), and 79.7 ml for particulate matter with an aerodynamic diameter of less than 2.5 μm ($PM_{2.5}$) (P=0.04). As a result, the percentage of 18-year-old participants with an attained FEV_1 below 80% of the predicted value was much higher in the most polluted zones than in the least polluted zones; for $PM_{2.5}$, for example, these values were 7.9% and 1.6%, respectively (P=0.002). Maternal exposure to pollutants during pregnancy may also affect the FEV_1 level in the offspring.[32]

It is notable that decreases in $PM_{2.5}$ and nitrogen dioxide contamination in California were associated with significant improvements in the FEV_1 in a 13-year follow-up.[33] As a result, the percentage of children with an FEV_1 below 80% of the predicted value at 15 years of age declined significantly, from 7.9% to 3.6%, as the air quality improved (P=0.001).

CHILDHOOD ASTHMA

Children with persistent asthma have been consistently shown to reach a lower FEV_1 plateau during the third decade of life than children without asthma.[34] Only a fraction of children with asthma go on to have fixed airflow limitation, but among those who do, three phases have been described[35] (Fig. 1). First, children with persistent asthma have a slightly but significantly lower maximal expiratory flow and respiratory-system compliance assessed shortly after birth than do those without asthma,[36] which suggests that factors impairing lung growth in utero confer a predisposition for the subsequent development of asthma. Second, longitudinal studies have shown that by the time they reach the early school years, children with subsequent persistent asthma already have a large percentage of the deficits in FEV_1 that they will show during the plateau phase of lung function.[37] Approximately 40% of the deficits in the maximal expiratory flow that were observed at 6 to 7 years of age in children with asthma were present at birth, whereas 60% of the deficits developed during the preschool years.[38] Taken together, these findings identify the period between birth and 6 years of age as critical for the development of airflow

limitation in children with persistent asthma. Third, further declines in FEV_1 occur during the school years[39] and in adult life[40] as part of the natural history of asthma, but these declines seem to be a much smaller fraction of the total impairment than those observed in early life.

As a result of these deficits in FEV_1 growth associated with childhood asthma, by the third decade of life approximately 17% of all patients with mild or moderate asthma reach stage 1 COPD, as established by the Global Initiative for Chronic Obstructive Lung Disease (GOLD) (i.e., postbronchodilator FEV_1:FVC ratio <0.70 and FEV_1 ≥80% of the predicted value), whereas 5% reach stage 2 COPD (<0.70 and <80%, respectively).[41] Moreover, among a group of patients in whom severe asthma had been diagnosed by the age of 10 years, 44% had stage 1 COPD at 50 years of age,[42] a rate that was independent of smoking. Most of the reduction in lung function that was observed in patients with COPD at 50 years of age and a history of severe childhood asthma was already established in the childhood years (Fig. 3).

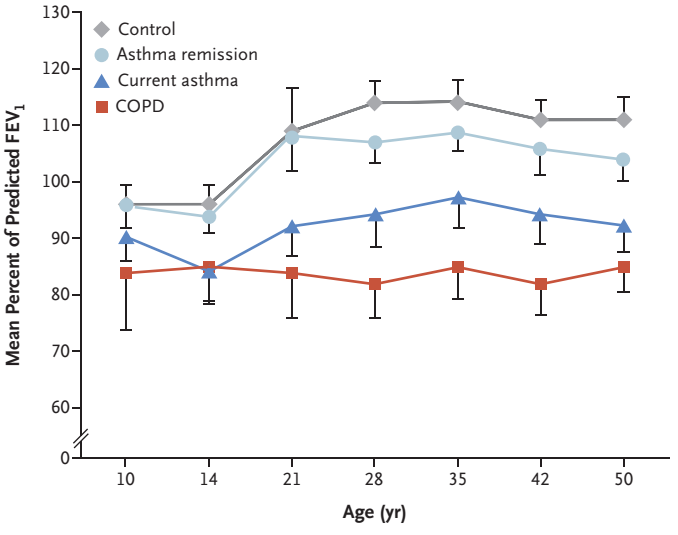

Figure 3. Percent of Predicted FEV_1 in Participants Followed between the Ages of 10 and 50 in the Melbourne Asthma Study.

Participants were classified at 50 years of age as follows: no asthma (control), current asthma, childhood asthma with remission in adult life, and stage 1 COPD, defined as an FEV_1 of 80% or more of the predicted value and an FEV_1:FVC ratio of less than 0.70. Among participants with COPD, airway obstruction that justified a definition of COPD was already present at 10 years of age. Adapted with permission from Tai et al.[42]

The mechanisms by which asthma causes nonreversible airflow limitation, especially during early childhood, are not well understood. Airway biopsy samples obtained from infants with recurrent wheezing show no evidence of airway remodeling, as assessed by the presence of a thickened reticular basement membrane in medium-size bronchi,[43] which is characteristic of school-age children with persistent asthma. However, incipient thickening can be observed in preschool children with wheezing[44] at approximately the age at which the largest deficits in airway growth are observed in children with subsequent persistent asthma.

For years, airway remodeling was attributed to chronic airway inflammation, but randomized trials did not show a better prognosis in children receiving long-term treatment with daily inhaled glucocorticoids than in those treated with placebo.[45,46] Sputum cells obtained from children with acute exacerbations of asthma and evidence of persistent airway obstruction have a lower expression of genes encoding proteins involved in the regulation of interferon type I responses and of genes involved in type 1 helper T cell (Th1)–like and cytotoxic responses than do those obtained from children with asthma and no evidence of persistent airway obstruction.[47] In vitro studies of epithelial cells from patients with asthma have shown abnormal airway repair after injury.[48] It is reasonable to surmise that these alterations may induce remodeling processes that lead to narrowed airways.

Bronchial hyperresponsiveness, which can be present as early as 6 years of age, is a hallmark of childhood asthma that persists into adult life,[49] and its corollary, recurrent airway smooth-muscle contraction, may increase compressive mechanical stress and activate airway repair and remodeling mechanisms independent of airway inflammation.[50] It is tempting to speculate that such processes may have stronger effects on airway structure during early childhood, a period of normally active lung and airway remodeling.

ACTIVE SMOKING DURING ADOLESCENCE

Active smoking during adolescence is associated with significant reductions in the FEV_1 level and the FEV_1:FVC ratio,[51] decreases that could have major effects on maximally attained lung func-

tion. Fortunately, a major public health success in the United States has been the dramatic reduction in the prevalence of active smoking among adolescents: whereas 18% of 10th graders reported regular daily smoking in 1996, only 3% did in 2014.[52] These trends coincided with major restrictions in tobacco advertisement and the allocation of funds from legal settlements between the federal government and tobacco companies to antitobacco campaigns.

Unfortunately, consumption of e-cigarettes has become increasingly more common among adolescents. The percentage who reported use of e-cigarettes in the past 30 days increased from 1.5% in 2010 to 17.2% in 2014.[53] This is a matter of great concern, because adolescents who try e-cigarettes are more likely to start smoking than those who do not.[52] If these trends persist, some of the gains made in the past 20 years in preventing adolescent smoking could be lost.

EARLY-LIFE EVENTS AND THE RATE OF LUNG-FUNCTION DECLINE

The above discussion focuses on the potential for early-life events to influence the maximally attained FEV_1 during the third decade of life. There is increasing evidence from longitudinal studies, however, that childhood events and exposures can accelerate the rate of decline in the FEV_1 level and induce early expression of chronic respiratory symptoms in both smokers and nonsmokers. Prenatal and postnatal parental smoking increases susceptibility to the ill effects of active smoking in adult life, with smokers who were exposed to parental smoking having greater deficits in FEV_1 than those whose parents did not smoke.[54] Smokers who had lower respiratory illnesses due to respiratory syncytial virus before 3 years of age are more likely to receive a diagnosis of asthma in the third decade of life than smokers without such an early-life history.[55] Women who as young girls lived through the so-called Dutch famine, a circumscribed episode of severe human starvation that occurred between October 1944 and May 1945 in the Netherlands, were more likely to be hospitalized for COPD before 60 years of age than their age peers who were not exposed to famine.[56] These effects were particularly noticeable among active smokers, suggesting that postnatal malnutrition may increase susceptibility to the deleterious effects of smoking.

Evidence from less reliable retrospective studies also suggests that childhood adversity may increase the risk of COPD. As compared with smokers without a history of pneumonia, smokers who recalled having had pneumonia during childhood were 40% more likely to have COPD, and they had an FEV_1:FVC ratio that was significantly lower (0.63 vs. 0.67, $P<0.001$).[57] In a European study, participants who recalled having "childhood disadvantage factors" (i.e., parental or childhood asthma, childhood respiratory infections, or maternal smoking) showed a steeper FEV_1 decline in adult life than those without such factors.[58]

CONCLUSIONS

There has been a major change in our understanding of the natural history and risk factors for COPD, a frequent cause of illness and death. Although smoking is still a major culprit, genetic, environmental, and developmental factors that are associated with diverse biologic mechanisms and that exert their effects during the growing years can both diminish the maximally attained FEV_1 and accelerate FEV_1 decline in adult life, thus increasing the risk of COPD. Prevention of prematurity, and especially of bronchopulmonary dysplasia, is a major public health priority that is made more urgent by its potential role in the pathogenesis of COPD. Promising advances in the development of vaccines against respiratory syncytial virus[59] and of prevention strategies for childhood asthma[60] could markedly decrease the risk of COPD. Efforts to decrease exposure to air contaminants during pregnancy and childhood and to preserve and expand the striking reduction in adolescent smoking during the past 20 years could also decrease the incidence of COPD.

Dr. Martinez reports receiving consulting fees from Compañía Agropecuaria Copeval and grant support from Johnson & Johnson. No other potential conflict of interest relevant to this article was reported.

Disclosure forms provided by the author are available with the full text of this article at NEJM.org.

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Asthma remission: what is it and how can it be achieved?

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Number 7 in the series "Innovations in asthma and its treatment"
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Shareable abstract (@ERSpublications)
As we are moving through a new era of highly effective targeted biologics, macrolides and precision medicine in asthma management, it is logical to consider a paradigm shift in the treatment goals from asthma control to asthma remission https://bit.ly/3N9nEqN

Cite this article as: Thomas D, McDonald VM, Pavord ID, *et al*. Asthma remission: what is it and how can it be achieved? *Eur Respir J* 2022; 60: 2102583 [DOI: 10.1183/13993003.02583-2021].

Received: 28 Sept 2021
Accepted: 17 March 2022

Abstract

Asthma treatment goals currently focus on symptom and exacerbation control rather than remission. Remission is not identical to cure, but is a step closer. This review considers the current definitions of remission in asthma, the prevalence and predictors, the pathophysiology of remission, the possibility of achieving it using the available treatment options, and the future research directions. Asthma remission is characterised by a high level of disease control, including the absence of symptoms and exacerbations, and normalisation or optimisation of lung function with or without ongoing treatment. Even in those who develop a symptomatic remission of asthma, persistent pathological abnormalities are common, leading to a risk of subsequent relapse at any time. Complete remission requires normalisation or stabilisation of any underlying pathology in addition to symptomatic remission. Remission is possible as part of the natural history of asthma, and the prevalence of remission in the adult asthma population varies between 2% and 52%. The factors associated with remission include mild asthma, better lung function, better asthma control, younger age, early-onset asthma, shorter duration of asthma, milder bronchial hyperresponsiveness, fewer comorbidities and smoking cessation or never smoking. Although previous studies have not targeted treatment-induced remission, there is some evidence to show that the current long-term add-on therapies such as biologics and azithromycin can achieve some criteria for asthma remission on treatment, at least in a subgroup of patients. However, more research is required. Long-term remission could be included as a therapeutic goal in studies of asthma treatments.

Introduction

Asthma is a common chronic respiratory disease affecting over 300 million people around the world with significant morbidity and mortality [1]. It is a heterogeneous disease characterised by variable airflow limitation, bronchial hyperresponsiveness (BHR), mucus hypersecretion and airway inflammation, leading to airway narrowing which causes symptoms of wheeze, breathlessness and chest tightness for people with the disease [2]. Persistent or repeated inflammation of the airways may lead to airway structural changes such as epithelial hyperplasia and metaplasia, changes in mucus-secreting cells, subepithelial fibrosis, muscle cell hyperplasia, and angiogenesis. These pathological alterations in the airways may result in a change in composition, distribution, thickness, mass or volume and/or the number of structural components observed in the airway wall. This is believed to contribute to a progressive irreversible loss of lung function referred to as airway remodelling [3–5]. Based on the underlying pathological changes and the corresponding physiological consequences in the airways, people may experience mild, moderate, severe or refractory asthma. Some of these individuals also might be at risk of irreversible airway remodelling and/or fixed airflow limitation.

A fundamental feature of asthma is variability, characterised by inconsistency in the expression of the disease. While it is well recognised that variability in asthma may cause asthma attacks, it is less well understood that some people may spontaneously become asymptomatic with or without resolving their underlying pathological airway impairment and enter into a symptom-free state [6, 7]. This is referred to as "asthma remission". These individuals who remit or resolve the manifestations of asthma constitute a very interesting cohort who have recently gained much attention among the research community [8, 9]. Understanding the mechanisms of remission may shed light on novel therapeutic approaches to asthma. In addition, a proportion of people who have achieved asthma remission or have "grown out of asthma" may relapse later in life. The reason for relapse is unclear, but it might be due to the ongoing dysfunction of underlying pathology. Consequently, these symptom-free patients have a certain risk of relapse, which may occur at any time, and this capricious phenomenon of diminution of disease is called remission [9–11].

Although the term remission is rarely used in the current management of asthma, it is well defined in other chronic inflammatory conditions such as rheumatoid arthritis, Crohn's disease, ulcerative colitis and systemic lupus erythematosus [8, 12]. In asthma, the treatment goals are still minimisation of acute attacks and achievement of symptom control, with less attention paid to achieving remission. Furthermore, although remission in childhood asthma is a common phenomenon [13, 14], remission in adults with asthma is a relatively new concept and a less researched area that has, however, recently gained attention. Since remission is possible as part of the natural history of the disease (*e.g.* childhood asthma remission), it might also be possible to induce remission with treatments. Developing or identifying a treatment that can induce remission in asthma will be a paradigm shift in the asthma management goals, a step closer to the cure. Such success has already been achieved in rheumatoid arthritis, largely with the widespread use of effective targeted therapy using monoclonal antibodies [12, 15]. Effective monoclonal antibody therapies are now increasingly used in asthma [16, 17], raising the hope of asthma remission as a realistic therapeutic goal. In this review, we critically evaluate the current concept of asthma remission, what it constitutes, the prevalence and predictors of remission, the pathophysiology of remission, the possibility of achieving remission in the current clinical environment, and future research directions (figure 1).

Asthma remission: what is it?

Remission is not cure. Cure requires reversing to the normal pathological state of the airways in addition to prolonged absence of symptoms, typically in the absence of ongoing treatment requirements. An objective demonstration of normal airway function, normal airway responsiveness and the absence of any airway pathology suggestive of asthma are required to establish that asthma has been cured. Once cured, no treatment is required. Suboptimal treatment outcomes in asthma are mainly attributed to the fact that a

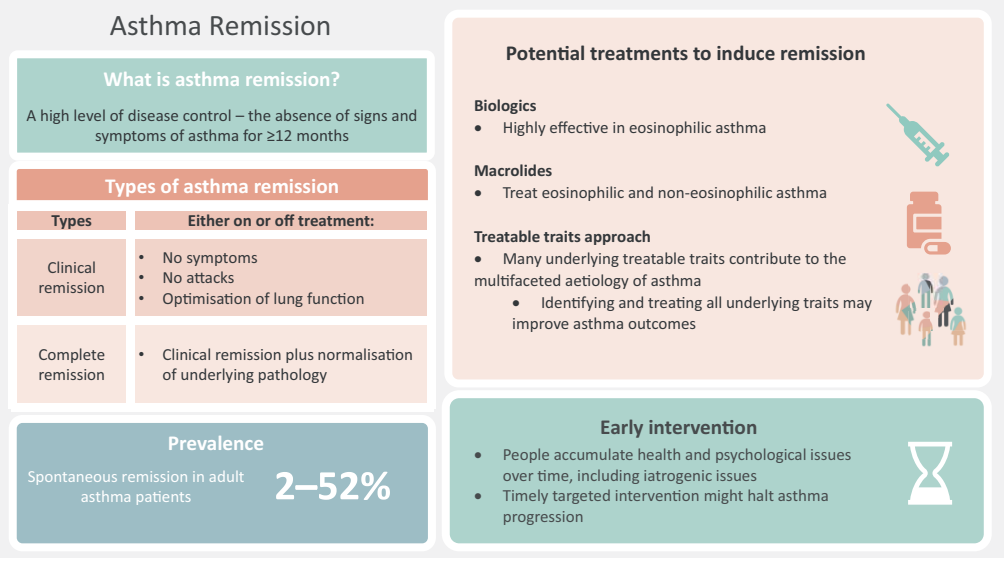

FIGURE 1 A visual summary of remission. Content has been reproduced with permission from the Centre of Excellence in Treatable Traits, originally developed as part of the Centre of Excellence in Treatable Traits (https://treatabletraits.org.au).

large number of factors contribute to the clinical features of asthma and some are not modifiable. Moreover, it is far from clear whether currently available treatments normalise the underlying pathology such as airway remodelling. Hence, a cure for asthma may not be achievable in the current environment.

We may need a more realistic and achievable goal. The absence of signs and symptoms of a disease for a prolonged time with or without normalising underlying pathology may be referred to as remission. It can be a complete remission or partial remission based on the rigidity of the definition (*e.g.* no symptoms, absence of pathology, *etc.*) with or without background treatment [18]. In rheumatology, there was a shift in disease management after the introduction of disease-modifying anti-rheumatic drugs and targeted anti-tumour necrosis factor therapies from a treatment model aiming at disease control to disease remission [19–21]. It is now a logical time to consider a similar paradigm shift in the asthma management goals with the increased understanding of the pathobiology of asthma (*e.g.* the role of epithelial-derived cytokines in asthma) and the introduction of targeted biological therapies (*e.g.* mepolizumab, omalizumab, benralizumab, dupilumab and reslizumab) [9, 22–24].

Current definition

Menzies-Gow *et al.* [8] proposed a generalised framework for asthma remission based on the evidence from other chronic inflammatory diseases with remission definitions (rheumatoid arthritis, Crohn's disease, ulcerative colitis and systemic lupus erythematosus) and considering the components of published definitions of spontaneous asthma remission. The framework derived from the literature was subject to a modified Delphi survey to gain expert consensus on the core elements of an asthma remission definition.

The authors produced four subtypes of asthma remission definitions with varying degrees of rigour in criteria [8]. Clinical remission requires stabilisation of lung function and patient/clinician agreement on remission in addition to absence of significant asthma symptoms and acute attacks for a minimum duration of 12 months, whereas complete remission also requires normalisation of underlying pathology (*e.g.* resolution of airway inflammation) in addition to clinical remission. Both are further subdivided into remission on treatment and off treatment.

Strengths and limitations of current definition

The definition of asthma remission derived by Menzies-Gow *et al.* [8] is comprehensive and covers the complex nature and impacts of the disease, including symptoms, acute attacks and airways pathology, and is applicable across the range of asthma severity. The definition also includes remission on treatment, which is a pragmatic and valuable goal in more severe asthma cases, albeit none of the previous studies that evaluated spontaneous asthma remission included remission on treatment. There are also four independent definitions of remission provided (table 1), and researchers and clinicians have the flexibility to adapt a definition that is achievable and measurable based on their clinical setting and study population.

The Menzies-Gow *et al.* [8] definition for asthma remission is lenient on reliever (rapid-onset β_2-agonist) use, which might be a questionable element. Rapid-onset β_2-agonists are mainly used on an *ad hoc* basis

TABLE 1 Types and measures of asthma remission

Type	Criteria	Assessments
Clinical remission	No symptoms	Sustained absence of significant asthma symptoms established using a validated instrument (*e.g.* ACQ score ⩽1 or ACT score ⩾20); the use of relievers is not permitted during the remission period
	No exacerbations	The use of systemic corticosteroids for exacerbation treatment is not permitted during the remission period; hospitalisation or emergency department visit or unscheduled doctor visit for asthma exacerbation management are also not permitted during the remission period
	Optimisation of lung function	Example: post-bronchodilator FEV_1 ⩾80% predicted
Complete remission	Clinical remission plus normalisation of underlying pathology	No evidence of current inflammation established using either blood eosinophil count (<300 cells·μL^{-1}), sputum eosinophil count (<3%) or F_{ENO} (<40 ppb) [97, 98]; other measures of underlying pathology may include a negative bronchial hyperresponsiveness test (*e.g.* histamine or methacholine provocation tests) or degree of subepithelial fibrosis (subepithelial thickness)

Both clinical and complete remission can be achieved either on treatment or off treatment. ACQ: Asthma Control Questionnaire; ACT: Asthma Control Test; FEV_1: forced expiratory volume in 1 s; F_{ENO}: exhaled nitric oxide fraction. With acknowledgement of [8].

to relieve symptoms when people experience symptoms. Since the absence of symptoms is one of the main criteria for remission in all four definitions, we propose the absence of reliever use also should be included. The authors acknowledge that the framework needs to be further tested in prospective studies, and streamlined based on input from experts, professional bodies and patients, so the opportunity exists to further develop this definition.

Assessment of remission

Recognition of remission requires an assessment of asthma status. Key variables may include evaluation of asthma symptoms and exacerbations, and assessment of lung function and underlying pathology. Table 1 summarises the key variables of asthma remission and measures to assess them.

Several validated tools exist for the assessment of symptoms in asthma; however, these have seldom been applied to the definition of remission. The Asthma Control Questionnaire (ACQ) is a useful tool for reliably assessing symptoms in asthma and has established criteria for symptom control. Mean scores range from 0=no impairment to 6=maximum [25]. HARVEY *et al*. [26] assessed super-response to mepolizumab therapy in severe asthma patients using a cut-off of <1 using the five-item ACQ (ACQ-5), indicating the value of an asthma control measure to address possible remission of symptoms in asthma. The Asthma Control Test (ACT) is another widely used tool to assess symptom control [27]. The total scores range from 5=poor asthma control to 25=complete asthma control. An ACT score >19 indicates well-controlled asthma. The PROSPERO (Prospective Observational Study to Evaluate Predictors of Clinical Effectiveness in Response to Omalizumab) study classified responders as an ACT score ⩾20 [28], whereas TUOMISTO *et al*. [29] used a cut-off of 25 in the Seinäjoki Adult-onset Asthma Study (SAAS). These studies indicate the potential to use asthma control measurements when assessing remission in future work.

Exacerbations, or attacks, are a key outcome in asthma. There are many criteria available to assess exacerbations such as attacks causing hospitalisation, emergency department visit or unscheduled doctor visit. The most widely used is the oral corticosteroid (OCS) burst, which records OCS treatment to manage acute attacks of asthma [26, 30]. Use of OCS for acute attack or long-term disease control is not permitted during the remission period.

Normal or stabilised lung function (*e.g.* normal spirometry assessments) and absence of airway inflammation (*e.g.* reduced blood or sputum eosinophils, exhaled nitric oxide fraction (F_{ENO}), *etc.*) and BHR (*e.g.* histamine or methacholine provocation tests) are the other important factors that researchers and clinicians could consider including in the remission definition.

A broad range of symptom-free periods has been used in previous studies, ranging from 6 months to 3 years with an average of 1 year [10]. The minimum duration of 12 months seems to be reasonable, which will cover the seasonality of the disease activity. The majority of the clinical trials have a 12-month follow-up and hence it is feasible to use in research. However, wherever possible, a longer duration should be considered as disease stabilisation and relapse may depend on the length of remission [31].

The risk of relapse depends on how strict remission is defined. For example, the risk of relapse might be minimal for those who achieved “complete remission off treatment” with an ACT score of 25 for a long period of time [11]. However, it might not be feasible to achieve complete remission in all severities of asthma [10], and adapting a feasible and achievable remission definition based on the study population is important.

The super-responder model

UPHAM *et al*. [32] recently developed an international consensus-based definition for severe asthma super-responders using a modified Delphi process. The definition encompassed two domains: major and minor criteria. The major criteria include an absence of exacerbation, a large improvement in asthma control and cessation of maintenance OCS or weaning to adrenal insufficiency. The minor criteria were composed of a 75% exacerbation reduction, having well-controlled asthma and ⩾500 mL improvement in forced expiratory volume in 1 s (FEV_1). Improvement in three or more criteria (including at least two major criteria) assessed over 12 months is required to meet the super-responder definition. The proposed super-responder definition is more relaxed and looking for improvement rather than normalisation, and hence not meeting the requirements for remission. Two recent studies have identified severe asthma super-responders to biological agents, but they did not use the composite outcome remission [26, 32, 33]. EGER *et al*. [34] identified super-responders after 2 years of treatment with anti-interleukin (IL)-5 agents. The authors used a composite outcome (no chronic OCS use, no OCS bursts in the past 3 months, ACQ score <1.5, FEV_1 ⩾80% predicted, F_{ENO} <50 ppb and complete control of comorbidities, such as chronic

rhinosinusitis, nasal polyps, chronic otitis, allergic rhinoconjunctivitis and atopic dermatitis). Although this definition resembles remission, it was more lenient on OCS use, *i.e.* assessing OCS bursts only in the past 3 months and considering only the current use of maintenance OCS [34]. Remission represents an important step beyond super-response, eliminating exacerbations and asthma symptoms for a sustained period rather than reducing the number of attacks.

Prevalence and predictors of asthma remission

Many studies have evaluated the incidence of spontaneous remission in asthma. The rate varied considerably across studies mainly because of the clinical heterogeneity between study populations, designs, definitions and assessments. For example, age: these studies evaluated a range of age groups including children, adolescents and adults; used several design types such as birth cohort, cross-sectional surveys, extended follow-up of clinical trials and national databases; and applied variable rigour to the definition of remission, *e.g.* clinical and complete, length of remission (6 months to 3 years) and assessment methods (*e.g.* questionnaire-based and objective assessment) [8, 11]. The majority of these studies included a mix of childhood and adult asthma, which most likely caused an overestimation of the remission rate since the rate of remission in childhood asthma is much higher than that in adult asthma [35–37]. The overall incidence rate reported in these studies ranged from 2% to 74% and there was a higher incidence of clinical remission compared with complete remission [11].

Remission in the adult asthma population

A handful of studies (n=14) have evaluated the remission rate and factors associated with remission in the adult asthma population. Of these, five studies exclusively included new adult-onset asthma [29, 38–41]. Although the remaining nine studies included both adult- and childhood-onset asthma [42–50], the participants were adults at the time of enrolment. Tupper *et al*. [39] included 62% adult-onset asthma and 38% childhood-onset asthma, and their report includes only data from the former group. The studies were predominantly conducted in Europe. A majority of the studies (n=11) were longitudinal studies with a baseline assessment and a follow-up assessment. Two studies identified the asthma population from medical records and conducted a follow-up interview [43, 50], and one study was a cross-sectional survey [44]. The characteristics of the studies are presented in table 2.

The follow-up duration ranged from 5 to 33 years. All the adult-onset asthma studies used objective assessments to confirm asthma diagnosis, whereas only one study in the mixed adult/childhood-onset group used objective assessments [46]. The remission period evaluated ranged from 6 months to 3 years. The majority of studies assessed clinical remission (no symptoms and no asthma medications) for a duration of ⩾12 months.

The incidence of remission in the adult-onset asthma population ranged from 2% to 17%, whereas it ranged from 6% to 52% in the other group. Three studies reported a remission rate <10%, eight reported 10–20% and four reported >20%. Four studies included some objective measures of remission and the rate ranged from 2% to 17%. All of these studies assessed spontaneous remission. It is important to note that none of the studies assessed treatment-induced remission, which identifies a significant knowledge gap that needs to be addressed.

The predictors of asthma remission are summarised in table 2. Baseline factors such as mild asthma [41, 46–49], better lung function [29, 38, 41], better asthma control [40, 45], younger age [40, 43], early-onset asthma [43, 44], shorter duration asthma [39, 44], milder BHR [38, 40], no/few comorbidities [40, 41, 43, 44, 49] and smoking cessation or never smoking [41, 46, 48] were consistently associated with remission. The majority of the listed factors are well established due to the fact that these are also inversely related to uncontrolled asthma [2]. An important factor which may influence remission might be the severity of the disease. The disease activity stays much the same over a longer period of time in moderate-to-severe asthma, while mild asthma cases are likely to experience remission [11]. This will have a major impact on the ability to achieve remission based on the population or asthma severity studied.

Pathophysiology of remission

Asthma is a heterogeneous disease. The aetiology and pathophysiology of asthma are complex and poorly understood. Moreover, there is a paucity of research that has sought to understand the pathophysiology of asthma remission. However, some previous research suggests that people with remitted asthma might still have some degree of ongoing inflammation, BHR and airway remodelling [10, 11, 51, 52], which may determine the future risk of relapse [53, 54].

TABLE 2 Summary of studies that have evaluated adult asthma remission: study characteristics, prevalence and predictors of remission

First author [ref.]	Study characteristics	Remission definition	Remission	Associated with remission	Comments
Adult-onset asthma					
Almqvist [38]	n=205; follow-up: 15.3 years; Sweden	No symptoms or asthma medications in past 12 months	11.2%	Better lung function and less severe BHR	Objective assessment of asthma at baseline; population: asthma onset after 20 years; prospective longitudinal study
Tupper [39]	n=78; mean follow-up: 33.3 years; Denmark	No symptoms or asthma medications in past 12 months; F_{ENO} <50 ppb, no bronchodilator reversibility, no airway hyperresponsiveness and no airflow limitation	17%	Shorter duration of asthma at baseline	Objective assessment of asthma both at baseline and follow-up; prospective longitudinal study
Westerhof [40]	n=170; follow-up: 5 years; the Netherlands	No symptoms or asthma medications in past 12 months	15.9%	Independent predictors of remission include less BHR and no nasal polyps; remission was associated with younger age, better asthma control, lower doses of ICS and lower levels of blood neutrophils	Clinically assessed asthma at baseline; prospective longitudinal study
Tuomisto [29]	n=203; follow-up: 12.2 years; Finland	No asthma symptoms, ACT score 25, no asthma medication in last 6 months, no use of oral prednisolone in past 2 years; objective assessment of normal lung function	Clinical: 3%; normalisation of lung function: 2%	Better lung function and lower IgE level both at baseline and follow-up visit	Objective assessment of asthma at baseline and follow-up; asthma onset after adulthood (mean±SD baseline age 46±14 years); prospective longitudinal study
Rönmark [41]	n=250; follow-up: 5.8 years; Sweden	Clinical: no asthma symptoms and no asthma medications in past 12 months; complete: no medications, no symptoms, FEV_1 >80% predicted and PC_{20} >8 mg·mL^{-1}	Clinical: 4.8%; complete: ~3%[#]	Mild disease, normal lung function at onset, absence of allergic sensitisation, rhinitis and being a non-smoker	Objective assessment of asthma; asthma onset after 20 years; prospective longitudinal study
Adult at the time of enrolment					
Traulsen [42]	n=239; follow-up: 9 years; Denmark	No current use of asthma medications and no asthma symptoms in past 12 months	28%	Use of medication at baseline	Questionnaire-based asthma diagnosis and remission; age at enrolment 20–44 years; 19% had asthma and COPD; prospective longitudinal study
Sözener [43]	n=160; follow-up: 7 years; Turkey	Clinical: no current use of asthma medications and no asthma symptoms in past 2 years; complete: no BHR (assessed in 50% of clinical remission cases)	Clinical: 11.3%; complete: of the 9 with BHR assessment, 3 achieved BHR normalisation	Younger age, younger onset, atopy, allergic rhinitis and few comorbidities	Asthma cohort identified from medical records and questionnaire survey at follow-up; mean±SD age at onset 35.8±11.4 years; retrospective cohort followed-up after 7 years
Pesce [44]	n=38 596; follow-up: 70 years; Italy	No asthma attacks in past 2 years and no current use of medications	52%	Presence of hay fever, age at onset and time since asthma onset	Questionnaire-based asthma diagnosis; age at enrolment 20–84 years; cross-sectional cohort

Continued

TABLE 2 Continued

First author [ref.]	Study characteristics	Remission definition	Remission	Associated with remission	Comments
Cazzoletti [45]	n=214; follow-up: 9 years; Italy	No current use of asthma medication, no asthma-like symptoms and no asthma attacks in past 12 months	29.7%	Asthma control at baseline	Questionnaire-based asthma diagnosis and remission; age at enrolment 21–47 years; prospective longitudinal study
Lindström [50]	n=119; follow-up: 20 years; Finland	No asthma symptoms and using no asthma medication in past 3 years	11.8%	Exercise test and spirometry results	Military servicemen in 1987–1990; age at enrolment 18–27 years (mean±SD 20.1±1.4 years); asthma diagnosis based on medical records (symptoms, medication use, lung function and allergy tests); retrospective cohort followed-up after 20 years
Ekerljung [49]	n=295; follow-up: 10 years; Sweden	No symptoms or asthma medications in past 12 months	14.6%	Mild disease and no rhinitis	No objective assessments; population-based questionnaire surveys 10 years apart; age at enrolment 20–69 years; prospective longitudinal study
Holm [48]	n=1153; follow-up: ~8.5 years; Iceland, Norway, Sweden, Sweden, Estonia	No asthma symptoms in 2 consecutive years and no current use of asthma medications	18.6%	Quitting smoking and presence of mild disease at baseline	No objective diagnosis of asthma (*i.e.* self-reported questionnaire-based asthma at baseline); age at enrolment 26–53 years; prospective longitudinal study
de Marco [47]	n=586; follow-up: 9 years; Europe, North America and Oceania	No symptoms and no use of medications in past 12 months	11.9%	Less severe asthma and lowest increase in body mass index	No objective diagnosis of asthma (*i.e.* self-reported questionnaire-based); age at enrolment 20–44 years (mean±SD 34±7 years); prospective cohort study
Rönmark [46]	n=267; follow-up: 10 years; Sweden	No asthma symptoms and no asthma medications in past 12 months	6%	Mild disease and smoking cessation	Objective assessment of asthma; age at enrolment 35–66 years; prospective longitudinal study

BHR: bronchial hyperresponsiveness; F_{ENO}: exhaled nitric oxide fraction; ICS: inhaled corticosteroid; ACT: Asthma Control Test; FEV_1: forced expiratory volume in 1 s; PC_{20}: provocative concentration causing a 20% fall in FEV_1; COPD: chronic obstructive pulmonary disease. #: visually presented in a figure.

Previous reviews have reported that the level of airway inflammation is related to the development of asthma remission over time [10, 55]. Carpaij *et al*. [11] reviewed the studies comparing inflammatory markers in people with asthma remission and current asthmatic and/or healthy individuals. The authors noted that inflammatory markers were generally lower in remission compared with current asthma, but higher compared with healthy individuals, albeit some studies report no difference between groups [11, 56, 57]. Likewise, some previous studies have shown that BHR persists in a considerable proportion of people with symptomatic asthma remission [54, 56, 58]. Chronic or frequent inflammation in asthma may damage the surface epithelium of the airways leading to airway remodelling. A specific characteristic of airway remodelling is thickening of the subepithelial reticular basement membrane resulting from subepithelial fibrosis. It can even occur in the early stages of life and is more prominent in severe disease [59]. The consequences of airway remodelling may include increased BHR, fixed airflow limitation and irreversible loss of lung function. The exact sequence of events that take place during the remodelling process is hard to disentangle and the mechanisms regulating these changes are poorly understood; studies assessing airway remodelling in asthma remission are also scarce. Available evidence suggests that basement

membrane thickening is still present in asthma remission [11, 53, 60]. These findings indicate that some aspects of asthma pathophysiology remain during remission. However, the results should be interpreted with some caution, carefully considering the clinical heterogeneity between studies. For example, factors such as asthma severity of the included population, duration of asthma, duration of remission and rigour of remission definition may modify the underlying pathology and affect the study results [57].

The question remains, how will this ongoing airway inflammation and remodelling affect asthma remission, and how it can be effectively treated to prevent relapse in those who are in clinical remission? Currently, there are no effective treatments that halt or reverse the changes of airway remodelling and its effects on lung function. Existing asthma treatments are aimed at controlling airway inflammation and may subsequently reduce the progress of airway remodelling. However, this approach is only partially successful in treating airway remodelling. The search for novel therapies that can directly target individual components of the remodelling process should be made a priority, which may provide a more effective strategy to prevent or reverse structural changes and restore lung function. The interrelationship between airway remodelling, inflammation and BHR should be clearly evaluated and demonstrated. A clear understanding of the molecular events leading to airway remodelling and factors influencing each of the components of remodelling may facilitate the development of new treatment approaches [5].

Another important element contributing to asthma pathobiology is genetic. Although previous genome-wide association studies have identified multiple genes associated with asthma origin and remission, more research is required in this area [61].

Enablers and challenges of achieving asthma remission

The multifaceted aetiology and the complex pathology make asthma a difficult disease to cure. Although advances in asthma treatments have improved the symptoms of asthma and reduced the frequency of attacks and the overall burden experienced by people with asthma, the treatments and advances in care still fail to cure the disease, and to date, asthma treatment has mainly focused on disease control over cure. A few studies have identified super-responders to biological agents [26, 33], a step closer to remission [32], but these failed to evaluate their effectiveness in inducing remission. Previous work which demonstrated that a certain percentage of individuals spontaneously outgrow the disease for a certain period of time, or the rest of their lives, is promising. The question is whether the proportion of individuals attaining remission can be increased or is it possible to prolong the remission period with treatment?

The lack of disease-modifying agents in asthma is a weakness. Reversing the remodelling that has already occurred might be a challenge that requires the development of new therapies. Although the person with asthma may be asymptomatic in remission, the underlying disease might be active and continuing to progress, and treatment with ongoing anti-inflammatory agents might be useful [18]. This is an important lesson in asthma, since history reveals that minimising symptoms with β-agonists without addressing the underlying airway inflammation is associated with severe acute attacks and asthma deaths. This lesson would argue that a proposed clinical definition of remission, and seeking treatments to induce remission, may be risky without an accompanying measure of airway pathophysiology in asthma.

Treatments to induce remission

The introduction of inhaled corticosteroids (ICS) in the 1980s has revolutionised asthma treatment and currently they are the cornerstone of asthma therapy. Although ICS are generally very effective in reducing asthma symptoms, preventing exacerbations and improving lung function in mild-to-moderate asthma, their ability to alter the natural history of asthma and effectiveness on disease progression are questionable [62, 63]. Likewise, the introduction of a combination of inhaled ICS and long-acting β_2-agonist (LABA) further improved asthma management, but their effectiveness on disease progression needs to be evaluated [64].

Biologics

Over the past decade, greater awareness of the underlying biology of asthma has led to the development of a new range of treatment targets. Researchers understood that the multifaceted aetiology of asthma includes many phenotypes caused by a variety of pathophysiological mechanisms referred to as endotypes. Two inflammatory subtypes are currently identified: the type 2 (T2)-high and T2-low inflammatory pathways. The inflammatory cascade is often initiated by inhaled allergens, microbes or pollutants that interact with the airway epithelium. This interaction leads to the activation of mediators such as thymic stromal lymphopoietin (TSLP), IL-25 and IL-33. Subsequent immune cell activation (type 2 innate lymphoid cells, T-helper 2 cells, *etc.*) releases IL-4, IL-5 and IL-13, leading to the attraction and activation of basophils, eosinophils, mast cells and immunoglobulin class switching and secretion of IgE by B-cells. This inflammatory process leads to bronchoconstriction, BHR, mucus production and subsequent airway

remodelling [65]. Current biological agents such as omalizumab, mepolizumab, benralizumab, reslizumab and dupilumab mainly act on the effector molecules of the T2 inflammatory cascade (*i.e.* IL-4, IL-5, IL-13 and IgE) [66]. Newer agents targeting the upstream targets of T2 inflammation (*i.e.* TSLP, IL-25 and IL-33) are under development [67]. Biologics are a promising tailored treatment approach for eosinophilic asthma with a high potential to achieve disease remission in at least a subset of patients [26, 30]. The improved efficacy achieved when biologics are added to previous treatment may meet the criteria for remission. However, it is challenging to evaluate the effectiveness of biologics in inducing remission using the current evidence since the majority of the previous studies aimed for disease control rather than remission.

Menzies-Gow *et al.* [9] evaluated the efficacy of biologics in inducing remission using the current evidence. The authors critically reviewed the current studies, and retrospectively extracted and constructed data related to remission. They used the remission definition "clinical remission on treatment". The authors concluded that biologics are highly effective in reducing exacerbations and symptoms, and in improving lung function. These treatments achieve some, but in most cases not all, criteria for remission.

A recent *post hoc* analysis of dupilumab found that 20% of the dupilumab-treated participants achieved clinical remission (no exacerbation, ACQ-5 score <1.5 and post-bronchodilator FEV_1 ⩾80%) at 12 months [68]. Hence, dupilumab has the potential to achieve asthma remission in a subset of people with severe asthma. However, the study was a *post hoc* analysis and it is important to include this outcome as an objective in future study designs.

The Harvey *et al.* [26] super-responder study found that 31% of severe asthma patients treated with mepolizumab achieved well-controlled asthma (ACQ-5 score <1). Of those, 67% were free of OCS burst and 79% were free of maintenance OCS therapy. Although the study failed to identify the proportion of patients achieving the composite outcome remission, it provided an insight into the potential of mepolizumab in achieving remission in a subset of patients with severe asthma. The Eger *et al.* [34] super-responder model was more or less similar to the proposed remission definition and 14% (16 out of 141) of patients treated with anti-IL-5 agents achieved the composite criteria. Shorter asthma duration, higher FEV_1 and adult asthma onset predicted super-response.

Biologics were initially used in rheumatology for end-stage severe disease conditions, but were later integrated at an earlier stage of disease management to modify disease progression, which resulted in a durable and treatment-free remission in some patients [69, 70]. Asthma clinicians and researchers can learn from this experience and identify the future role of biologics in the management of asthma. Achieving remission in mild-to-moderate cases may help to hinder the further deterioration of airway remodelling and lung function decline, leading to more severe and uncontrolled asthma. Conceptually, timely introduction of biologics therapy may weaken the inflammatory process at the earlier stages of disease activity, restricting the exposure of inflammatory mediators to the airway wall, lowering the potential for airway remodelling and halting the disease progression.

Since it is believed that underlying inflammation has a role in relapse, continuing the treatment with biologics might be an option to prolong remission and prevent relapse [71], although this needs to be evaluated in clinical trials. The dosing frequency of biologics is convenient, requiring administration only once a fortnight, month or every 2 months, depending on the treatment. It might also be logical to think about and explore the possibility of stopping all asthma treatments except biologics in a patient who has achieved remission with biological therapies. Continuing ongoing treatment with biologics may help to control underlying inflammation and subsequent relapse. In the COLUMBA (Open-label Long Term Extension Safety Study of Mepolizumab in Asthma Subjects) trial, 33% of participants did not experience exacerbation during the average 3.5-year study period [72]. Moreover, the COMET study assessed the clinical impact of stopping mepolizumab after 3 years of use. The authors concluded that stopping mepolizumab was significantly associated with a shorter duration to first clinically significant exacerbation and reduced asthma control [73].

The effect of biologics on airway remodelling needs to be investigated. Previous research reported some beneficial effects [74–76]. A recent study found anti-IL-13 monoclonal antibody lebrikizumab reduced the degree of subepithelial fibrosis and T2-high biomarkers in addition to improved lung function [75]. Anti-IgE monoclonal antibody omalizumab also improved airway structure and decreased inflammatory markers in many previous studies [76–81], although one recent small study reported no benefit on airway remodelling [82]. Larger studies with longer follow-up are needed to show whether biologics can truly maintain improved airway structure.

Additionally, these agents are relatively newer therapies and their long-term safety profiles are still emerging. Moreover, the long-term consequences of blocking a biological pathway for the lifetime in an individual need to be explored.

Macrolide antibiotics

Low-dose long-term azithromycin (a macrolide antibiotic) significantly reduced asthma attacks in both eosinophilic asthma and non-eosinophilic asthma [83, 84]. Although azithromycin meets some criteria for remission (*e.g.* more than half of the patients in the azithromycin group did not experience attacks during the 12-month study period and azithromycin treatment improved asthma control, measured using ACQ-6), further research is required to evaluate its effectiveness in inducing sustained remission. Azithromycin has anti-inflammatory and immunomodulatory properties and is cost-effective, and current guidelines recommend its use in severe asthma [23, 85, 86]. Azithromycin also could be considered for non-eosinophilic asthma in which no other promising treatment options are currently available. However, concerns such as the potential for anti-microbial resistance and side-effects, including cardiac, sensory and gastrointestinal effects, may limit its widespread use [86, 87].

Although the exact mechanism of action of azithromycin in asthma is yet to be determined, it is believed that azithromycin operates by non-T2 pathways [88]. The alternate mechanism of action of azithromycin provides an opportunity to combine it with biologics, and that might provide additional benefits and may address residual disease burden (ongoing exacerbations despite treatment) of individual treatment.

The treatable traits concept

Many underlying treatable conditions contribute to the multifaceted aetiology of asthma, deterioration of health-related quality of life in patients and suboptimal response to treatment [89]. The most prominent underlying risk factors in severe asthma include airflow limitation, eosinophilic and non-eosinophilic inflammation (also termed T2-high and T2-low disease), obstructive sleep apnoea, vocal cord dysfunction, inhaler device polypharmacy and non-adherence, upper airway disease, physical inactivity and obesity, systemic inflammation, anxiety and depression, and being exacerbation prone [90–92]. These treatable underlying conditions are referred to as treatable traits [93, 94]. Researchers identified an average of 10 treatable traits in severe asthma patients [89, 91, 92]. The treatable traits concept has several useful implications for asthma remission. Since traits such as comorbidity and smoking are associated with remission, and some underlying comorbid conditions may trigger asthma-like symptoms and/or worsen asthma severity, it is possible that targeting treatable traits may increase the chance of asthma remission; however, this approach should be assessed in clinical studies. Remission includes the absence of asthma symptoms. It is hard to disentangle the symptoms caused by airflow limitation and other comorbid conditions, such as vocal cord dysfunction, and this may lead to inappropriate asthma assessment or symptom misattribution [95]. A treatable traits assessment will identify these other conditions, and identify and treat symptom misattribution, leading to a more accurate assessment of remission. Current treatments are mainly focused on airflow limitation, airway inflammation and exacerbation control, and the other traits are largely neglected. To achieve remission in severe asthma patients, we propose identifying and treating all underlying behavioural and biological treatable risk factors, so that their effect on asthma is minimised. It is crucial considering the absence of comorbidities was one of the key variables associated with remission in multiple spontaneous remission studies.

The trajectory of remission

Complete remission off treatment is the ultimate goal of asthma management and it essentially constitutes a cure. It might not always be achievable and measurable in the current environment, but nevertheless should remain as a goal. Aiming for complete remission in populations with early-onset and mild asthma, and aiming for clinical remission on or off treatment in more severe and treatment-refractory asthma populations, might be a practical and achievable approach.

The exact trajectory of remission still needs to be explored and defined. However, in most cases, achieving clinical remission on treatment might be the initial step and then aiming for complete remission. Hence, the initial aim of achieving remission is to eliminate the exacerbations and symptoms, and halt disease progression and further damage to the airway wall, which will help the people to live a normal life. Subsequently steps should then aim to normalise the impairment already caused by the disease.

Future research should explore the possibility of withdrawing treatment after achieving remission and it may not be possible in more severe cases, at least in the current scenario. Identifying the predictors and markers of successful treatment withdrawal will assist clinicians in trying deprescribing.

TABLE 3 Asthma remission: future research questions

Definition	
1	Does the definition of remission require measurement of inflammation?
2	How do asthma control and severity relate to asthma remission?
Treatment-induced remission	
3	Is treatment-induced remission possible?
4	How does treatment-induced remission compare with spontaneous asthma remission, based on prevalence, predictors and risk of relapse?
5	Is it possible to increase the proportion of people achieving remission with treatment?
6	Is it possible to prolong the remission period with treatment?
7	What are the rates of remission with MART and step therapy?
8	Does prolonging asthma treatment after control is achieved increase the chance of asthma remission?
Trajectory of remission	
9	How long should the treatment be continued to achieve remission? When to move from one stage to another?
Pathophysiology	
10	What are the molecular events leading to airway remodelling?
11	Can airway remodelling be treated?
12	Does treating airway remodelling induce remission?
Relapse	
13	How does ongoing airway inflammation and remodelling affect relapse?
14	How can inflammation/remodelling be effectively treated to prevent relapse in those who are in clinical remission?
Biologics and asthma remission	
15	What is the prevalence of remission after biologics therapy for asthma?
16	Are biologics more effective than inhaled preventers at achieving asthma remission?
17	Does the early introduction of biologics modify the trajectory of asthma and halt the disease progression?
18	Does continuing the treatment with biologics prolong remission and prevent relapse?
19	Is it possible to stop all asthma treatments except biologics in a patient who has achieved remission with biological therapies?
Macrolide therapy	
20	What is the prevalence of remission after macrolide therapy for asthma?
Treatable traits	
21	Does targeting treatable traits increase the chance of asthma remission?

MART: maintenance and reliever therapy.

The way forward

The question arising is in what direction do scientists need to delve to discover a definitive cure for asthma? Is there a silver bullet to treat asthma? The current answer is no. However, observations of spontaneous asthma remission (table 2) offer hope that this may be possible. An analysis of patients treated with available treatment options such as biologics and macrolides might be useful to assess the proportion of people meeting the criteria for remission with these treatments. Better characterisation of patients responding to available therapies will help to streamline the treatment (*e.g.* some may respond to a particular biological agent and some may respond to macrolides or emerging therapies). Treatment should also target other modifiable underlying risk factors. A combination of therapies could also be considered to cover the multifaceted aetiology of asthma.

It is also important to identify the areas of remission that are met and not met with current treatments, which will help to identify the unmet areas for further research [9]. Additionally, it is also crucial to identify the factors associated with lung function decline and develop treatments that could alter disease progression. Furthermore, evaluating the effect of early intervention to halt the onset of asthma or disease progression is also important as an early intervention might halt or delay the progression of the disease [96]. This is vital as people accumulate health and psychological issues over time, including iatrogenic issues, and timely intervention might radically modify this process. Table 3 presents a number of critical research questions associated with asthma remission. Of those, the priority might be producing an international consensus on the remission definition.

Asthma guidelines in the future should also include a definition for remission as a treatment goal that could be implemented by researchers. Nevertheless, by acknowledging the aforementioned factors and evaluating the promising research skyline, we are much closer to reaching the treatment goal of remission in asthma management.

Previous articles in this series: *No. 1:* Asher MI, García-Marcos L, Pearce NE, *et al.* Trends in worldwide asthma prevalence. *Eur Respir J* 2020; 56: 2002094. *No. 2:* Hinks TSC, Levine SJ, Brusselle GG. Treatment options in type-2

low asthma. *Eur Respir J* 2021; 57: 2000528. *No. 3:* O'Byrne PM, Reddel HK, Beasley R. The management of mild asthma. *Eur Respir J* 2021; 57: 2003051. *No. 4:* Pijnenburg MW, Frey U, De Jongste JC, *et al.* Childhood asthma: pathogenesis and phenotypes. *Eur Respir J* 2022; 59: 2100731. *No. 5:* Riccardi D, Ward JPT, Yarova PL, *et al.* Topical therapy with negative allosteric modulators of the calcium-sensing receptor (calcilytics) for the management of asthma: the beginning of a new era? *Eur Respir J* 2022; 60: 2102103. *No. 6:* Mortimer K, Reddel HK, Pitrez PM, *et al.* Asthma management in low- and middle-income countries: case for change. *Eur Respir J* 2022; 60: 2103179.

Conflict of interest: D. Thomas has nothing to disclose. V.M. McDonald reports grants and personal fees from GSK and AstraZeneca, and personal fees from Menarini, outside the submitted work. I.D. Pavord reports speaker's honoraria for sponsored meetings from AstraZeneca, Boehringer Ingelheim, Aerocrine, Almirall, Novartis, Teva, Chiesi, Sanofi, Regeneron and GSK; payments for organising educational events from AstraZeneca, GSK, Sanofi, Regeneron and Teva; honoraria for attending advisory panels with Genentech, Sanofi, Regeneron, AstraZeneca, Boehringer Ingelheim, GSK, Novartis, Teva, Merck, Circassia, Chiesi, Knopp, Almirall, Dey Pharma, Napp Pharmaceuticals, RespiVert and Schering-Plough; sponsorship to attend international scientific meetings from Boehringer Ingelheim, GSK, AstraZeneca, Teva, Sanofi, Regeneron and Chiesi; grants from Chiesi to support a Phase II clinical trial in Oxford; and is co-patent holder of the rights to the Leicester Cough Questionnaire and has received payments for its use in clinical trials from Merck, Bayer and Insmed. P.G. Gibson reports grants and personal fees from GSK and AstraZeneca, and personal fees from Novartis, Chiesi and Sanofi, outside the submitted work.

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Published in:
Pharmacology & Therapeutics

DOI:
10.1016/j.pharmthera.2019.05.002

Publication date:
2019

Pharmacology & Therapeutics 201 (2019) 8–24

Contents lists available at ScienceDirect

Pharmacology & Therapeutics

journal homepage: www.elsevier.com/locate/pharmthera

A review on the pathophysiology of asthma remission

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ARTICLE INFO

Available online 8 May 2019

Keywords:
Clinical asthma remission
Complete asthma remission
Predictors
Prevalence
Biomarkers
Airway remodeling

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.

Contents

Abbreviations: ALL, Acute lymphocytic leukaemia; BHR, Bronchial hyperresponsiveness; BMI, Body mass index; $FEF_{25-75\%}$, Forced expiratory flow at 25-75% of the expired forced expiratory volume; FEV_1, Forced expiratory volume in one second; ICS, Inhaled corticosteroids; LRTI, Lower respiratory infection; PC_{20}, Provocative concentration (e.g. histamine, methacholine, adeno-'5-monophosphate) causing 20% a drop of FEV_1; SABA, Short-acting beta-agonist; SNP, Single nucleotide polymorphism; SPT, Skin prick test.

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https://doi.org/10.1016/j.pharmthera.2019.05.002
0163-7258/

1. 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 (Bateman et al., 2018). Current treatments fail to cure the disease. Nevertheless, it has been reported that asthma can go into spontaneous remission (Carpaij et al., 2017; Vonk et al., 2004), 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 (Broekema et al., 2011). In fewer cases, subjects go into complete asthma remission, additionally having no pulmonary function impairment or bronchial hyperresponsiveness (Carpaij et al., 2017; Vonk et al., 2004). To date, most discussion of asthma treatment goals revolves around disease control (Bateman et al., 2008; Taylor, Cowan, Greene, Willan, & Sears, 2005; Upham & James, 2011), 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.

2. 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 (Bronnimann & Burrows, 1986; Butland & Strachan, 2007; Carpaij et al., 2017; Vonk et al., 2004). This resembles "remission" of cancer (National Cancer Institute, 2018), 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 (Tiippana-Kinnunen, Paimela, Laasonen, & Leirisalo-Repo, 2010;Upham & James, 2011), inflammatory bowel disease (Vidal et al., 2006) and multiple sclerosis (Steinman, 2014). 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.

2.1. Symptom perception

The asthmatic's perception of the severity of symptoms has a dominant important role in the effective management of asthma (Banzett, Dempsey, O'Donnel, & Wamboldt, 2000). 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 (Still & Dolen, 2016). 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 (Still & Dolen, 2016). In other words, individuals with remitted asthma could experience symptoms differently than family and associates would judge them.

2.2. The 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 (Upham & James, 2011). 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 (Upham & James, 2011). 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 (van den Toorn, Overbeek, Prins, Hoogsteden, & De Jongste, 2002), while healthy non-asthmatic individuals would not experience dyspnea (Basoglu et al., 2005). 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.

2.3. 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.

2.4. 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 (Vonk et al., 2004). Defining complete asthma remission results in fewer subjects who meet these criteria (Carpaij et al., 2017; Panhuysen et al., 1997; Sears et al., 2003; Vonk et al., 2004; Wang, Datta, Weiss, & Tantisira, 2018). 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 (Broekema et al., 2011; Vonk et al., 2018). Second, the risk of asthma relapse is lower in complete remission subjects:

Table 1
Various definitions, prevalence rates and factors associated with asthma remission.

Ordered in quality of design, age of enrollment and follow-up

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 (Covar et al., 2010; Wang et al., 2018)	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 (Assar, Idani, Monajemzadeh, Ganai, & Rahim, 2013)	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
Outpatient cohort of Hacettepe University Pediatric Allergy and Asthma Unit, Turkey (Sekerel et al., 2006)	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(Arshad et al., 2014; Soto-Ramírez et al., 2013; Zhang et al., 2018)	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
					Clinical remission, i.e. PC_{20} methacholine <8mg/ml	11% (18Y)	- Less BHR at baseline - Less atopy
					Complete remission, i.e. PC_{20} methacholine ≥8mg/ml	10% (18Y)	
Outpatient cohort of Marmara University Pediatric Allergy and Immunology Department, Turkey (Aydogan et al., 2013)	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
					Clinical remission, i.e. PC_{20} methacholine <8mg/ml, negative SPT	16% (16Y)	- Less BHR at baseline - Absence of rhinitis - Higher childhood FEV_1
					Complete remission, i.e. PC_{20} methacholine ≥8mg/ml, negative SPT	34% (16Y)	- Higher childhood $FEF_{25-75\%}$
Obstructive Lung Disease in Northern Sweden Studies (OLIN), Sweden (Andersson et al., 2013; Bjerg & Rönmark, 2008)	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	Prospective cohort with **21**-year clinical FU. Asthma: registered at outpatient clinic, ≥3 episodes of wheezing.	55	5-14Y	No signs of: asthma symptoms Medication use: no	Remission	**16**% (30Y)	- Male sex

(Kjellman & Gustafsson, 2000)	High quality: reasonably defined asthma diagnosis, well-defined and clinically assessed asthma remission diagnosis.			asthma-medication Period: 1 year			
Outpatient cohort of Pediatric pulmonology department of the University Medical Center Groningen, The Netherlands (Vonk et al., 2004)	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 Clinical remission, i.e. PC_{20} methacholine ≤16mg/ml or FEV_1 % pred. ≤90% Complete remission, i.e. PC_{20} methacholine and >16mg/ml, FEV_1 % pred. >90%	**52**% (32-42Y) 30% (32-42Y) 22% (32-42Y)	- Higher baseline FEV_1 - Higher increase in FEV_1 - Less pack years in adulthood
Outpatient cohort of Pediatric pulmonology department of the University Medical Center Groningen, The Netherlands (Carpaij et al., 2017)	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 Clinical remission, i.e. FEV_1 % pred. ≤90% or PC_{20} methacholine ≤9.8mg/ml Complete remission, i.e. FEV_1 % pred. >90% and PC_{20} methacholine >9.8mg/ml	**18**% (25Y) **40**% (49Y) 11% (25Y) 30% (49Y) 7% (25Y) 10% (49Y)	- Not wheezing during cold - No pneumonia in childhood - Dusty house in childhood - Leukemia in family history - No FEV_1 <80% in childhood - Having pets in childhood - Not described - Not described - Not wheezing during cold - No maternal atopy - Leukemia in family history - Having a higher FEV_1/FVC - SPT positivity to mould - Not found
Adult-Onset Asthma and Inflammatory Subphenotypes (ADONIS), The Netherlands (Westerhof et al., 2018)	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 Clinical remission, i.e. PC_{20} methacholine <4mg/ml Complete remission, i.e. FEV_1 % pred. >80% and PC_{20} methacholine >4mg/ml	**16**% (+5Y) 6% (+5Y) 10% (+5Y)	- Lower ICS dosage at onset - Less BHR at baseline - No nasal polyps - Less blood neutrophils
Outpatient cohort of *Department of Chest Diseases, Ankara University School of Medicine, Turkey* (Sözener, Aydın, Mungan, & Mısırlıgil, 2015)	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 Clinical remission, i.e. positive methacholine test Complete remission, i.e. negative methacholine test	**11**% (53Y) 4% (53Y) 2% (53Y)	- Younger age of onset - Not described - Not described
Dunedin Multi-disciplinary Health and Development Study (DMHDS), New Zealand (Sears et al., 2003)	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 Clinical remission, i.e. PC_{20} methacholine ≤8mg/ml or reversibility ≥10% at any assessment from 9-21 Y Complete remission, i.e. PC_{20} methacholine >8mg/ml and reversibility <10% at any assessment from 9-21 Y	**15**% (26Y) 10% (26Y) 5% (26Y)	- Not described
Childhood Asthma Study (CAS), USA (Limb et al., 2005)	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 immuno-therapy*	**15**% (23Y)	- Lower blood IgE - Fewer positive SPT's
Melbourne Asthma Study (MAS), Australia (Horak et al., 2003; Martin, McLennan, Landau, & Phelan, 1980; Tai et al., 2014; Williams & McNicol, 1969)	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.	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)	- Mild symptoms in childhood - Male sex - Mild symptoms in childhood - No childhood hayfever

(continued on next page)

Table 1 (*continued*)

Ordered in quality of design, age of enrollment and follow-up

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
	Moderate quality: debateable asthma definition, well-defined and clinically assessed asthma remission diagnosis, multiple follow-up visits.						- No childhood eczema - - No SPT positivity
Outpatient cohort Asthma clinic at Beatrix-oord hospital Haren, The Netherlands (Panhuysen et al., 1997)	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* Clinical remission, i.e. PC_{20} histamine ≤4mg/ml or FEV_1 % pred. ≤90% Complete remission, i.e. PC_{20} histamine >4mg/ml and FEV_1 % pred. >90% **5% used pulmonary medication*	**40**% (48Y) 25% (48Y) 11% (48Y)	- Male sex - Younger age of onset - Higher baseline FEV_1 - Less BHR at baseline
Military service men in 1987–1990 referred to the Central Military Hospital, Finland (Lindström et al., 2012)	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
European Community Respiratory Health Survey II (ECRHSII), Europe, North America, Oceania (de Marco et al., 2006)	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 (Traulsen, Halling, Bælum, Davidsen, & Miller, 2018)	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 (Tuomisto et al., 2016)	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 Clinically assessed, i.e. FEV_1 % pred. >80%, FEV_1/FVC >70%, reversibility <12%, FeNO ≤20ppb	**6**% (58Y) 3% (58Y)	- Higher baseline FEV_1/FVC - Lower blood IgE - Not described
Lung Disease in Northern Sweden study (OLIN), Sweden (Rönmark et al., 1999)	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* Clinically assessed, i.e. FEV_1 % pred. ≥80%* **Including few subjects with persistent wheeze and medicine use*	**6**% (+10Y) 4% (+10Y)	- Younger age of onset - Mild asthma in adulthood - Cessation of smoking
Environment and Childhood Asthma (ECA) Study in Oslo, Norway (Carlsen et al., 2006)	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 (Guerra et al., 2004)	Prospective cohort with **16**-year clinical FU. Asthma: reporting presence of wheezing >3 episodes in	166	2Y	No signs of: wheeze Medication use: not	Remission	**42**% (13-16Y)	- No obesity - No early onset of puberty

	previous year in at least on survey or physician diagnosed. Lower quality: debatable definition of asthma, undefined medication use in asthma remission.			defined Period: 1 year			- No childhood sinusitis - No positive SPT
Comprehensive medical record database of the Olmsted Medical Center, USA (Javed et al., 2013)	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 (Bacopoulou et al., 2009)	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 (Taylor et al., 2005)	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) 25% (26Y)	- Older age of onset - Higher baseline FEV_1 - Higher baseline FEV_1/FVC - Less BHR at baseline - Less reversibility - Not described
Comprehensive medical evaluation for eligibility for national service between 1999 and 2008, Israel (Cohen et al., 2015)	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 (Bronnimann & Burrows, 1986)	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 (Blair, 1977)	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
European Community Respiratory Health Survey I (ECRHSI), Europe, North America, Oceania (de Marco et al., 2004)	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	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

(continued on next page)

Table 1 (*continued*)

Ordered in quality of design, age of enrollment and follow-up

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
	record of asthma.						
Tasmanian Longitudinal Health Study (TAHS, Australia (Burgess et al., 2011)	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 (Settipane, Greisner, & Settipane, 2000)	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 (Pesce et al., 2015)	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
Italian Study on Asthma in Young Adults (ISAYA), Italy (Cazzoletti et al., 2014)	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 (Holm et al., 2007)	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 (Omori et al., 2017)	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

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

one quarter compared to two-third in clinical asthma remission subjects (Carpaij et al., 2017).

3. Prevalence of asthma remission

3.1. The prevalence of clinical and complete 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 (Bateman et al., 2018), 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 (Martinez, 2002).

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 (World Health Organization, 2017), the remission proportion is highest in this age sub-group (Bronnimann & Burrows, 1986; de Marco, Pattaro, Locatelli, & Svanes, 2004; Pesce et al., 2015), and elucidating this phenomenon within this population is of highest prognostic value (Wang et al., 2018). 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 (Westerhof, Coumou, de Nijs, Weersink, & Bel, 2018). 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 (Aydogan et al., 2013; Westerhof et al., 2018) . 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.

4. 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 1younger 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 (Bateman et al., 2018). In the following section, some of these factors are further discussed.

4.1. Male sex

One of the acknowledged factors associated with asthma remission is male sex (Andersson et al., 2013; Arshad et al., 2014; Burgess et al., 2011; Kjellman & Gustafsson, 2000; Panhuysen et al., 1997; Tai et al., 2014). 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 (Postma, 2007). 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 (Jenkins et al., 2006) and early-onset menarche (McCleary, Nwaru, Nurmatov, Critchley, & Sheikh, 2018; Postma, 2007; Salam, Wenten, & Gilliland, 2006). Additionally, 30–40% of female asthmatics experience perimenstrual asthma worsening (Melgert, Ray, Hylkema, Timens, & Postma, 2007; Vrieze, Postma, & Kerstjens, 2003). The TRIALS study assessed associations of transition though puberty with asthma remission in 2,230 male and female subjects (Vink, Postma, Schouten, Rosmalen, & Boezen, 2010). 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 (Melgert et al., 2007); 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 (Castro-Rodríguez, Holberg, Morgan, Wright, & Martinez, 2001). And last, methacholine hyperresponsiveness is more severe in the post-pubertal female asthmatics compared to their male peers (Tantisira et al., 2008). 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 (Salam et al., 2006), while another found no association (Jenkins et al., 2006). In contrast, oral contraceptive pills have also been associated with an increased risk for asthma (Macsali et al., 2009; Salam et al., 2006), 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 (Guthikonda et al., 2014).

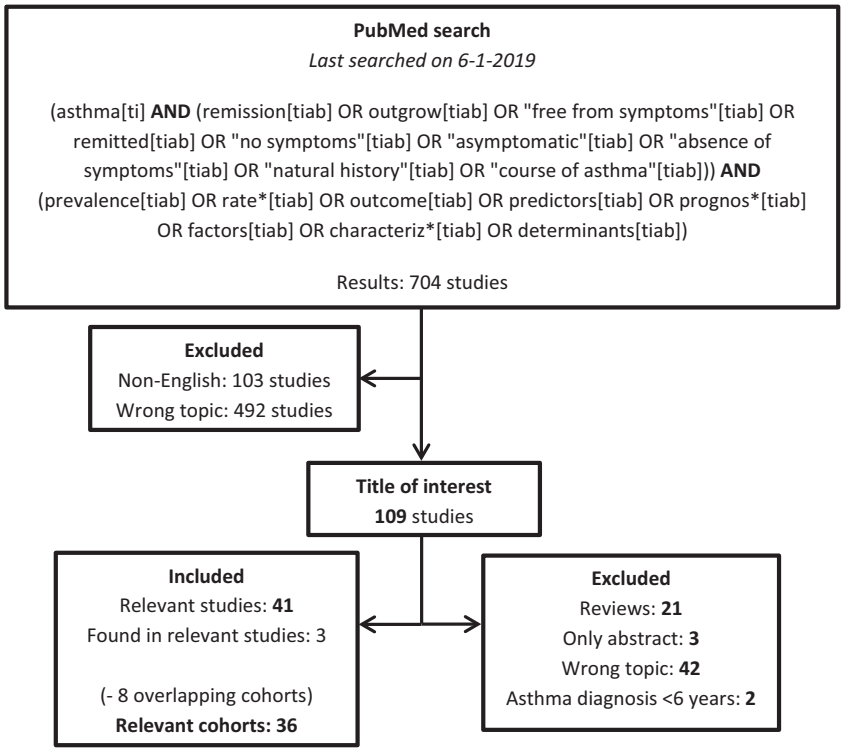

Fig. 1. PubMed search algorithm.

4.2. Severity of asthma

The severity of asthma-onset is a recognized factor influencing the outcome of asthma remission (Bronnimann & Burrows, 1986; Rönmark, Jönsson, & Lundbäck, 1999; Tai et al., 2014). In moderate and severe cases of asthma, the disease activity stays much the same over long periods of time (Chen, FitzGerald, Lynd, Sin, & Sadatsafavi, 2018; Sears et al., 2003; Tai et al., 2014), 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 (Tai et al., 2014). 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.

Very recently, authors of the CAMP-trial assessed whether clinical features in childhood asthmatics could predict asthma outcome later in life (Wang et al., 2018). 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 (Carpaij et al., 2019).

4.3. 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 (Burgess et al., 2011; Carpaij et al., 2017; Strachan, Butland, & Anderson, 1996). This is in agreement with the hypothesis that childhood lower respiratory tract infections (LRTI), such as pneumonia, could influence asthma persistence (Burgess et al., 2012; Castro-Rodríguez et al., 1999) or trigger its inception (Thomas, Lemanske, & Jackson, 2014). 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 (Castro-Rodríguez et al., 1999). 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 (Burgess et al., 2012). These findings suggest a balance between infections and asthma persistence in children.

Other pediatric studies relied on radiologically diagnosed infiltrations (Backman, Piippo-Savolainen, Ollikainen, Koskela, & Korppi, 2014; Castro-Rodríguez et al., 1999; Clark, Coote, Silver, & Halpin, 2000), 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 (Castro-Rodríguez et al., 1999). Here, children with pneumonia had lower levels of FEV_1 and $FEF_{25-75\%}$ 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 (Coumou, Westerhof, de Nijs, Amelink, & Bel, 2018). 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 (Gern & Busse, 2002; Matricardi et al., 2000). 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 (Clark et al., 2000). It is highly probable that LTRIs are merely a sign of underlying asthma susceptibility (Backman et al., 2014). 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 (Martinez, 2005; Openshaw, Dean, & Culley, 2003). 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.

4.4. Leukemia

We have previously shown that another factor associated with asthma remission is having a positive family history for leukemia (Carpaij et al., 2017). 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) (Schüz, Morgan, Böhler, Kaatsch, & Michaelis, 2003; Wen et al., 2000). Various studies found a reduced chance to develop childhood asthma while affected by ALL (Jourdan-Da Silva et al., 2004; Rudant et al., 2010; Wen et al., 2000). Children born by caesarean delivery – causing a deviant immune-maturation - developed more asthma and ALL than children delivered via the vaginal route (Sevelsted, Stokholm, Bønnelykke, & Bisgaard, 2015). A meta-analysis found a significant pooled odds-ratio of ALL in atopy (0.69 ,95% CI 0.54 – 0.89) (Linabery, Jurek, Duval, & Ross, 2010), 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 ($P = 0.45$) (Zhou & Yang, 2015). 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 (Wiemels, 2012), 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 (Wiemels, 2012). 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 (Palmieri et al., 2003) , 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 (Weigel et al., 2000). 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.

5. Interventions 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

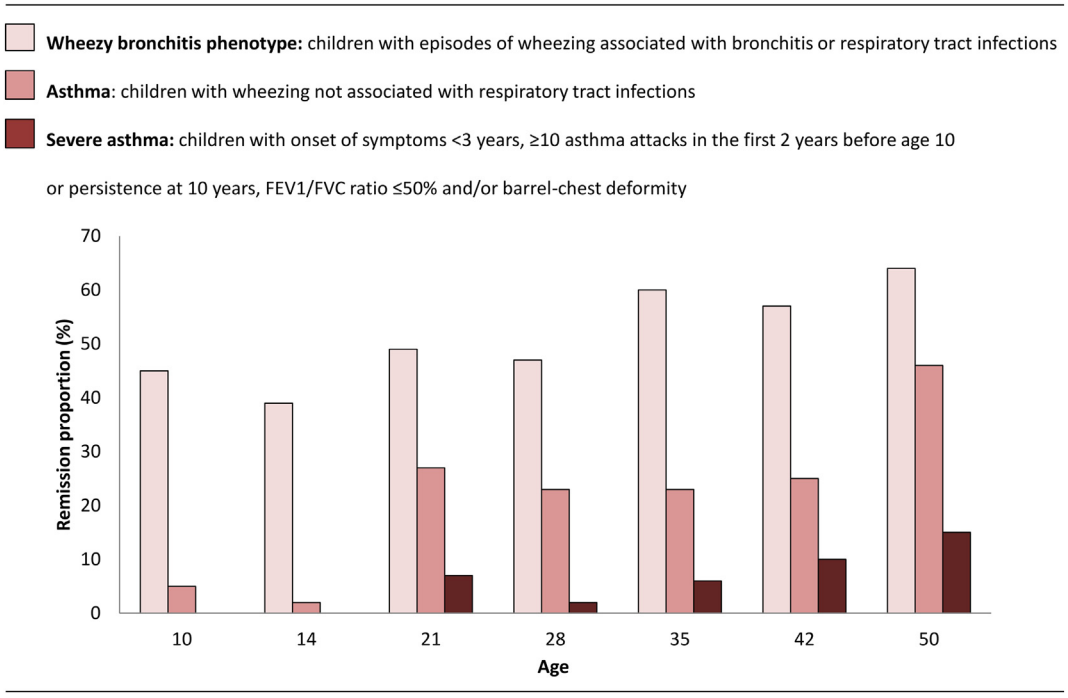

Fig. 2. Remission percentages at age 10 to 50 in children, enrolled at age 7 and divided in three asthma severity levels. This figure shows that less severe asthma is associated with a higher chance of remission later in life. Reproduced, with permission, from Tai et al., 2014.

been linked to asthma remission as well (Schwerk, Ballmann, & Hansen, 2008; Wirtz, Kroegel, Caffier, & Bittner, 2005), still this controversial intervention followed by immunosuppression is not discussed in this review.

5.1. Weight loss

The asthma-obesity relationship is well described, although the mechanisms underlying it are not well understood (Ulrik, 2016). It is hypothesized that this relation is affected by a different type of inflammation (Telenga et al., 2012) and by other comorbidities such as gastro-esophageal reflux and diabetes mellitus type 2 (Carpaij & van den Berge, 2018; Bateman et al., 2018). Weight loss has shown positive effects on several measures of asthma-control (Juel, Ali, Nilas, & Ulrik, 2012). (Scott et al., 2013; Stenius-Aarniala et al., 2000)(Scott et al., 2013) (Schatz et al., 2015), 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 (de Marco et al., 2006). 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 (Boulet, Turcotte, Martin, & Poirier, 2012a)(van Huisstede et al., 2015)(Baltieri et al., 2018; van Huisstede et al., 2015)(Guerron et al., 2018; van Huisstede et al., 2015), 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 (Macgregor & Greenberg, 1993). The United Kingdom National Bariatric Surgery Registry analyzed the prevalence of comorbidities after this surgical intervention over five years (Miras et al., 2018). 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.

5.2. Immunotherapy

Various studies found that negativity of skin prick tests (SPTs) was associated with asthma remission (Andersson et al., 2013; Covar et al., 2010; Limb et al., 2005; Tai et al., 2014). Allergen avoidance is also related to asthma remission (Froidure, Vandenplas, D'Alpaos, Evrard, & Pilette, 2015), 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 (Lee et al., 2017). All participants had documented symptoms, were either bronchial hyperresponsive to methacholine (PC_{20} ≤25 mg/ml) or reversible to salbutamol (≥12% and ≥200 ml), and had a 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 (Nuhoglu et al., 2007). 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 (Bağ et al., 2013). 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 (Adkinson et al., 1997). 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.

6. 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 (Fuchs, Bahmer, Rabe, & von Mutius, 2017; Upham & James, 2011). Figure 3 illustrates a theory in which asthma severity is correlated to whether a subject experiences symptoms or experiences lung function impairment.

In accordance with figure 3, individuals with remitted asthma might still have ongoing airway inflammation (Broekema et al., 2011). 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 endobronchial 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 (Broekema et al., 2011), 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 (Broekema et al., 2011). 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 (Tomiita et al., 2015; van Den Toorn et al., 2001; van Den Toorn, Prins, Overbeek, Hoogsteden, & de Jongste, 2000).

7. Airway remodeling in asthma remission

Chronic inflammation of the airways may lead to altered structure in the airway wall, referred to as remodeling (Bousquet, Jeffery, Busse, Johnson, & Vignola, 2000). Airway remodeling, such as increased basement membrane thickness, can occur early in childhood and is associated with an increased risk of developing clinical asthma (Bonato et al., 2018). Studies investigating airway remodeling in asthma

remission are scarce (Broekema et al., 2011; van Den Toorn et al., 2001). A cross-sectional study assessing remodeling enrolled 54 never-smoking adolescents aged 18–25 in three groups; asthmatics, asthma remission subjects and healthy controls (van Den Toorn et al., 2001). Endobronchial 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 (11.5 μm ±1.5 versus 10.9 μm ±1.3 respectively), both were significantly thicker than those in the healthy controls (7.9 μ ±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 (Broekema et al., 2011). 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 (5.3 μm [2.8-8.2] versus 5.7 μm [2.8-12.6] and 6.5 μm [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"(Broekema et al., 2011). 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.

8. 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 (Dijkstra et al., 2011; Panhuysen et al., 1997), 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 (Bucková, Izakovicová Hollá, & Vácha, 2002; Pampuch et al., 2006), and both the development and the severity of asthma (Cho et al., 2001; Cho, Ryu, & Oh, 2004; Pampuch et al., 2006). 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 (Portelli, Hodge, & Sayers, 2015), 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 (Vonk et al., 2018). 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 (Chen et al., 2014), potentially contributing to the resolution of inflammation (Chen et al., 2014). CCT2 has been associated with cell growth (Amit et al., 2010) and maintenance of cell proliferation (Izawa, Goto, Kasahara, & Inagaki, 2015). 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 (Akhabir et al., 2014; Akhabir & Sandford, 2011). This could imply that these variants play a role in regulating type 2 inflammation (Vonk et al., 2018).

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

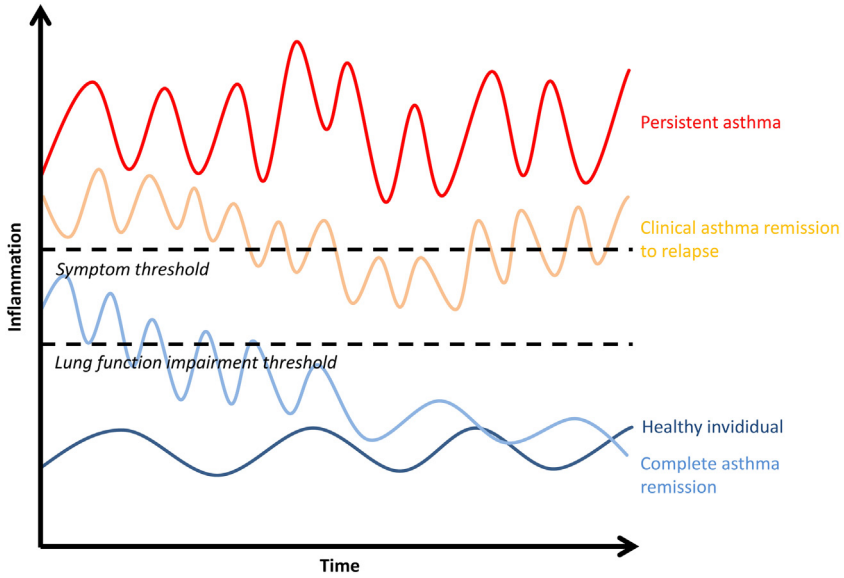

Fig. 3. Theoretical trajectory of persistent asthma, asthma remission and relapse over time. Adapted, with permission, from Upham & James, 2011.

Table 2
Biomarkers associated with clinical and complete asthma remission in cross-sectional studies.

Markers in asthma remission	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, Turcotte, Plante, & Chakir, 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, Turcotte, Plante, et al., 2012
Blood IgE	Kim et al., 2018 Noma et al., 1999 van Den Toorn et al., 2001		Waserman et al., 2012 Boulet, Turcotte, Plante, 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, Turcotte, Plante, 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, Turcotte, Plante, et al., 2012[ComR]		Boulet, Turcotte, Plante, et al., 2012[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, Turcotte, Plante, et al., 2012[ComR]		Waserman et al., 2012 Volbeda et al., 2010[A]	Boulet, Turcotte, Plante, 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., 2001
Sputum neutrophils			Boulet, Turcotte, Plante, et al., 2012[ComR] Broekema et al., 2011 Volbeda et al., 2010			Boulet, Turcotte, Plante, et al., 2012[ComR] Broekema et al., 2011 Waserman et al., 2012 Volbeda et al., 2010 Arshad et al., 2014
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., 2001
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		
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

Table 2 (*continued*)

Markers in asthma remission	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
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

Study groups (number of asthma/remission/control)
Arshad et al., 2014: n = 108/45/0, mean age 18, Andersson et al., 2013: n = 84/43/0, age range 16–17, Boulet, Turcotte, Plante, et al., 2012: n = 29/42/15 mean age 32, Broekema et al., 2011: n = 103/62/0, mean age 49, range 18–75, Kim et al., 2014: n = 31/30/31, mean age 8, Noma et al., 1999: n = 6/7/7, age range 6–35, Obase et al., 2001: n = 20/20/80, mean age, range 20–29, Panhuysen et al., 1997: n = 161/201/0 mean age 48, range 35-71, Tomiita et al., 2015: n = 18/15/14, mean age 21, van Den Toorn et al., 2001, 2000: n = 19/18/17, mean age 22, range 18–25, Volbeda et al. 2010: n = 46/7/0 mean age 49, range 18–70, Warke et al., 2002: n = 0/25/35, mean age 7, Waserman et al., 2012: n = 15/15/15, mean age 14, range 12–18, Xu et al., 2000: n = 0/20/30, age range >18.

Legend

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

of asthma (Liu, 2015). A cross-sectional cohort of 30 children with asthma remission, 31 with persistent asthma and 30 controls were studied in the KOREA study (Kim et al., 2018). 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 (Kim et al., 2018).

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.

9. 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 (Brussino, Heffler, Bucca, Nicola, & Rolla, 2018; Kips et al., 2003). 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 (Upham & Chung, 2018): Dupilumab (Zayed et al., 2018), a monoclonal antibody that blocks the common receptor for IL-4 and IL-13, Tezepelumab (Corren et al., 2017), a monoclonal antibody directed against thymic stromal lymphopoeitin which is produced by epithelial cells and affects multiple immune cells, and Fevipiprant (Kao & Parulekar, 2019), 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.

10. 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 (Soares et al., 2018), functional CT imaging by parametric response mapping of the lungs (Bell et al., 2019), optical coherence tomography of the airway walls (Goorsenberg et al., 2018), and further characterization of the histology in endobronchial 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 (Regev et al., 2017).

Conflict of interest statement

O.A. Carpaij, J.K. Burgess, H.A.M. Kerstjens, M.C. Nawijn and M. van den Berge, report no relationship/conditions/circumstances that present a potential conflict of interest for this submitted work.

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ORIGINAL ARTICLE

Longitudinal Asthma Phenotypes from Childhood to Middle-Age
A Population-based Cohort Study

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Abstract

Rationale: Asthma is a heterogeneous condition, and longitudinal phenotyping may provide new insights into the origins and outcomes of the disease.

Objectives: We aimed to characterize the longitudinal phenotypes of asthma between the first and sixth decades of life in a population-based cohort study.

Methods: Respiratory questionnaires were collected at seven time points in the TAHS (Tasmanian Longitudinal Health Study) when participants were aged 7, 13, 18, 32, 43, 50, and 53 years. Current-asthma and ever-asthma status was determined at each time point, and group-based trajectory modeling was used to characterize distinct longitudinal phenotypes. Linear and logistic regression models were fitted to investigate associations of the longitudinal phenotypes with childhood factors and adult outcomes.

Measurements and Main Results: Of 8,583 original participants, 1,506 had reported ever asthma. Five longitudinal asthma phenotypes were identified: early-onset adolescent-remitting (40%), early-onset adult-remitting (11%), early-onset persistent (9%), late-onset remitting (13%), and late-onset persistent (27%). All phenotypes were associated with chronic obstructive pulmonary disease at age 53 years, except for late-onset remitting asthma (odds ratios: early-onset adolescent-remitting, 2.00 [95% confidence interval (CI), 1.13–3.56]; early-onset adult-remitting, 3.61 [95% CI, 1.30–10.02]; early-onset persistent, 8.73 [95% CI, 4.10–18.55]; and late-onset persistent, 6.69 [95% CI, 3.81–11.73]). Late-onset persistent asthma was associated with the greatest comorbidity at age 53 years, with increased risk of mental health disorders and cardiovascular risk factors.

Conclusions: Five longitudinal asthma phenotypes were identified between the first and sixth decades of life, including two novel remitting phenotypes. We found differential effects of these phenotypes on risk of chronic obstructive pulmonary disease and nonrespiratory comorbidities in middle age.

Keywords: asthma phenotypes; longitudinal phenotypes; trajectories; chronic obstructive pulmonary disease; comorbidities

(Received in original form August 18, 2022; accepted in final form May 17, 2023)

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Supported by funds from the National Health and Medical Research Council of Australia (grant 1021275); the University of Melbourne; the Clifford Craig Foundation; the Victorian, Queensland, and Tasmanian Asthma Foundations; the Royal Hobart Hospital Research Foundation; the Helen MacPherson Smith Trust; GlaxoSmithKline; Asthma Australia; and a National Health and Medical Research Council Postgraduate Scholarship and Royal Australian College of Physicians Woolcock Scholarship (D.J.T.). The funding bodies had no direct role in the conduct of the study, statistical analysis or interpretation of the data, and preparation or approval of the manuscript.

Author Contributions: D.J.T., E.H.W., C.J.L., J.L.P., and S.C.D. conceived and designed the study. S.C.D., E.H.W., G.S.H., P.S.T., R.W.-B., and M.J.A. acquired the data. S.C.D., J.L.P., E.H.W., G.S.H., P.S.T., R.W.-B., and M.J.A. obtained funding for the study. D.J.T., D.S.B., J.L.P., and S.C.D. performed the statistical analysis. All authors interpreted the data and critically revised the manuscript for intellectual content and approved the submission of the manuscript. D.J.T., S.C.D., J.L.P., D.S.B., and G.B. had access to the raw data.

Am J Respir Crit Care Med Vol 208, Iss 2, pp 132–141, Jul 15, 2023

Originally Published in Press as DOI: 10.1164/rccm.202208-1569OC on May 20, 2023
Internet address: www.atsjournals.org

At a Glance Commentary

Scientific Knowledge on the Subject: Longitudinal modeling of current asthma has been limited to children and young adults.

What This Study Adds to the Field: Modeling of asthma data collected over five decades identified five common pathways (longitudinal phenotypes) that asthma follows from childhood to middle age. These phenotypes were differentially associated with childhood exposures and adult outcomes. Most asthma patients, including some with remitted disease, were at substantially increased risk of chronic obstructive pulmonary disease and nonrespiratory comorbidities in middle age.

Asthma is a common chronic respiratory disease in both children and adults and affects an estimated 300 million people worldwide (1). It is associated with substantial personal and community health burdens across all age groups, and although mortality has decreased in recent decades as management has improved, morbidity remains high (1). Asthma is known to be a heterogeneous condition, with multiple subgroups and varied natural histories over the life course (2, 3). Better characterization of these subgroups and their distinct etiologies and outcomes may help facilitate novel prevention and improved management strategies.

In recent years, substantial effort has been invested in phenotyping asthma, and the findings have advanced our understanding of the disease (4, 5), especially in severe asthma (6, 7). These studies have led to new therapeutic options and targeted management, including the "treatable traits" approach to airway disease (8). To date, most research has focused on cross-sectional asthma phenotypes (9, 10), though some prospective studies have also characterized distinct patterns of asthma activity over time (10–13). These "longitudinal phenotypes," also called asthma or wheeze "trajectories," have the potential to provide novel insights into the natural history of asthma across different age groups.

To date, longitudinal asthma phenotypes have generally been characterized using two approaches. Earlier studies used manual classifications to define phenotypes on the basis of *a priori* clinical criteria, such as in the Dunedin Study, which described seven early wheeze phenotypes from ages 7–26 years (14). More recently, studies have used data-driven techniques such as latent class analysis (LCA) and group-based trajectory modeling (GBTM) to identify distinct subgroups (10–13). These studies have consistently identified four phenotypes from childhood to adulthood ("never or infrequent," "early transient," "early persistent," and "late-onset wheeze") but have had limited follow-up of participants into only their mid-20 s. In contrast, although the TAHS (Tasmanian Longitudinal Health Study) characterized trajectories of "asthma and allergic diseases" over a longer follow-up period, the study did not delineate asthma-specific phenotypes (15), and a major gap remains in our understanding of asthma transition over the life course.

We applied a data-driven approach to respiratory data collected on participants at seven time points in the TAHS from ages 7–53 years. We aimed to characterize the longitudinal phenotypes of asthma from childhood to middle age and investigated associations with early-life exposures and adult clinical outcomes. Some of the results of these studies have been previously reported in the form of an abstract (16).

Methods

Study Design and Data Collection

The TAHS is a population-based cohort of children born in 1961 and attending school in Tasmania, Australia, in 1968 (17). Respiratory questionnaires were first collected when participants were aged 7 years (baseline) and subsequently in follow-up studies conducted at ages 13, 18, 30, 43, 50, and 53 years. All studies were approved by the human ethics review committees of the relevant institutions, and written informed consent was obtained from all participants.

Procedures

Pre–bronchodilator (BD) spirometry was measured at all but one follow-up time point (age 30 yr), in accordance with American Thoracic Society and European Respiratory Society guidelines (18–20). In the studies at ages 45 and 53 years, post-BD spirometry was measured 15 minutes after administration of an inhaled BD (salbutamol 300 μg). Predicted values for spirometry were derived from Global Lung Initiative reference values (21). Lung function trajectories of FEV_1 growth and subsequent decline over the course of the study were developed as previously described (22). An overview of this process is also provided in the online supplement (*see* Methods E1).

Definitions

At each time point, we defined "ever asthma" as an affirmative response to the question "Have you, at any time in your life, suffered from attacks of asthma or wheezy breathing?" In participants who met the definition of ever asthma, we defined "current asthma" as an affirmative response to the question "Have you had an attack of asthma or wheezy breathing in the last 12 months?" These questions were answered by parents when participants were aged 7 and 13 years and by the participants themselves at the follow-up visits at ages 18, 30, 43, 50, and 53 years. These questions have been previously validated against physician assessments of asthma (23).

Childhood exposures (eczema, allergic rhinitis, food allergy, bronchitis, pneumonia, breastfeeding, parental asthma, and smoking) were assessed using parents' responses to the baseline study questionnaire. Adult outcomes (respiratory symptoms, healthcare use, comorbidities) were assessed using participants' responses to the age 53 questionnaire. The specific questions corresponding to these variables are presented in Methods E2. Chronic obstructive pulmonary disease (COPD) was defined spirometrically as a post-BD FEV_1:FVC ratio below the lower limit of normal (21).

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This article has a related editorial.

This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org.

Statistical Analysis

To ensure that only participants with sufficient data were included, we limited the study sample to participants with asthma data (current asthma, ever-asthma) in at least three of the three whole-cohort studies conducted at ages 7, 43, and 53 years. Among these participants, those who reported ever asthma at any of the seven follow-up time points were assigned to the "ever-asthma sample." The remaining participants who did not report ever asthma at any follow-up time point were assigned to the reference "never-asthma sample."

In the ever-asthma sample, we used GBTM to identify participants whose current asthma status followed similar patterns over time. GBTM is a form of finite fixture modeling whose aim is to explain population heterogeneity by identifying distinct subgroups within the population that follow similar patterns over time (24). Models with an increasing number of subgroups (longitudinal phenotypes) were developed, and a final model was selected using maximum likelihood estimation and a minimum class membership of 5% (25). GBTM was used to estimate the population prevalence of each subgroup and the posterior probability of each participant belonging to each subgroup. GBTM also allowed the retention of participants with incomplete data by imputing missing observations (24). Assignment of participants to a single phenotype was based on the modal method (the subgroup with the highest posterior probability for that individual). Further details on the process used to select the final model are provided in the online supplement (*see* Methods E3). Using the output of the GBTM models, we then calculated the prevalence of current asthma within the cohort (whole sample) stratified by asthma phenotype at each time point (*see* Methods E4).

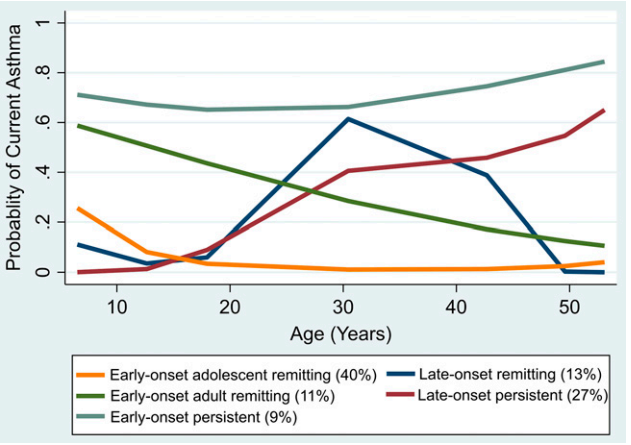

Figure 1. Longitudinal asthma phenotypes from ages 7–53 years (ever-asthma sample): probability of current asthma at each time point.

We examined associations between each asthma phenotype and TAHS childhood factors and adult outcomes, using never asthma as the reference category. Linear regression was used for continuous outcomes, logistic regression for binary outcomes, and multinomial logistic regression for nominal outcomes. Models were adjusted for a minimum set of confounders selected using directed acyclic graphs. Interactions between the longitudinal phenotypes and adult smoking and weight status were tested. In a *post hoc* analysis, we also examined the association of each phenotype with SNPs in 45 genes known to be associated with asthma or atopic sensitization. Methods for the genotyping process are provided in the online supplement (*see* Methods E5). We used a complete case analysis approach for tests of associations between the asthma phenotypes and early-life exposures and adult outcomes. Analyses were performed using Stata version 16 (StataCorp) with a GBTM plug-in (26).

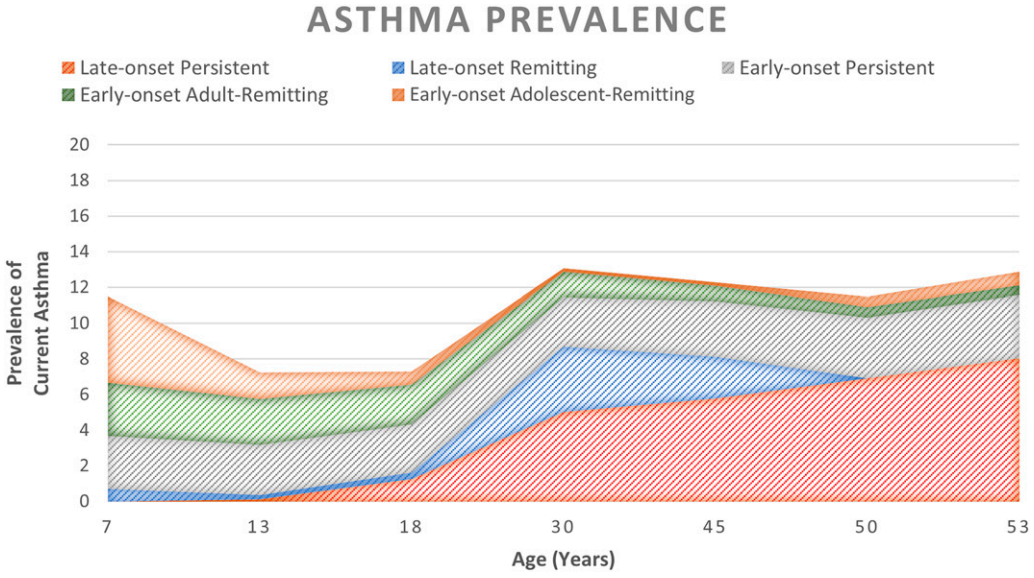

Figure 2. Contribution of each phenotype to asthma prevalence at each time point from ages 7–53 years (whole sample).

Table 1. Characteristics of the Longitudinal Asthma Phenotypes

	Never Asthma ($n = 1,743$)	Early-Onset Adolescent-Remitting ($n = 822$)	Early-Onset Adult-Remitting ($n = 95$)	Early-Onset Persistent ($n = 123$)	Late-Onset Remitting ($n = 110$)	Late-Onset Persistent ($n = 356$)
Childhood characteristics at age 7 yr						
Female	867 (50%)	413 (50%)	28 (29%)*	56 (46%)	67 (61%)†	224 (63%)*
Weight status						
Normal	1,422 (85%)	685 (85%)	72 (81%)	101 (84%)	93 (85%)	286 (84%)
Underweight	68 (4%)	22 (3%)	4 (4%)	4 (3%)	3 (3%)	10 (3%)
Overweight or obese	192 (12%)	96 (11%)	13 (14%)	15 (13%)	13 (12%)	43 (13%)
Socioeconomic status						
First quintile (highest)	438 (26%)	178 (23%)	17 (18%)	33 (29%)	27 (26%)	83 (25%)
Second quintile	132 (8%)	55 (7%)	5 (5%)	11 (10%)	8 (8%)	25 (7%)
Third quintile	453 (27%)	249 (32%)	26 (28%)	29 (25%)	28 (27%)	105 (31%)
Fourth quintile	449 (27%)	206 (26%)	30 (33%)	28 (24%)	25 (24%)	85 (25%)
Fifth quintile (lowest)	191 (11%)	91 (12%)	14 (15%)	14 (12%)	16 (15%)	37 (11%)
Smoking status at major follow-up time points						
Age 7 yr (parental)	1,094 (64%)	548 (68%)†	66 (70%)	88 (73%)	63 (59%)	244 (70%)†
Age 13 yr (personal)	35 (2%)	49 (7%)*	5 (5%)	4 (4%)	3 (3%)	14 (4%)†
Age 45 yr (personal)	393 (23%)	192 (23%)	22 (23%)	33 (27%)	35 (32%)†	108 (31%)‡
Age 53 yr (personal)	268 (15%)	127 (15%)	17 (18%)	20 (16%)	19 (17%)	83 (23%)*
BMI ($kg \cdot m^{-2}$) at major follow-up time points						
Age 7 yr	16.0 (1.5)	16.1 (1.4)	16.0 (1.6)	16.0 (1.4)	16.0 (1.2)	16.2 (1.6)†
Age 13 yr	18.9 (2.5)	19.1 (2.4)	18.7 (3.0)	20.0 (3.6)	19.1 (1.6)	19.4 (2.8)
Age 45 yr	27.0 (5.3)	28.3 (6.1)‡	27.7 (4.6)	28.7 (5.8)†	27.6 (4.6)	29.5 (7.3)*
Age 53 yr	28.2 (10.4)	29.0 (9.1)†	28.1 (5.0)	29.5 (6.1)	28.2 (4.8)	30.2 (6.6)*
Reproductive history (female subjects only)						
Age at menarche	13.0 (1.6)	12.9 (1.7)	12.4 (1.5)	12.7 (2.0)	13.2 (1.7)	12.8 (1.8)
Age at menopause	52.8 (2.3)	52.6 (3.0)	52.0 (4.0)	51.8 (4.1)	51.6 (5.6)	52.1 (3.0)
Menarche (age < 12 yr)	117 (14%)	74 (18%)†	8 (29%)†	13 (23%)†	12 (18%)	42 (19%)
Hormonal contraceptive (ever)	765 (89%)	371 (90%)	23 (82%)	51 (91%)	61 (91%)	196 (88%)
Pregnancy (ever)	762 (88%)	357 (86%)	22 (76%)	51 (91%)	62 (93%)	198 (88%)

Definition of abbreviation: BMI = body mass index.
Data are *n* (%) or mean (SD). The reference group was never asthma. Reproductive data were available for 1,637 (99%) of the female sample. Overweight and obese status was defined using age- and sex-specific thresholds from Cole and colleagues (41). Groups were compared using logistic regression for dichotomous variables, multinominal logistic regression for nominal variables, and linear regression for continuous variables.
*$P < 0.001$.
†$P < 0.05$.
‡$P < 0.01$.

Results

Of the original 8,583 TAHS participants, 3,249 (38%) had asthma status defined for at least the three whole-cohort follow-up time points at ages 7, 43, and 53 years. Of these, 1,506 (46%) reported ever asthma for at least one follow-up visit and formed the ever-asthma sample. The remaining 1,743 (54%) participants formed the reference never-asthma sample. The participants included had similar baseline characteristics to those not included, except for more female participants, more with childhood allergies and lung diseases, and fewer with smoking parents (*see* Table E1).

Longitudinal Asthma Phenotypes

The best-fitting model identified five longitudinal asthma phenotypes within the ever-asthma sample (Figure 1). On the basis of age of onset and age of remission over the follow-up period, these asthma phenotypes were labeled early-onset adolescent-remitting (40% of the ever-asthma sample), early-onset adult-remitting (11%), early-onset persistent (9%), late-onset remitting (13%), and late-onset persistent (27%). The mean posterior probability for each cluster in the final model ranged from 0.60 to 0.85, indicating reasonable model accuracy. In an assessment of model stability across different five-class models (*see* Methods E3), the most stable clusters were the early-onset persistent (posterior probability = 0.85 to 0.87), late-onset persistent (0.66–0.86), and early-onset adult-remitting (0.65–0.84) phenotypes. Posterior probabilities for early-onset adolescent-remitting (0.55–0.78) and late-onset remitting (0.50–0.60) asthma were comparatively lower across all models, indicating higher uncertainty in the allocation of participants to these groups.

The prevalence of current asthma stratified by phenotype at each time point is shown in Figure 2. At age 7 years, the overall prevalence of current asthma in the total population was 11.5%, composed primarily of the three early-onset phenotypes

Table 2. Adjusted Associations between Longitudinal Asthma Phenotypes and Childhood Factors

	Early-Onset Adolescent-Remitting ($n = 822$)	Early-Onset Adult-Remitting ($n = 95$)	Early-Onset Persistent ($n = 123$)	Late-Onset Remitting ($n = 110$)	Late-Onset Persistent ($n = 356$)
Childhood characteristics at age 7 yr					
Female	1.02 (0.86–1.20)	0.42 (0.27–0.66)*	0.84 (0.59–1.22)	1.57 (1.06–2.34)†	1.71 (1.36–2.17)*
Ever eczema	2.02 (1.57–2.59)*	4.00 (2.47–6.48)*	7.12 (4.67–10.84)*	1.71 (0.97–3.00)	1.90 (1.35–2.66)*
Ever allergic rhinitis	2.81 (2.17–3.64)*	8.23 (5.15–13.16)‡	10.22 (6.66–15.67)*	1.22 (0.62–2.42)	1.85 (1.28–2.68)‡
Ever food allergy	1.94 (1.39–2.70)*	2.86 (1.48–5.53)*	4.83 (2.85–8.17)*	0.77 (0.28–2.16)	1.55 (0.97–2.48)
Ever bronchitis	2.95 (2.46–3.55)*	8.22 (4.50–15.00)*	16.13 (8.07–32.35)*	1.65 (1.09–2.48)†	1.37 (1.08–1.76)†
Ever pneumonia	2.18 (1.70–2.79)*	3.98 (2.46–6.43)*	4.66 (3.03–7.17)*	1.78 (1.00–3.18)†	1.45 (1.00–2.09)†
Weight					
Normal	Ref	Ref	Ref	Ref	Ref
Underweight	0.69 (0.42–1.14)	1.36 (0.47–3.88)	1.05 (0.37–2.96)	0.76 (0.23–2.47)	0.79 (0.40–1.56)
Overweight or obese	1.00 (0.76–1.32)	1.49 (0.78–2.85)	1.04 (0.57–1.93)	0.90 (0.47–1.73)	1.01 (0.70–1.47)
Breastfeeding					
Breastfed only	Ref	Ref	Ref	Ref	Ref
Bottle only	1.11 (0.89–1.39)	1.39 (0.81–2.38)	1.27 (0.80–2.01)	0.80 (0.46–1.40)	0.80 (0.46–1.40)
Breast and bottle	1.04 (0.85–1.28)	1.21 (0.73–2.00)	0.61 (0.37–1.02)	1.11 (0.70–1.74)	1.22 (0.90–1.67)
Parental characteristics at age 7 yr					
Maternal asthma	2.14 (1.60–2.86)*	3.59 (2.07–6.25)*	3.14 (1.86–5.30)*	2.50 (1.40–4.49)‡	2.28 (1.57–3.32)*
Paternal asthma	1.69 (1.27–2.25)*	4.07 (2.42–6.83)*	5.84 (3.77–9.06)*	2.50 (1.43–4.35)‡	1.36 (0.90–2.05)
Maternal smoking	1.29 (1.08–1.55)‡	1.13 (0.72–1.77)	1.14 (0.75–1.72)	1.06 (0.69–1.64)	1.10 (0.85–1.42)
Paternal smoking	1.04 (0.87–1.24)	1.12 (0.71–1.74)	1.41 (0.94–2.12)	0.87 (0.58–1.32)	1.34 (1.04–1.72)†

Definition of abbreviation: Ref = reference.
Data are relative risk ratio (95% confidence interval). The reference group was never asthma. Groups were compared using logistic regression for dichotomous variables and multinominal logistic regression for nominal variables. For eczema, allergic rhinitis, food allergy, and bronchitis, models were adjusted for sex, parental asthma, childhood socioeconomic status, parental smoking, and breastfeeding. For pneumonia and pleurisy, the model was adjusted for sex, childhood socioeconomic status, parental smoking, and breastfeeding. For childhood weight status, the model was adjusted for sex, childhood socioeconomic status, parental smoking, breastfeeding, and childhood pneumonia and pleurisy. For breastfeeding, the model was adjusted for parental asthma, childhood socioeconomic status, and parental smoking. For parental asthma, the model was adjusted for parental smoking and childhood socioeconomic status. For parental smoking, models were adjusted for parental asthma and childhood socioeconomic status.
*$P < 0.001$.
†$P < 0.05$.
‡$P < 0.01$.

(adolescent-remitting, 4.8%; adult-remitting, 3.0%; persistent, 3.0%). Between childhood and middle age, current asthma prevalence ranged from 11.5% to 13.1%, except in adolescence, when prevalence was lower (7.2–7.3%) because of remission of the early-onset adolescent-remitting phenotype. After adolescence, the prevalence of current asthma increased in line with the emerging late-onset phenotypes, peaking by age 30 years (13.1%). Thereafter, asthma prevalence remained stable, and remission of the early-onset adult-remitting and late-onset remitting phenotypes was offset by an increasing prevalence of late-onset persistent asthma.

Characteristics of the Longitudinal Asthma Phenotypes

The early-onset adult-remitting phenotype had more male subjects ($P < 0.001$); the early-onset adolescent-remitting and early-onset persistent phenotypes had equal male-to-female sex ratios (Table 1); and in contrast, the late-onset remitting ($P = 0.02$) and late-onset persistent ($P < 0.001$) phenotypes had more female subjects. Compared with never asthma, three asthma phenotypes were characterized by increased exposure to cigarette smoke at different time points: for early-onset adolescent-remitting asthma and late-onset remitting asthma, cigarette smoke exposure was more common when subjects were most symptomatic (ages 7 and 13 years for early-onset adolescent-remitting and age 45 years for late-onset remitting). For the late-onset persistent phenotype, cigarette smoke exposure and higher body mass index were more common at multiple time points from ages 7–53 years.

For female reproductive history, early menarche (<12 yr) was more common among the three early-onset asthma phenotypes compared with the never asthma reference ($P < 0.05$ for all). Hormonal contraceptive use (ever) and history of pregnancy (ever) were reported at similar rates among all five longitudinal phenotypes.

Childhood Risk Factors

We identified differential associations between childhood factors and the longitudinal phenotypes when compared with never asthma (Table 2). All phenotypes were independently associated with maternal asthma, childhood bronchitis, and pneumonia, and except for late-onset remitting asthma, all phenotypes were also associated with childhood eczema, allergic rhinitis, and food allergy. These associations were much stronger for the early-onset persistent and early-onset adult-remitting phenotypes. We found that maternal smoking was an independent risk factor for early-onset adolescent-remitting asthma ($P = 0.01$), whereas paternal smoking was an

Table 3. Adjusted Associations between Longitudinal Asthma Phenotypes and Spirometric Outcomes at Age 53

	Early-Onset, Adolescent-Remitting ($n = 822$)	Early-Onset Adult-Remitting ($n = 95$)	Early-Onset Persistent ($n = 123$)	Late-Onset Remitting ($n = 110$)	Late-Onset Persistent ($n = 356$)
Pre-BD lung function (% predicted)					
FEV_1	−1.7 (−4.1 to 0.8)	−4.6 (−10.7 to 1.5)	−14.8 (−20.3 to −9.3)*	−4.2 (−9.5 to 1.2)	−10.6 (−14.0 to 7.2)*
FVC	−0.3 (−3.0 to 2.3)	−2.6 (−9.4 to 4.3)	−7.6 (−13.8 to −1.5)†	−2.4 (−8.3 to 3.6)	−6.5 (−10.2 to 2.7)‡
FEV_1:FVC ratio	−1.0 (−1.7 to −0.4)‡	−2.2 (−3.9 to −0.6)‡	−6.6 (−8.0 to −5.1)*	−1.8 (−3.2 to −0.3)†	−3.8 (−4.6 to −2.9)*
Post-BD lung function (% predicted)					
FEV_1	−2.3 (−4.4 to −0.3)†	−4.6 (−9.8 to 0.7)	−12.7 (−17.3 to −8.0)*	−4.0 (−8.6 to 0.5)	−8.8 (−11.7 to −6.0)*
FVC	−1.3 (−3.5 to 0.9)	−2.5 (−8.0 to 3.1)	−5.5 (−10.5 to −0.4)†	−1.8 (−6.7 to 3.1)	−4.9 (−8.0 to −1.8)‡
FEV_1:FVC ratio	−1.1 (1.84 to −0.3)‡	−2.1 (−4.0 to −0.2)†	−7.6 (−9.4 to −5.9)*	−2.3 (−4.0 to −0.6)‡	−4.2 (−5.2 to −3.1)*
Spirometric COPD, OR (95% CI)	2.00 (1.13 to 3.56)†	3.61 (1.30 to 10.02)†	8.73 (4.10 to 18.55)*	1.86 (0.54 to 6.35)	6.69 (3.81 to 11.73)*

Definition of abbreviations: BD = bronchodilator; CI = confidence interval; COPD = chronic obstructive pulmonary disease; OR = odds ratio.
Data are mean difference (95% CI) except as indicated. The reference group was never asthma. Groups were compared using logistic regression for dichotomous outcomes and linear regression for continuous outcomes. Models were adjusted for sex, parental asthma, parental smoking, childhood socioeconomic status, childhood pneumonia and pleurisy, and childhood body mass index.
*$P < 0.001$.
†$P < 0.05$.
‡$P < 0.01$.

independent risk factor for late-onset persistent asthma ($P = 0.02$).

We identified modest associations between SNPs in several asthma-related genes and the longitudinal phenotypes (*see* Table E2). SNPs in the HLA-DR-DQ (rs6903608) and filaggrin (rs41370446) genes were associated with a slightly increased risk of early-onset adolescent-remitting asthma, whereas an SNP in the TLR6 (Toll-like receptor 6) (rs1039559) gene was protective. SNPs in the IL4R (IL-4 receptor) region (rs2057768 and rs4787948) were associated with early-onset adult-remitting asthma, SNPs in the HLA-DR-DR gene (rs9268614) and TGFBR2 (transforming growth factor β receptor 2) gene (rs11924422) were associated with early-onset persistent asthma, SNPs in CD14 (cluster of differentiation 14) gene (rs2569190 and rs2915863) were associated with late-onset remitting asthma, and an SNP in IRF2 (IFN regulatory factor 2) gene (rs724528) was associated with late-onset persistent asthma.

Clinical Outcomes: Lung Function and COPD

We also identified differential associations between the longitudinal phenotypes and spirometric outcomes at age 53 years (Table 3). Although all asthma phenotypes were associated with pre- and post-BD spirometric changes consistent with obstructive deficits, the magnitude of the deficits were far greater for persistent asthma (both early-onset and late-onset). We identified a multiplicative interaction between the effects of persistent asthma and personal smoking on pre- and post-BD FEV_1:FVC ratio at age 53 years (Table 4).

All longitudinal phenotypes, excluding late-onset remitting asthma, were associated with an increased risk of spirometrically defined COPD at age 53 years. Consistent with the spirometric findings, the highest odds of developing COPD were observed for early-onset persistent (odds ratio, 8.73 [95% confidence interval (CI), 4.10–18.55]) and late-onset persistent asthma (odds ratio, 6.69 [95% CI, 3.81–11.73]).

For the relationship between asthma phenotypes and FEV_1 trajectories (22), we found that early-onset persistent and late-onset persistent asthma were associated with three abnormal lung function trajectories (Table 5). These trajectories were characterized by subnormal FEV_1 in childhood and subnormal maximally attained FEV_1 in early adulthood. Among

Table 4. Interaction between Longitudinal Phenotypes and Smoking Status on FEV_1:FVC Ratio at Age 53 Years

	Smoking Status	Pre-BD FEV_1:FVC Ratio	*P* Value for Interaction	Post-BD FEV_1:FVC Ratio	*P* Value for Interaction
Never asthma (control)	Nonsmoker	Ref	—	Ref	—
	Smoker	−2.9 (−3.9 to −2.0)*	—	−4.9 (−6.1 to −3.8)*	—
Early-onset persistent	Nonsmoker	−6.0 (−7.5 to −4.5)*	—	−7.6 (−8.5 to −5.0)*	—
	Smoker	−13.1 (−16.6 to −9.5)*	0.05	−19.9 (−24.3 to −15.5)*	<0.001
Late-onset persistent	Nonsmoker	−2.6 (−3.5 to −1.6)*	—	−2.6 (−3.8 to −1.5)*	—
	Smoker	−9.4 (−11.0 to −7.7)*	<0.001	−13.7 (−15.8 to −11.6)*	<0.001

Definition of abbreviations: BD = bronchodilator; Ref = reference.
Data are mean difference (95% confidence interval). Groups were compared using linear regression. Models were adjusted for sex, parental asthma, parental smoking, childhood socioeconomic status, childhood pneumonia and pleurisy, and childhood body mass index.
*$P < 0.001$.

these, the strongest associations were observed with a trajectory ("early below average, accelerated decline") also characterized by accelerated FEV_1 decline during adulthood (early-onset persistent: relative risk ratio, 28.98 [95% CI, 12.48–67.27]; late-onset persistent: RRR, 10.04 [95% CI, 5.13–19.63]). Although to a lesser extent, the three remitting phenotypes also appeared to be associated with these same lung function trajectories. However, these associations did not always reach statistical significance (Table 5).

Clinical Outcomes: Adult Comorbidities

Late-onset persistent asthma was associated with multiple nonrespiratory comorbidities at age 53 years, including mental health disorders (anxiety and depression), cardiovascular risk factors (diabetes and high cholesterol) and gastroesophageal reflux disease (GERD) (Table 6). Similarly, late-onset remitting asthma was associated with high cholesterol, depression, and GERD. In contrast, the early-onset phenotypes were associated with anxiety (early-onset adult-remitting), GERD (early-onset persistent), or both anxiety and GERD (early-onset adolescent-remitting). None of the phenotypes was associated with hypertension.

Discussion

This study is the first to characterize longitudinal asthma phenotypes from childhood to middle age. Using a data-driven approach, on the basis of respiratory histories collected at seven time points from ages 7–53 years, we identified five distinct asthma phenotypes distinguished by age of asthma onset and remission. This study extends the current framework of longitudinal asthma phenotypes (limited in previous studies to ages in the mid-20 s) to the mid-50 s. In this study, we described two novel phenotypes: early-onset adult-remitting and late-onset remitting asthma. We also found that longitudinal asthma phenotypes were differentially associated with childhood factors and adult outcomes.

Several studies have now characterized asthma-wheeze phenotypes over limited periods from childhood to early adulthood in prospective cohorts, including LCA and GBTM analyses of the population-based BAMSE (Barn/Child, Allergy, Milieu, Stockholm, Epidemiology) birth cohort (10) (follow-up 1–24 years, $n = 4{,}089$), Pelotas birth cohort (13) (follow-up 4–22 years, $n = 5{,}249$), and Study Team for Early Life Asthma Research (STELAR) consortium of five United Kingdom birth cohorts (follow-up from birth to 18 years, $n = 7{,}719$) (11).

Table 5. Association between Longitudinal Asthma Phenotypes and Lifetime Lung Function (FEV_1) Trajectories

	Early-Onset Adolescent-Remitting (*n* = 602)	Early-Onset Adult-Remitting (*n* = 69)	Early-Onset Persistent (*n* = 90)	Late-Onset Remitting (*n* = 88)	Late-Onset Persistent (*n* = 248)
Persistently high	1.09 (0.77–1.53)	0.41 (0.12–1.41)	1.07 (0.41–2.80)	1.14 (0.54–2.41)	1.03 (0.61–1.76)
Average	1 (Ref)	1 (Ref)	1 (Ref)	1 (Ref)	1 (Ref)
Early low, accelerated growth, normal decline	1.36 (0.91–2.02)	2.35 (0.99–5.53)	1.10 (0.31–3.91)	0.92 (0.34–2.47)	1.00 (0.51–1.94)
Below average	1.49 (1.16–1.91)*	1.13 (0.59–2.17)	2.03 (1.06–3.89)†	1.63 (0.94–2.81)	2.02 (1.40–2.92)‡
Persistently low	1.80 (1.05–3.06)†	2.47 (0.86–7.13)	5.47 (2.14–14.00)‡	1.84 (0.60–5.60)	5.34 (2.96–9.66)‡
Early below average, accelerated decline	2.02 (1.01–4.02)†	3.22 (0.87–11.91)	28.98 (12.48–67.27)‡	4.95 (1.70–14.47)*	10.04 (5.13–19.63)‡

Definition of abbreviation: Ref = reference.
Data are relative risk ratio (95% confidence interval). The reference group was never asthma. Lung function trajectory data were available for 2,255 (69%) of the study sample. Groups were compared using multinominal logistic regression. Models were adjusted for sex, parental asthma, parental smoking, childhood socioeconomic status, childhood pneumonia and pleurisy, and childhood body mass index.
*$P < 0.01$.
†$P < 0.05$.
‡$P < 0.001$.

Table 6. Adjusted Associations between Longitudinal Asthma Phenotypes and Nonrespiratory Comorbidities at Age 53 Years

Comorbidities (Ever)	Early-Onset Adolescent-Remitting ($n = 822$)	Early-Onset Adult-Remitting ($n = 95$)	Early-Onset Persistent ($n = 123$)	Late-Onset Remitting ($n = 110$)	Late-Onset Persistent ($n = 356$)
Hypertension	1.08 (0.89–1.32)	0.89 (0.53–1.49)	1.29 (0.84–1.97)	0.73 (0.43–1.21)	1.20 (0.92–1.58)
Diabetes	1.40 (0.99–1.99)	1.29 (0.55–3.06)	1.74 (0.87–3.45)	1.83 (0.89–3.78)	1.81 (1.16–2.82)*
High cholesterol	1.09 (0.88–1.34)	1.36 (0.82–2.23)	1.25 (0.80–1.97)	1.97 (1.27–3.05)*	1.42 (1.07–1.89)†
GERD	1.44 (1.08–1.91)†	1.60 (0.83–3.11)	2.33 (1.38–3.95)*	2.99 (1.79–5.01)‡	1.89 (1.31–2.71)*
Anxiety	1.40 (1.12–1.76)*	1.75 (1.02–3.03)†	1.30 (0.78–2.15)	1.19 (0.70–2.03)	1.62 (1.20–2.18)*
Depression	1.11 (0.89–1.38)	1.20 (0.69–2.09)	1.55 (0.99–2.42)	1.89 (1.21–2.95)*	1.69 (1.28–2.23)‡

Definition of abbreviation: GERD = gastroesophageal reflux disease.
Data are odds ratio (95% confidence interval). The reference group was never asthma. Comparisons between groups were assessed using logistic regression. Models were adjusted for sex, parental smoking, childhood socioeconomic status, and childhood body mass index.
*$P < 0.01$.
†$P < 0.05$.
‡$P < 0.001$.

These studies have identified four similar longitudinal wheeze phenotypes: never or infrequent, early transient, early persistent, and late-onset wheeze. In our TAHS cohort, the present asthma phenotypes followed similar trajectories up to age 18 years but later diverged on the basis of persistence or remission of asthma symptoms during adulthood. Consequently, we identified two additional remitting asthma-wheeze phenotypes between childhood and middle age. Importantly, it should be noted that our GBTM analysis was conducted within an ever-asthma sample rather than an unselected cohort, as used in previous studies. This approach was chosen so that distinct subgroups could be identified without the interference of a large never-asthma subgroup, which constituted 80%, 71%, and 54% of the BAMSE, Pelotas, and STELAR cohorts, respectively.

Recently, a secondary analysis of the STELAR consortium cohorts applied a novel partition-around-medoids (PAM) clustering approach to multidimensional wheeze variables on the basis of six wheeze characteristics and identified an additional "intermittent wheeze" phenotype (12), but this was not evident in the present study. The PAM approach appeared to reduce within-class heterogeneity compared with a conventional LCA model on the basis of binary (yes or no) wheeze data. Although differences in statistical approach (GBTM vs. PAM) likely explain the absence of this phenotype within our model, it is also possible that longer periods between follow-up time points in TAHS (seven time points from ages 7–53 yr) compared with the STELAR cohorts (five time points from ages 1–18 yr) may have limited our ability to identify this phenotype.

The findings of our multivariable analyses suggest that the longitudinal phenotypes identified are relevant from both etiological and clinical perspectives. Although most phenotypes shared common childhood risk factor profiles, there was evidence of differential associations among them. Familial factors, childhood allergies, and childhood lung conditions appeared to play a greater role in the natural history of early-onset and persistent phenotypes and consequently may hold prognostic value. These findings are consistent with those from prior studies of early-life factors associated with early-onset versus late-onset asthma (27), and persistence versus remission of asthma (28, 29), including previous analyses conducted in TAHS. We also found some evidence that phenotypes were associated with different asthma-related genetic polymorphisms, although this is of uncertain clinical relevance.

Our study provides new insights into the relationship between asthma and lung function across the life course into middle age. In particular, our findings add to growing evidence indicating that even remitted asthma is an important yet often underrecognized cause of lung function impairments in adulthood (30–32). All three remitting phenotypes were associated with significant obstructive deficits in middle age, and both early-onset remitting phenotypes were also associated with an increased risk of established COPD. This is consistent with a recent prospective study by Miura and colleagues (30), in which remitted childhood asthma was shown to be an independent risk factor of accelerated lung function decline in middle-aged adults. These findings raise concerns regarding the optimal follow-up and management of individuals with apparently remitted asthma, who represented an important >30% of the general population at age 53 years in TAHS (27). This highlights a need to identify high-risk subgroups at least in early adult life.

Our study is unique in its comparison of longitudinal asthma phenotypes and lung function trajectories, both characterized from ages 7–53 years. We found that the spirometric deficits associated with persistent asthma phenotypes were at least in part related to abnormal lung function trajectories characterized by subnormal FEV_1 in childhood, subnormal maximally attained FEV_1 in early adulthood, and, in some cases, accelerated FEV_1 decline during adulthood. Consistent with other studies, we showed that the effects of persistent asthma (both early onset and late onset) were exacerbated by adult personal smoking with a multiplicative interaction (33–35). Our findings reinforce the importance of smoking cessation and good asthma control in preserving lung function across the life course in individuals with asthma. This is supported by findings of a recent systematic review showing that inhaled corticosteroid use attenuates the adverse effects of asthma on lung function (36), with different effects in children and adults.

Of the phenotypes described in this study, late-onset persistent asthma was associated with the most nonrespiratory comorbidities in middle age, including multiple cardiovascular risk factors, mental

ill health, and GERD. Biological mechanisms proposed to underlie associations of asthma with cardiovascular disease include systemic inflammation and T-helper cell type 1 inflammatory responses (37, 38) and, for mental health disorders, chronic illness reactions, use of systemic steroids, and T-helper cell type 2-inflammatory responses (39, 40). Shared risk factors such as personal smoking and obesity are likely also involved and were characteristics of the late-onset persistent phenotype, though we did not identify statistical evidence for a direct interaction.

Our study has a number of major advantages. The comprehensive respiratory data collected at seven time points from childhood to middle age in TAHS allowed us to extend the current framework of longitudinal asthma phenotypes from the mid-20 s in previous studies to the mid-50 s in TAHS. The population-based nature of TAHS also allowed us to examine the phenotype-specific prevalence of current asthma at each time point and to examine associations with a range of childhood, lifetime, and adult characteristics.

However, there were also several limitations. First, as data on current asthma at seven different time points were used to characterize our longitudinal phenotypes, participants with asthma symptoms occurring between follow-up time points (e.g., relapsing-remitting asthma) may have been misclassified. In addition, the interval between follow-up time points in TAHS was also longer compared with other prospective cohorts (10, 11, 13).

Second, our phenotypes were characterized using data at seven time points from ages 7–53 years, including at age 53 years when outcomes (COPD and comorbidities) were assessed. This approach limited our ability to determine the temporality (and thus causality) of the relationship between asthma phenotypes and clinical outcomes.

Third, although we aimed to examine the association between asthma phenotypes and early-life exposures and adult outcomes, we did not test for between-phenotype differences in these analyses. The small sample sizes for some phenotypes may have also resulted in a lack of statistical power within these groups.

Fourth, we did not statistically adjust for multiple testing in our analyses (e.g., via Bonferroni or Šidák corrections) and instead considered biological plausibility in the interpretation of our results. Replication of our analyses in other cohorts is also required to further examine the stability and generalizability of the identified phenotypes.

Finally, our use of self-reported data to determine asthma status is another potential limitation. However, these definitions have been validated with sensitivity and specificity of 80% (58–93%) and 97% (90–99%) against respiratory physician assessments (23), respectively.

Conclusions

This study is the first to characterize longitudinal asthma phenotypes over the life course from childhood to middle age. Using a data-driven GBTM approach, we identified five distinct phenotypes distinguished by age of asthma onset and asthma remission, of which two remitting phenotypes are novel. These phenotypes were differentially associated with childhood risk factors and adult outcomes. We would emphasize that asthma at any age should be taken seriously and treatment titrated to achieve good clinical control. Clinicians should also be aware that clinically remitted asthma may be an important risk factor for COPD in later life. Future research should focus on linking longitudinal asthma phenotypes with biological pathways and further exploring genetic associations. Studies should also determine whether long-term preventive treatment and smoking cessation can alter disease trajectory and improve outcomes for patients with asthma. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

Acknowledgment: The authors acknowledge the participants and previous investigators of the TAHS. The authors thank TAHS investigators who are not coauthors of this paper for their assistance with obtaining funds and data collection. The authors also acknowledge all the study site coordinators and respiratory scientists who collected the data; the research interviewers and data entry operators; and the organizational roles of Cathryn Wharton and Dr. Desiree Mészáros. Furthermore, the authors thank the late Dr. Stephen Morrison (University of Queensland) for his assistance with obtaining funds and data collection. Finally, the authors thank the Archives Office of Tasmania for providing data from the 1968 and 1974 TAHS questionnaires and copies of the school medical records.

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Viral infections and atopy in asthma pathogenesis: new rationales for asthma prevention and treatment

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Prospective birth cohort studies tracking asthma initiation and consolidation in community cohorts have identified viral infections occurring against a background of allergic sensitization to aeroallergens as a uniquely potent risk factor for the expression of acute severe asthma-like symptoms and for the ensuing development of asthma that can persist through childhood and into adulthood. A combination of recent experimental and human studies have suggested that underlying this bipartite process are a series of interactions between antiviral and atopic inflammatory pathways that are mediated by local activation of myeloid cell populations in the airway mucosa and the parallel programming and recruitment of their replacements from bone marrow. Targeting key components of these pathways at the appropriate stages of asthma provides new opportunities for the treatment of established asthma but, more crucially, for primary and secondary prevention of asthma during childhood.

Asthma is a common disease affecting millions of children and adults. In its chronic form, asthma has a complex pathology that is characterized by airway inflammation accompanied by alterations in patterns of vascularization, innervation and airway smooth muscle growth and disturbances of the epithelial-mesenchymal trophic unit throughout the conducting airways[1]. These changes collectively compromise an individual's capacity to maintain normal respiratory functions, particularly when exposed to ubiquitous airborne environmental stimuli. This pathological state stems from repeated episodes of airway inflammation and ensuing cycles of tissue repair and regeneration occurring over a period of years (**Fig. 1**, top). This results in cumulative structure and function changes in the lung that eventually exceed critical thresholds beyond which the 'persistent asthma phenotype' comes to represent the new norm (**Fig. 1**, bottom).

In terms of asthma therapeutics, although effective symptom-relieving treatments are available, attempts to develop disease-modifying drugs have met with limited success. This slow pace of drug development is a reflection of the complexity of the underlying pathogenic mechanisms of asthma. However, this complexity is not universally acknowledged, and in the quest for new drugs, the strong tendency is still to view the disease process in unidimensional terms and focus exclusively on established asthma. This view is exemplified by the approach to the most common form of this disease, atopic asthma, in which the identity of the perceived primary pathogenic mechanism is enshrined in the name of the disease itself. Superficially, it seems that it could not be more straightforward: atopic sensitization leads to airway inflammation, which results in asthma. This sequence of events is readily demonstrable in experimental models, findings from which have driven the development of a range of therapeutic agents, such as a monoclonal antibody (mAb) to interleukin-5 (IL-5), that are effective in blocking their specific allergy-associated targets in short-term trials in subjects with established atopic asthma but do not achieve long-term disease modification. We do not share the view held by some in this field that the underlying experimental approach to understanding asthma pathogenesis that led to the development of these drugs is flawed; however, we believe that much of the knowledge gained from the experimental approach continues to be applied clinically in suboptimal contexts. In this regard, we share the growing view that whereas aeroallergen sensitization is one of the strongest asthma risk factors, it rarely *per se* leads directly to persistent asthma. Instead, this sensitization most frequently acts in synergy with other proinflammatory environmental cofactors, most notably respiratory viral infections, to drive disease development. The nature of the interactions between antiviral and atopic pathways and how they may be potentially targeted to treat and/or prevent asthma is the focus of this review.

Mechanisms driving asthma initiation: birth cohort studies

The potential role of respiratory viral infections as an initiating factor in asthma pathogenesis has been recognized for many years[2], but a more comprehensive understanding of the magnitude of the impact of these infections has only become evident relatively recently in light of emerging results from long-term birth cohort studies in the United States[3,4], Australia[5,6] and Europe[7] that span decades (reviewed in ref. 8).

These studies have collectively showed that whereas wheeze is common during infancy—particularly in association with respiratory infection—expression of this phenotype is usually transient and resolves spontaneously by age ~3 years (**Box 1**). In a subset of children, however, wheeze consolidates into a persistent clinical pattern that is indicative of early

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Published online 4 May 2012; doi:10.1038/nm.2768

onset asthma, and this phenotype is strongly associated with early sensitization to aeroallergens. In addition, lower respiratory viral infections (LRIs) can predispose to the development of early asthma, conferring a slightly higher risk than atopy alone. However, the most severe childhood asthma, and the type that confers the highest ensuing risk for progression to persistent asthma, is encountered when LRIs occur against a background of pre-existing aeroallergen sensitization[5,6,8–10], in particular during the period when postnatal lung growth and differentiation are proceeding most rapidly (**Box 1**). Under these circumstances, affected children are tenfold to 30-fold more likely to develop persistent and/or severe disease[6,9–11], and this outcome is a reflection of both the LRI frequency and intensity and the amounts of aeroallergen-specific IgEs that are present at the time of these episodes[5].

A key underlying issue is the relative strength of the associations between different respiratory viruses and early asthma pathogenesis. Earlier studies emphasized the key role of respiratory syncytial virus (RSV)[12], however, recent attention has shifted toward rhinovirus[3,10,13], especially the frequently encountered rhinovirus type C[14]. However, RSV is a major pathogen in infants[15], and the relative roles of these viruses in driving asthma beyond childhood are not completely understood[16].

Progression of asthma beyond early childhood

The question of what sustains asthma progression beyond the preschool years is central to the development of improved treatments, and cohort studies have again been instructive in this area. The strength of the association between atopy and asthma in schoolchildren was first recognized in the 1980s, but the clinical relevance of this association was not initially widely appreciated. These findings have proven to be extremely robust over time, as exemplified by recent studies in teenagers, particularly the clear quantitative associations between the strength of T helper type 2 (T_H2) cell immunity against indoor aeroallergens and asthma severity[17]. This connection, however, is not absolute—whereas the majority of teenagers with asthma are atopic, only a minority of these individuals with atopy develop asthma[17]. This suggests that although atopy manifesting as an inhalant allergy constitutes a strong risk factor for asthma in this age group, it is usually insufficient to trigger full-blown disease on its own, and additional cofactor(s) must be involved.

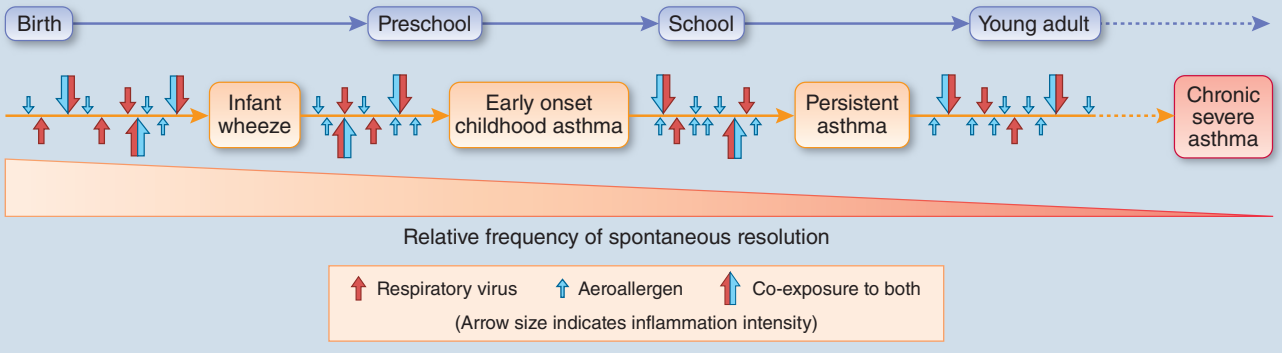
Epidemiological clues as to the nature of these cofactor(s) have come from studies on environmental triggers that precipitate wheezing attacks. Controlled exposure of aeroallergen-sensitized individuals with asthma to aerosolized allergens can provoke wheeze accompanied by T_H2 cell–associated airway inflammation[18], mirroring the murine asthma models. However, the extent to which direct aeroallergen-induced triggering explains spontaneous asthma attacks is unknown, as a clear clinical history of acute asthma after allergen exposure is rarely obtained. At the severe end of the asthma exacerbation spectrum, as exemplified by episodes that are associated with school absenteeism or hospital admission (reviewed in ref. 8), the most notable clinical feature is the concomitant presence of viral LRI. A variety of evidence suggests that this susceptibility is determined in part by aberrations in host antiviral immunity involving type 1 and/or type 3 interferons (IFNs)[19–22]. However, it is noteworthy that >80% of affected children are also atopic, and this comorbidity is a prominent feature of severe asthma exacerbations among adults as well[8]. This pattern is also reflected in children with less severe asthma, among whom the severity and duration of respiratory-virus–associated illnesses and the accompanying loss of asthma control are associated with an underlying atopy[11]. Emerging evidence suggests that additional features of these more severe asthma episodes are covert interactions between virally-triggered and allergen-triggered inflammatory pathways, as discussed below.

Viral-induced perturbation of immunological homeostasis

As noted above, viral infections perturb a range of mechanisms that are central to the maintenance of immunological homeostasis in the airways.

BOX 1 Initiation, progression and persistence of asthma in individuals with atopy

Normal lung function develops along percentiles[76,77] in a fashion similar to normal height, and disturbance to lung growth in early life can alter lung function permanently. The relevant lung growth processes, which proceed most rapidly during the first 2–3 years of life, include (i) gross structural alterations, such as progressively increasing airway diameter; (ii) more subtle processes, including alveolarization and associated changes in the airway-lining epithelia; and (iii) establishment of neural control of airway smooth muscle and local irritant receptor systems[78]. It has become apparent that environmental exposures that interfere with normal lung growth during this crucial period have the potential to limit an individual's capacity to attain optimal respiratory function[79,80]. The result of this interference is that the lung growth trajectory of an affected individual is dislodged, potentially permanently, onto a lower percentile line, and, thus, the individual is at an increased risk for asthma. A classic example is maternal tobacco smoke exposure during pregnancy that results in reduced respiratory function in her offspring at birth, which subsequently 'tracks' into later childhood and adulthood. Additionally, postnatal inflammatory insults to the growing lung that are associated with viral infection[4] and acute asthma exacerbations[81] also exert potent deleterious effects on the development of healthy respiratory function that can persist into later life. The precise mechanisms underlying this tracking phenomenon are probably multifactorial in nature and may include tissue remodeling changes associated with alterations in airway epithelial and myelofibroblast populations (the epithelial-mesenchymal trophic unit[82,83]).



A key cell population in this regard comprises the airway mucosal dendritic cells (AMDCs), which express the immature antigen-surveillance phenotype that they normally maintain until migration to the regional lymph nodes (RLNs), where they deliver a signal that is weakly T_H2 polarized that precedes the induction of tolerance[23]. During the course of viral infection, murine respiratory tract dendritic cell populations go through a variety of changes that potentially influence the tonus of the local immune surveillance mechanisms. One such change is the accumulation of plasmacytoid dendritic cells (pDCs)[24], which normally comprise a minor subpopulation in resting airway mucosa. These cells are postulated to play a crucial part in mucosal tolerance[25] and are also a major source of the IFNs that mediate the primary defense against viral infection.

A feature of ongoing immune surveillance within the airway mucosa is the orderly turnover of resident myeloid dendritic cells (mDCs), as replacements for emigrating antigen-bearing mDCs trafficking to the RLNs are continuously recruited from bone marrow[23]. In rats, this process is markedly accelerated during parainfluenza infection[26]. Comparable AMDC mobilization has been confirmed in mouse models[24,27] and in virally infected children[28]. Notably, expansion of the AMDC population persists well beyond parainfluenza clearance[26], and similar findings apply to RSV infection[24,27,28]. The mechanistic basis for these persistent postviral effects on AMDCs remains to be determined.

It is noteworthy that marked changes have been observed in other lung cell populations in infected mice that also persist after viral clearance[29]. Prominent among these populations are macrophages expressing the 'alternative activation' phenotype that is dependent on IL-4 or IL-13, and similar cells have been reported in lung biopsies from a small sample of patients with asthma and chronic obstructive pulmonary disease[29] and from bronchoalveolar lavage fluid derived from subjects with pulmonary fibrosis[30]. More detailed study of the activation phenotype of lung macrophages in individuals with asthma after exacerbation merits high priority given the long life span of these cells and, thus, their potential to influence the local tissue milieu for prolonged periods after a transient activation event.

Linking antiviral and atopic inflammatory pathways

Recent investigations have suggested that in addition to changes in the population dynamics of lung myeloid cell populations, viral infections may also profoundly influence their functional phenotype. Of particular relevance to this discussion are findings from Grayson and colleagues showing type 1 IFN–dependent induction of the high-affinity IgE receptor (FcεR1) on lung dendritic cells in the wake of murine parainfluenza infection[31,32]. The authors suggested that subsequent crosslinking of this receptor by viral-specific IgE may lead to the chemokine (C-C motif) ligand 28 (CCL28)-mediated recruitment and subsequent local activation of virus-specific T_H2 memory cells, thus providing a secondary source of proinflammatory cytokines in the already inflamed airways. The potential implications of a similar mechanism operating against a background of conventional aeroallergen sensitization were not explored in this model. However, a more recent study on the effects of influenza in mice sensitized to house dust mites confirmed upregulation of FcεR1 in infected lung tissues and linked this upregulation with an increased responsiveness to concomitant exposure to aerosolized house dust mites, which manifested as enhanced T_H2 cell–associated airway inflammation and decreased lung function[33].

Severe asthma exacerbations in children: recent insights

As has been previously noted[8], a small subset of asthma exacerbations in children are sufficiently severe to require hospitalization, and the children with these severe exacerbations typically have ongoing high-level inhalant allergy together with a concurrent respiratory viral infection. We recently

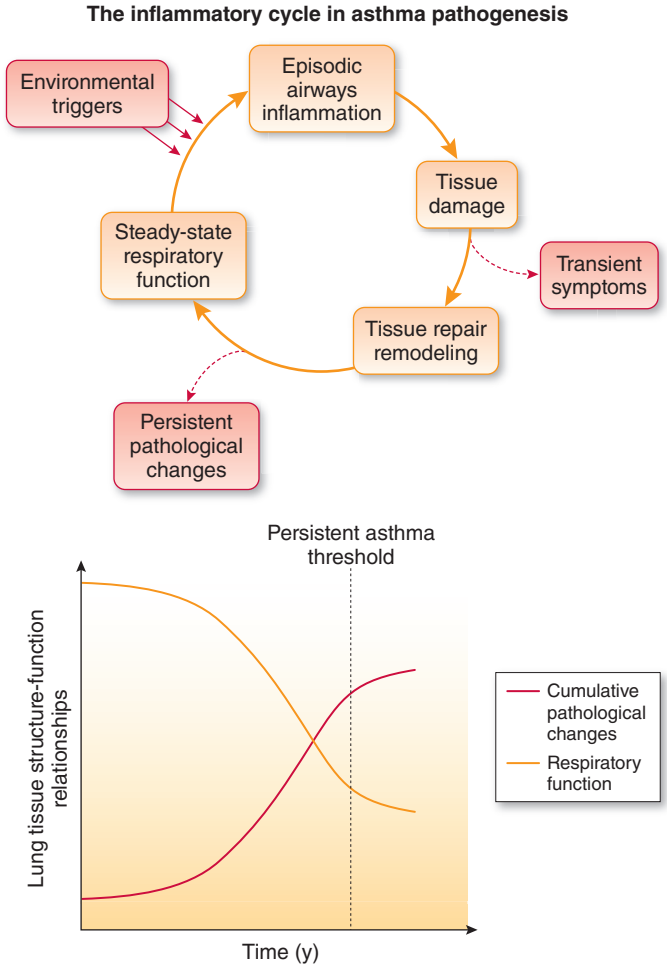


Figure 1 The inflammatory cycle in asthma pathogenesis. Asthma development is driven by repeated cycles of inflammation triggered by airborne irritant stimuli (top). Symptoms are initially intermittent and are associated with acute inflammation and edema and intermittent airway narrowing. Over time, the resolution of inflammation between clinically apparent episodes of asthma becomes less complete. Persistent inflammation leads to repeated cycles of tissue repair and regeneration, which may themselves be aberrant, and can lead to pathological changes that persist for long periods. As these changes accumulate, they lead to progressive deterioration in respiratory function (bottom). Once these changes exceed a critical threshold, they may not be reversible and may result in persistent asthma, with persistent symptoms that are not easily controlled by currently approved medications.

studied a cohort of such subjects[34] using an approach that was derived from earlier literature, which established that challenge sites in the lung signal to the bone marrow to recruit replacements for the myeloid cells that are engaged in antigen clearance during inflammatory episodes[35]. This process also involves selective preprogramming of effector functions that are dictated by the mix of incoming inflammatory signals reaching the bone marrow, thus equipping emigrating cells to deal optimally with the specific challenge involved[36]. Sampling blood-borne cell populations during inflammation as opposed to at baseline may provide some insight into the nature of the signals emanating from the challenge site and the resultant functional programming of migratory effector cells without directly accessing the challenge site itself.

Adopting this approach, we profiled circulating peripheral blood mononuclear cells from children with atopy who were hospitalized for severe

viral-induced asthma using a combination of microarray, quantitative RT-PCR and flow cytometry, comparing samples collected at admission to those collected during the subsequent convalescence[34]. The most prominent exacerbation-associated signatures in these subjects were within circulating myeloid populations (monocytes, mDCs and pDCs) and were indicative of IL-4– or IL-13–dependent activation and type 1 IFN signaling. Moreover, strong quantitative relationships were observed between the expression of individual genes and the intensity of the exacerbation[34]. These included *CCR2*, encoding chemokine (C-C motif) receptor 2, a major chemokine receptor directing migration to inflamed lung[37], together with *IL13RA2* (encoding IL-13R) and *CD1d*; upregulation of these genes was reproduced *in vitro* by the incubation of monocytes with recombinant IL-4 or IL-13 (ref. 34). The myeloid exacerbation signature also included FcεR1-γ, which was inducible *in vitro* by type 1 IFN, and parallel upregulation of FcεR1-α was shown both on monocytes and on the major dendritic cell subsets using flow cytometry[34].

The cytokine signals that are relevant to this systemic mechanism are probably transmitted from the airways to the bone marrow in a cell-associated form (**Fig. 2**). Indeed, the bone marrow is now recognized as a key repository for migratory T memory cells[38], and the *in situ* activation of these cells by immigrant antigen-bearing dendritic cells has also been formally shown[39]. Additionally, preactivation of these T memory cells may occur at sites of allergic inflammation, as has been inferred in human studies that identified IL-5–secreting T_H2 cells in bone marrow aspirates immediately after bronchial allergen challenge[18]. Type 1 IFN–dependent preprogramming of lung-homing leukocytes in bone marrow has also been shown during respiratory viral infection[40], and although the source of the IFN signal was not identified, the pathway through which tissue-derived dendritic cells can bypass the RLNs and traffic directly to bone marrow[39] may be involved.

It is noteworthy that FcεR1-α is known to be constitutively hyperexpressed on myeloid cells in individuals with atopy[41], and the translocation to the cell surface and stabilization of FcεR1-α as functional FcεR1-αγγ complexes is limited by the availability of the γ chain[42,43]. Consequently, upregulation of the γ chain gene in myeloid cells from individuals with atopy by type 1 IFN, as was shown in our *in vitro* studies[34], provides a potential mechanism for increasing the overall expression of functional FcεR1, similar to what has been reported in the atopic asthma mouse model[32,33] (**Fig. 2**). If this is the case, viral-triggered release of type 1 IFN in the infected airway may initiate this cascade through direct effects on resident AMDCs, and this cascade may be further amplified as inflammation associated with T_H2 cells develops in the mucosa and the resultant cell trafficking delivers the optimal combination of cytokine signals required to drive expression of both the FcεR1-α and γ chain genes in the myeloid precursor compartment. This mechanism may explain the presence in the circulation of monocytes and dendritic cells bearing functional FcεR1 in conjunction with gene signatures that are indicative of biomarkers of the alternative activation phenotype, which includes the FcεR1 γ chain itself plus the chemokine receptors required to facilitate their homing to the lung and airways to replenish local populations[34]. This systemic mechanism may function in virally infected children with atopy in parallel with the locally operating mechanisms described in murine parainfluenza models that stimulate the expression of the same range of T_H2-associated phenotypic markers on resident lung and airway myeloid cells[29,31,32].

One consequence of FcεR1-αγγ upregulation on AMDCs in virally infected individuals with atopy is suggested by observations relating to the pathogenesis of atopic dermatitis. Notably, studies on lesional sites in atopic dermatitis have indicated that if local dendritic cells armed with functional FcεR1 are coexposed to specific IgE and allergen, their capacity to capture, process and present activation signals to T_H2 memory cells is markedly amplified[44,45]. The preconditions of local availability of specific IgE and the relevant allergen would probably be met in the airways of many exacerbation-prone children with asthma among whom presensitization to perennial aeroallergens is common[8]. If both the specific IgE and relevant allergen are present, increased FcεR1-αγγ expression on the AMDCs triggered by virus should result in enhanced T_H2 cytokine release in the airway mucosa, increasing local inflammation in the infected airway (**Fig. 2**). A further consequence of increased FcεR1-αγγ expression may be a prolongation of the initiating infection itself through T_H2 cell–mediated antagonism of antiviral immunity, mirroring 'immune evasion' strategies that are used by a range of other pathogens[46,47]. This conclusion is consistent with a recent report on sputum-derived cells collected from children during severe viral-induced asthma exacerbations indicating the upregulation of T_H2-associated gene signatures, including *Fcer1a, IL5* and *IL13* with reciprocal downregulation of the T_H1-like/cytotoxic effector pathway[48]. It is also pertinent to mention a recent report that found that triggering of FcεR1 on pDCs attenuates their capacity to produce type 1 IFNs[49], thereby blunting the primary antiviral defense.

Protection against the spread of pathogens beyond the initial infection site is the primary role of type 1 IFNs, and the LRIs that are associated with asthma exacerbations reflect an initial failure of these primary defenses. Airway epithelial cells from individuals with asthma reportedly have a reduced capacity for the production of type 1 and type 3 IFNs[20,21], which may increase these individuals' susceptibility to infection. However, in terms of asthma exacerbations, the implications of variations in IFN-response capacity may be more complex and context dependent, given that several IFN-producing cell types (epithelia, neutrophils and pDCs) may be involved at various stages in the exacerbation cycle. Additionally, we have shown that although IFN-α efficiently activates myeloid FcεR1-γ gene transcription if present at sufficiently high concentrations, it can also provide feedback inhibition of IL-4– or IL-13–mediated FcεR1 α-chain induction[34], which is consistent with a longstanding reports in the literature regarding the allergy-antagonistic effects of this cytokine. This suggests that the same type 1 IFN signal that potentially initiates this FcεR1-dependent cascade through direct effects on resident AMDCs may eventually terminate the bone-marrow–dependent amplification loop that sustains the response, provided it accumulates in sufficient quantities in the bone marrow.

FceR1 expression on myeloid precursors during the 'atopic march'

Whereas the overall scenario described above (**Fig. 2**) may be restricted to the more severe manifestations of virus-associated asthma, the underlying mechanism involving communication between atopic lesional sites and myeloid precursor populations seems to operate covertly at lower intensities across a broad atopic disease spectrum. Three observations are consistent with this suggestion. First, blood monocytes from individuals with atopy constitutively hyperexpress FcεR1-α at levels that correlate strongly with their serum IgE titers[41], which can now be understood as precursor activation through a chronic drip feed of T_H2 cytokine signals from sites of allergic inflammation to the bone marrow. Second, IL-5 signals triggered in the airways by aeroallergen challenge of individuals with atopic asthma[18] (and sensitized mice[50]) leads to the activation of eosinophils in bone marrow and their subsequent trafficking back to the lung. And third, findings have indicated that during active flares of atopic dermatitis, rhinitis and atopic asthma, FcεR1-α expression is upregulated on dendritic cells in the respective target tissues (relative to when disease is quiescent) and that this transient upregulation is mirrored in dendritic cell populations in unaffected tissues in the same subjects[51]; this 'spillover' phenomenon is explicable via the operation of the atopic lesion–bone marrow axis, given that dendritic cell populations in all tissues are continuously renewed from the same precursor compartment.

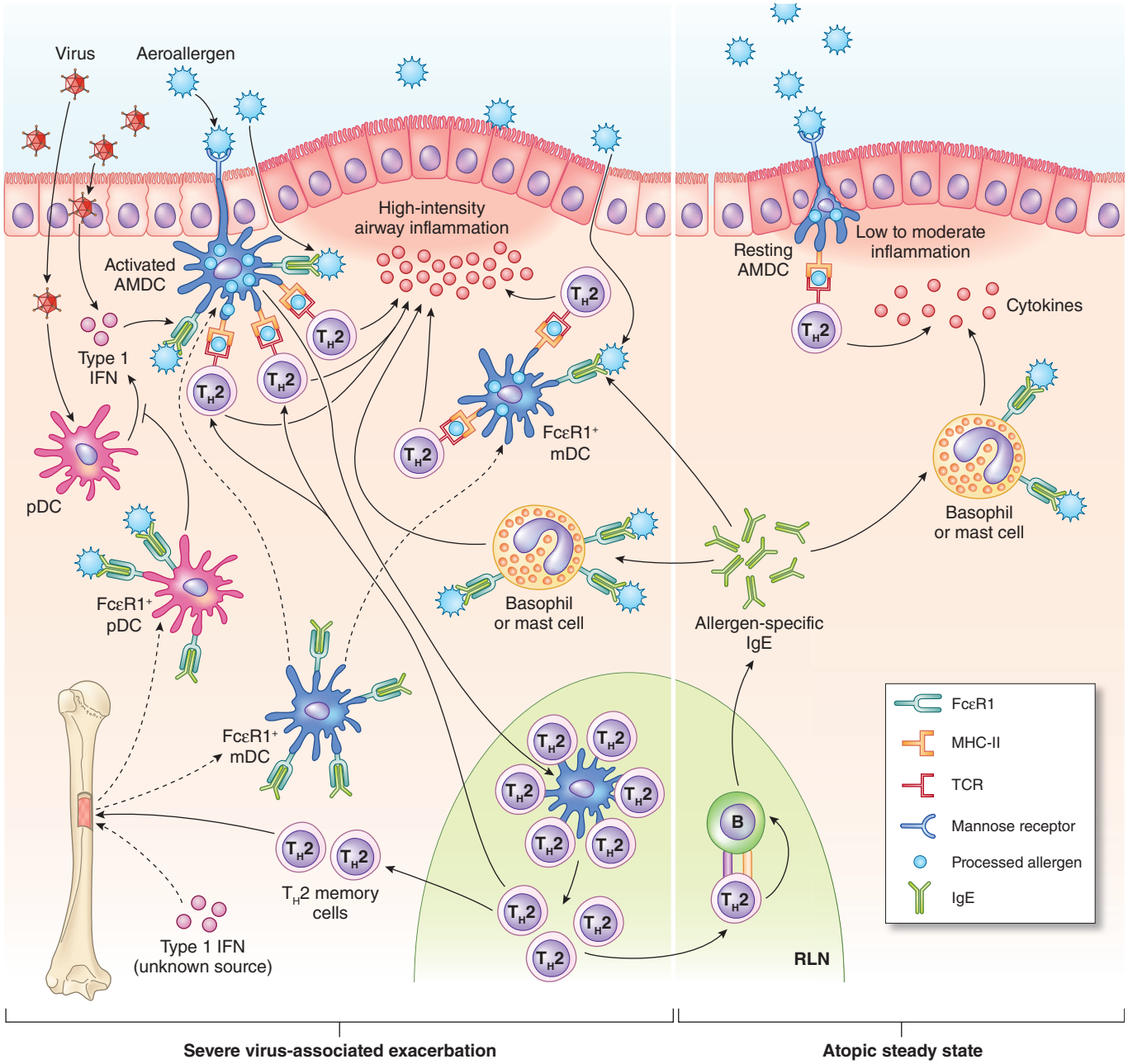


Figure 2 Cellular processes underlying virus-associated atopic asthma exacerbations. In individuals with stable atopic asthma, ongoing aeroallergen exposure sustains a state of relatively low level T cell–mediated and granulocyte-mediated inflammation in the airway epithelium that is punctuated occasionally by episodes of moderate intensity inflammation, for example, when aeroallergen exposure levels 'spike'; however, the potential for the local expression of high-intensity T_H2 cell–mediated inflammation is normally limited by restriction of the functional phenotype of the resident AMDCs to antigen uptake and processing with minimal presentation. This ongoing 'steady state' is perturbed by the local release of type 1 IFN in response to viral infection, which sets in motion a cascade that is initiated by the upregulation of turnover and FcεR1 expression on resident AMDCs, leading to increased recruitment and local (mucosal) activation of T_H2 effectors from the RLN and the subsequent expansion of T_H2 memory cell clones in the RLN by migrating AMDCs. The ensuing translocation of cytokine signals to the bone marrow by migrating T_H2 memory cells together with a source of type 1 IFN results in the release into the circulation of functionally upregulated myeloid populations, which home to the lung to further amplify local inflammatory responses. An additional short-term consequence is attenuation of local type 1 IFN production by pDCs through activation of their surface FcεR1, potentially contributing to viral persistence. The kinetics of this overall process are incompletely understood and may be highly variable; in particular, upregulation of myeloid cell populations in the lung and airways that persists long after viral clearance has been reported in both human and experimental systems, and the underlying mechanisms for persistence are unknown. B, b cell; MHC-II, major histocompatibility complex type 2.

These observations, together with those discussed in the previous section, suggest a plausible mechanism for what has been termed the 'atopic march', which is the characteristic pattern of atopic disease development during childhood involving the progression from initial atopic dermatitis and/or food allergy to rhinitis and eventually asthma[52] (**Fig. 3**). The operation of this axis provides a means by which active atopic disease in one organ system can facilitate the expression of disease in a secondary (previously unaffected) tissue by enhancing the efficiency of

the functions of local antigen-presenting cells through myeloid FcεR1 upregulation, thus optimizing the conditions for the local activation of allergen-specific T_H2 memory cells in the secondary tissue. The example in **Figure 3** draws on the literature showing, first, that aeroallergen sensitization in the absence of asthma symptoms is the rule rather than the exception[17] and, second, that allergic rhinitis is a strong risk factor for subsequent asthma[52]. In the scheme shown, active expression of allergic inflammation in the nose (rhinitis) increases the likelihood that inhalation of an aeroallergen will trigger clinically relevant levels of T_H2 cytokine secretion in the conducting airway mucosa by indirect upregulation of local FcεR1-dependent functions of the antigen-presenting cells. Over time, this may accelerate the progression toward expression of persistent airway disease (asthma), which is dependent on accumulation of pathological changes above a critical threshold in target tissues (**Fig. 1**, bottom).

It is worth noting that a related mechanism has been proposed based on murine experimental atopic dermatitis studies showing aggravation of asthma-like manifestations in the airways through hyperproduction of thymic stromal lymphopoietin (TSLP) by epidermal keratinocytes[53,54]; recent evidence suggests that this aggravation may also be mediated by effects in the bone marrow, possibly involving the stimulation of basophil hematopoesis[55].

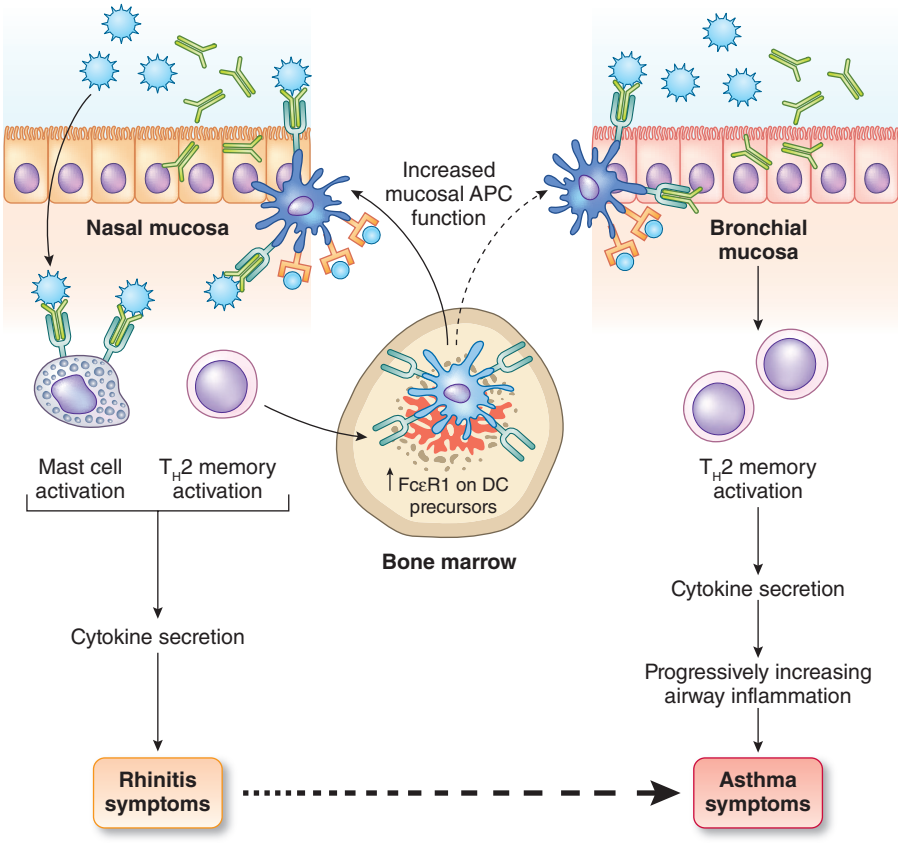


Figure 3 The atopic march unmasked? Findings summarized in the text provide a plausible mechanism for a longstanding but poorly understood clinical paradigm in the allergy field, notably the atopic march, which is exemplified by progression from allergic rhinitis to asthma. DC, dendritic cell.

The respiratory microbiome: a role for bacteria in asthma?

Opportunistic bacterial infections have long been recognized as potential contributors to the morbidity associated with viral LRIs, and this issue has arisen again in relation to the link between respiratory infections and asthma in light of two lines of recent evidence. First, using traditional culture methodology, it has been shown that early nasopharyngeal colonization with bacterial pathogens is associated with an increased risk for childhood asthma[56]. Recently, the application of sensitive metagenomic technology has revealed the presence in airway aspirates and tissue samples of a hitherto unrecognized microbiome comprising multiple bacterial strains[57,58]. These findings relate both to healthy individuals and individuals with asthma. Although bacterial diversity and density are increased in individuals with asthma, these are nevertheless multiple species that are also present in healthy individuals, suggesting that efficient homeostatic mechanisms must operate continuously in the lower respiratory tract to maintain a local steady state. Nevertheless, the constant presence of bacteria on the airway surface, particularly in atopic asthma, in which disturbance of epithelial integrity secondary to local inflammation is a characteristic feature, poses a series of quandaries relating to pathogenesis which have yet to be addressed.

From an immunological perspective, one window into the underlying relationships between a host and its respiratory microbiome is through an assessment of humoral immunity to index organisms, and some recent studies on the responses to protein antigens from *Haemophilus* and *Streptococcus* have provided interesting pointers for the future. Children who develop early asthma and atopy have lower serum concentrations of bacterial-specific IgG1 relative to the overall population[59,60], as do children who are susceptible to viral-triggered asthma exacerbations[61]. Reduced IgG1 concentrations in this context suggest an increased susceptibility to transmucosal bacterial incursion and may also connote underlying deficiencies in immune surveillance mechanisms. In this context, it is pertinent to note reports suggesting enhanced susceptibility to invasive pneumococcal disease in individuals with asthma[62,63].

Providing a second line of evidence, IgE responses against *Haemophilus* and *Streptococcus* are found in virtually all teenagers and, surprisingly, associate with a reduced susceptibility to asthma[64]; follow-up studies in 5-year-old subjects indicated a similar relationship[60]. Based on an extensive literature from other areas of inflammation biology, it seems probable that this bacterial-specific IgE may be a surrogate for underlying T_H2 cell immunity, especially for specific T_H2 memory cells producing IL-4 or IL-13. In this context, one of the most potent inflammatory responses involves interactions between bacteria and tissue macrophages, triggering the secretion of cytokines including IL-1 and tumor necrosis factor α (TNF-α), and this process is strongly inhibited by IL-4 or IL-13 (reviewed in ref. 64). Recirculating bacterial-specific T_H2 memory cells may serve this purpose in airway tissues, mitigating the inflammatory responses against low-level bacterial incursions across inflamed airway epithelial barriers that may be a relatively common occurrence. In this regard, it should be noted that bacterial-specific IgE concentrations increase in the wake of viral-associated asthma exacerbations[60], which is consistent with 'boosting' by an incoming bacterial antigen.

The question of why IgE against *Haemophilus* and *Streptococcus* do not apparently provoke mast-cell–mediated inflammation may reflect the fact that the relevant antigens are only released as membrane-associated

complexes that are accessible to phagocytes but are unsuitable for FcεR1 crosslinking. In contrast, IgEs against *Staphylococcus* superantigens that are secreted in a soluble form associate strongly with airway inflammation in the same children (reviewed in ref. 64).

New opportunities for prevention and treatment of asthma

Efforts over the last 20 years to develop improved therapeutics for atopic asthma have focused primarily on T_H2 effector mechanisms in subjects with established disease. These include biologics and small-molecule antagonists specific for T_H2 cytokines and the IgE antibody itself. Many of these drugs are highly effective at blanketing their respective targets, but in the contexts in which they have been tested in clinical trials, none have provided evidence of long-term disease modification. Part of the problem is that the pathological processes described in **Figure 1** become progressively less reversible over time. Additionally, it is becoming apparent that subphenotypes of established asthma exist that vary subtly with respect to the relative contributions of different inflammatory pathways to pathogenesis[65,66]. We argue, however, that an equally crucial issue is the lack of alignment of therapeutic strategies with the growing body of epidemiological and clinical information on how patterns of disease expression evolve over time under the influence of different classes of environmental triggers and, in particular, how interactions between the ensuing pathological processes contribute to symptom severity at different stages of disease. Bringing these issues to the forefront of the considerations regarding to target identification and clinical trial design provides new perspectives on drug development in asthma that might logically lead to new approaches to treatment and, more crucially, to asthma prevention (**Box 2**). Prominent among these approaches are a series of strategies based on using existing drugs in nonstandard clinical contexts that are outside the current treatment paradigms. From these we nominate below a high-priority list of treatment options that, based on available safety and efficacy data, are amenable to immediate testing.

Treatment of established atopic asthma. The most crucial targets in established asthma are the acute exacerbations triggered by a virus and/or aeroallergen. Current clinical practice involves the use of inhalers to relieve symptoms of airway narrowing together with nonspecific anti-inflammatory drugs (typically corticosteroids). However, this approach is essentially empirical and takes no account of the efficiency of these drugs in controlling individual rate-limiting steps in the cycle that drives the exacerbation. The scheme in **Figure 2** and the proof-of-concept studies cited in **Box 2** provide a rationale for the use of anti-IgE and a topical IL-4/IL-13Rα antagonist in this context, specifically targeting AMDC-driven or FcεR1-facilitated aeroallergen presentation and the ensuing activation of T_H2 memory cells in the airway mucosa and RLNs; these are the key steps in the underlying inflammatory cascade, and blocking either step sufficiently early in the cycle (meaning close to symptom onset) may halt its progression toward bone marrow amplification. Later in the cycle, an IL-4/IL-13Rα antagonist given systemically may be the ideal candidate drug to truncate the downstream events in the bone marrow, notably the IL-4– or IL-13–dependent generation and programming of $CCR2^+$ lung-homing alternatively activated macrophages and the $FcεR1^{hi}$ replacements for airway dendritic cells.

Halting the progression of atopic asthma during childhood. Atopic asthma progresses through recognizable stages during childhood (**Box 1**), and there is growing interest in early identification of children already on a trajectory toward asthma to institute treatments to reduce the frequency of the inflammatory episodes driving disease progression and consolidation. The priority targets are as follows.

(i) Virus-associated exacerbations. The most persuasive recent data supporting this approach are from the US Inner City Asthma Consortium showing that children on monthly anti-IgE therapy had significantly reduced rates of exacerbations, particularly during the common cold season[67]. The seasonality of respiratory viral infections (especially rhinovirus) leaves open the strong possibility of 'common cold season only' prophylactic treatment of high-risk individuals with atopy as a viable public health measure. An additional possibility meriting testing is treatment during the common cold season with oral immune enhancer (IE) preparations[68], particularly those can stimulate mucosal regulatory T (T_{reg}) cell activity[69,70], such as those described in **Box 2**.

(ii) Inhalant allergy: halting the atopic march. The burgeoning area of immunotherapy focuses primarily on 'curing' allergic diseases. However, in light of the evidence that during childhood one form of allergy can lead to another (shown in the link between allergic rhinitis and subsequent asthma in adults and children[71,72] and **Fig. 2**), early immunotherapy in appropriately selected subjects will also probably be useful for preventing disease progression, and this idea is supported by proof-of-concept studies on asthma prevention in children with rhinitis[73]. Notably, the key cellular process that is probably driving this example of the atopic march, namely mucosal dendritic cell trafficking, is highly sensitive to topical corticosteroids[74]; therefore, despite the failure of long-term topical steroid use by inhaler to ameliorate established wheeze[75], nasal steroids merit testing as an asthma prophylactic in the specific context of children with allergic rhinitis who are asthma free.

The 'holy grail': primary prevention of asthma. The ultimate goal is to identify high-risk subjects in the infancy to preschool age range and institute measures to subvert atopic and viral-associated inflammation early enough to block the initiation of disease. As noted in **Box 1**, the wheezy phenotype shows a high degree of plasticity at these early ages but becomes progressively less reversible thereafter, marking this period as a potentially ideal therapeutic window for long-term disease modification. The atopy pathway is already being tackled using prophylactic immunotherapy (**Box 2**). However, it is not yet possible to conduct trials for anti-IgE in this context because safety data is lacking for this age group. However, this agent merits testing as soon as these data become available in light of a recent report showing the quantitative nature of the relationship between aeroallergen-specific IgEs and LRIs in early asthma development. Notably, the strength of the synergism between IgEs and LRIs increased linearly with antibody titers across the full range of LRI frequencies, even at IgE concentrations below the conventional sensitization thresholds.

Tackling the viral pathway in this young age group has been problematic. Whereas an effective antibody treatment for RSV exists (**Box 2**), it has not yet been made available for testing in this clinical context, despite strong support (which we endorse) from senior investigators in this field[16]. Alternative approaches include the use of preparations from the IE class, given its potential for reducing both infection frequency and intensity in preschoolers[68], and, potentially, type 1 IFNs.

Future priorities

The cycles of episodic airway inflammation (**Fig. 2**) are driven by migrating T_H2 memory cells and myeloid cells responding to chemokine gradients through specific receptors such as CCR2 and the chemoattractant receptor-homologous molecule expressed on T_H2 cells (CRT_H2); targeting these players should be given high priority. Slightly over the horizon, rapid developments in relation to the airway microbiome are already creating expectations that antibacterials will soon join antivirals on the asthma agenda, despite justifiable concerns related to bacterial drug resistance. In our view, the key challenge is not the production of safe drugs that are effective against their designated targets (this will undoubtedly be achieved) but rather is to ensure that the clinical testing programs do justice to the upstream research and development efforts that led to their production. Achieving this for asthma will require a more disciplined approach to drug testing strategies than has been used in the past,

BOX 2 The development of rational strategies for asthma treatment and prevention

Respiratory infection resistance

Antivirals. A monoclonal antibody (mAb) for the treatment of RSV is available and has been shown to markedly reduce severe infections in infants[84].

Orally delivered immune enhancers (IE). This area is dominated by probiotics, and this approach has the potential for the mitigation of respiratory infection severity in children[85]. A related class of therapeutics derived from mixed bacterial extracts has been used extensively to enhance infection resistance in patients with chronic respiratory diseases, for example, chronic obstructive pulmonary disease[86,87] and has been shown more recently to reduce the frequency and intensity of respiratory infections in young children[68,88].

Type 1 IFN. The use of IFN therapy for the prevention of viral infection has a checkered history, particularly in terms of side effects. In a recent development, a nasal spray containing low-dose recombinant IFN-α-2b reduced influenza, parainfluenza and adenovirus infections in military recruits[89].

Airway inflammation

A topical antagonist to IL-4/IL-13Rα. Studies with an aerosolized antagonist reported significant reduction in the T_H2 cell–dependent late-phase response to aeroallergen challenge in individuals with atopic asthma[90].

MAb to IgE. An mAb to IgE has been tested in a variety of clinical contexts since its initial release over 10 years ago, but the clinical effects achieved in conventional trial designs have been modest; recent studies have shown that continued use of these mAbs can reduce the frequency of exacerbations[67,91,92].

T_{reg} cell stimulants. An unexpected recent finding in recent preclinical studies in rodent asthma models suggests that a major target for one class of the immune enhancer preparations is the expansion of mucosal-homing T_{reg} cell population(s) that dampen airway inflammation[69,70]. This may explain their effects in reducing the clinical symptoms associated with respiratory infection[68].

Myeloid cell activation in bone marrow

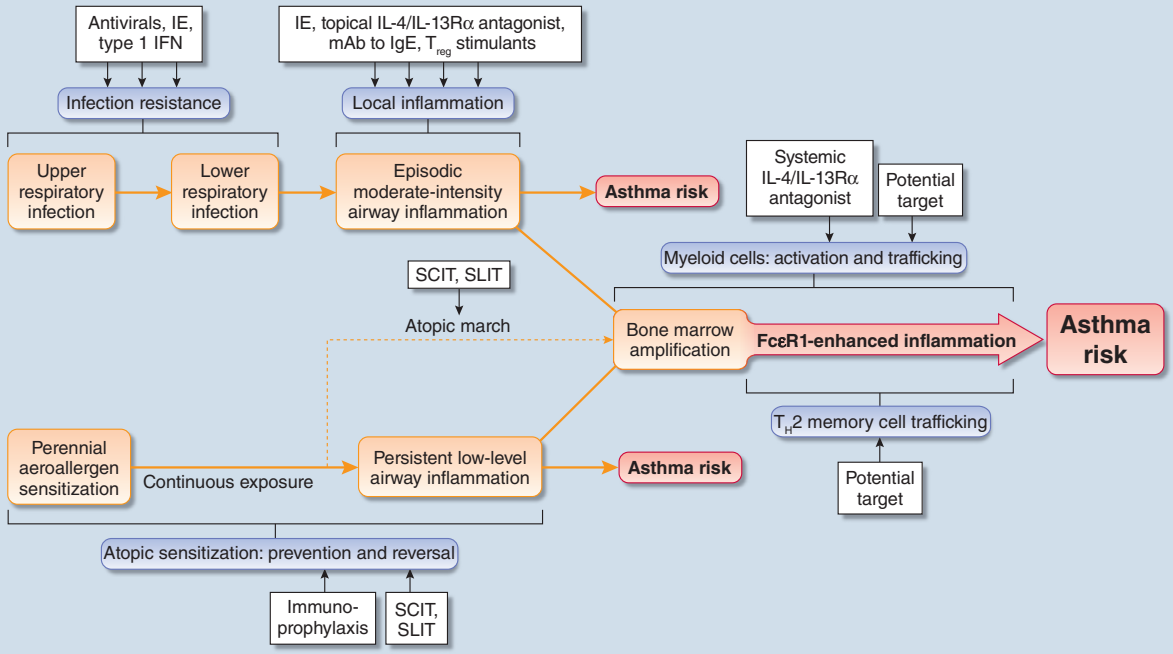
A systemic antagonist to IL-4/IL-13Rα. The agent detailed in reference 90 was equally potent in reducing the asthma late-phase response when delivered systemically in a form that would be bioavailable in bone marrow (and is also probably effective in airway RLNs).

The atopy pathway

The concept of immunoprophylaxis (primary prevention of sensitization) in infants and young children with oral or sublingual allergen is currently being tested in trials sponsored by the US National Institutes of Health; extensive literature exists on immunotherapy for sensitized children and adults, in particular, proof-of-concept studies showing that desensitization of children with rhinitis who are allergic to pollen using subcutaneous immunotherapy (SCIT) prevents the subsequent development of asthma[73] and studies on recently developed sublingual immunotherapy (SLIT[93]).

Gaps in the armor

Currently unavailable but highly relevant are drugs targeting the trafficking of myeloid and T_H2 cells. Also, as noted previously, this inflammatory cycle also has the potential for progression to a later phase that involves autocrine production of IL-13 by alternatively activated macrophages that may persist beyond viral clearance[29], and this cycle is potentially targetable with an mAb to IL-13 (ref. 94).



with a sharper focus on the clinical context within which individual targeted mechanisms contribute to symptoms at different stages of disease. In terms of existing drugs, specifically those targeting IgE and IL-4/IL-13Rα, we have only scratched the surface with respect to their optimal clinical use in asthma treatment, particularly from the perspective of preventing progression to a chronic state.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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Review article

The maternal gut microbiome during pregnancy and offspring allergy and asthma

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Environmental exposures during pregnancy that alter both the maternal gut microbiome and the infant's risk of allergic disease and asthma include a traditional farm environment and consumption of unpasteurized cow's milk, antibiotic use, dietary fiber, and psychosocial stress. Multiple mechanisms acting in concert may underpin these associations and prime the infant to acquire immune competence and homeostasis following exposure to the extrauterine environment. Cellular and metabolic products of the maternal gut microbiome can promote the expression of microbial pattern recognition receptors, as well as thymic and bone marrow hematopoiesis relevant to regulatory immunity. At birth, transmission of maternally derived bacteria likely leverages this *in utero* programming to accelerate postnatal transition from a T_H2- to T_H1- and T_H17-dominant immune phenotype and maturation of regulatory immune mechanisms, which in turn reduce the child's risk of allergic disease and asthma. Although our understanding of these phenomena is rapidly evolving, the field is relatively nascent, and we are yet to translate existing knowledge into interventions that substantially reduce disease risk in humans. Here, we review evidence that the maternal gut microbiome impacts the offspring's risk of allergic disease and asthma, discuss challenges and future directions for the field, and propose the hypothesis that maternal carriage of *Prevotella copri* during pregnancy decreases the offspring's risk of allergic disease via production of succinate, which in turn promotes bone marrow myelopoiesis of dendritic cell precursors in the fetus. (J Allergy Clin Immunol 2021;148:669-78.)

Key words: *Gut microbiome, fetal immunity, allergy, asthma*

The modern environment, lifestyle, and diet have altered the composition and function of the human gut microbiome,[1-11] which in turn plays a central role in early-life immune development.[12,13] Although most research regarding the gut microbiome and allergic disease has focused on the postnatal period,[13-15] the maternal gut microbiome during pregnancy promotes fetal immune development,[16,17] and appears to influence the expression of allergic disease in the offspring.[18,19] A range of mouse and human studies support the putative construct shown in Fig 1: In a traditional environment, adequate exposure to environmental microbes, the absence of antibiotics, adequate dietary fiber, and lower levels of maternal psychological stress and mental illness decrease the risk of offspring allergic disease and asthma via their impacts on the mother's gut microbiome and its products. The potential underlying pathways include the alignment between maternal and infant immunity; transplacental passage of microbial metabolites and components; and possible seeding of a fetoplacental microbiome. Collectively, these result in increased expression of microbe-associated molecular pattern (MAMP) recognition receptors, promotion of thymic regulatory T (Treg) cells, and the establishment

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Supported by National Health and Medical Research Council of Australia grants 1082307; 1147980; 1134812; 1166319.

Disclosure of potential conflict of interest: M. O'Hely, A.-L. Ponsonby, M. L. K. Tang, F. Collier, S. Ranganathan, and P. Vuillermin have a financial interest in the biotech company Prevatex Pty Ltd, which seeks to develop technology regarding maternal and infant carriage of *Prevotella copri* (PCT2016905378; PCT/AU2019/050878). M. L. K. Tang is an inventor on the patent "Methods and compositions for determining and for minimizing the likelihood of development of allergy in infants" (pending and licensed to Prevatex Limited).

Received for publication June 15, 2021; revised July 19, 2021; accepted for publication July 19, 2021.

Available online July 24, 2021.

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0091-6749/$36.00

https://doi.org/10.1016/j.jaci.2021.07.011

Abbreviations used
DC: Dendritic cell
EV: Extracellular vesicle
SCFA: Short-chain fatty acid
TLR: Toll-like receptor
Treg: Regulatory T

of dendritic cell (DC) networks in the gut, lung, and other tissues. Inoculation of the infant gut with the maternal microbiota during and after birth leverages the antenatal programming to accelerate the transition from T_H2 to T_H1, interferon regulatory transcription factor 7, and T_H17-dominant immunity required for pathogen clearance, and promotes DC induction of Treg cells in the gut and lung required to calibrate inflammatory responses.

There are however fundamental gaps in current knowledge. Few human studies have evaluated direct microbial transfer between mother and child, nor directly examined the relationship between the maternal gut microbiome and offspring allergic disease and asthma.[18,19] No such studies have incorporated metagenomic measures; nor have human studies demonstrated mediation pathways linking environmental factors, via maternal microbiome, and infant immunity to clinical outcomes. Relatively little is known about the multitude of factors driving fetal immune development, few human studies have shown that microbiome-related interventions during pregnancy alter offspring immunity, and overall, findings regarding probiotics supplementation during pregnancy are limited and conflicting. In this context, there is a clear need for improved evidence to progress and translate the potential of manipulating the maternal microbiome to prevent offspring allergic disease and asthma.

EXPOSURES IMPACTING BOTH MATERNAL GUT MICROBIOTA AND OFFSPRING ALLERGY

Farming environment

Studies in Europe, Asia, the United States, and Africa have shown that early-life exposure to a traditional farm environment is associated with reduced risk of allergic disease and asthma.[20-26] Although prenatal and postnatal exposures were highly correlated in each of these studies, the Prevention of Allergy Risk factors for Sensitization In children related to Farming and Anthroposophic Lifestyle (PARSIFAL) cohort was large enough to show that prenatal farm exposure may be more protective than postnatal exposure.[20]

Although the "farm effect" appears to relate to exposure to livestock[20,27,28] and a diverse range of environmental microbes,[29-32] several studies have shown that maternal consumption of unprocessed milk is also relevant.[27,33,34] Building on this, a recent study in mice showed that ingestion of raw versus pasteurized cow's milk increased butyrate-producing *Clostridiales* in maternal fecal samples, decreased *Proteobacteria*, and conferred protection against IgE-mediated food allergy in the offspring.[35] The risk of *Listeria monocytogenes* infection associated with ingestion of raw milk is an important consideration, particularly in pregnancy; thus, specific understanding of the underlying pathways is required to inform more targeted and safe interventions. Although traditional farming is becoming uncommon in Westernized populations, the same protection from allergic disorders has been observed in rural living environments, where extended biodiversity[36] has been suggested as a mediator.

Farm children demonstrate prenatal "priming" characterized in functional studies of cord blood by higher expression of the microbial pattern recognition Toll-like receptor (TLR)7 and TLR8 receptors,[37] equipping them to respond to viral-based signals during early infancy, as well as increased Treg-cell number and function, required to control inflammatory responses to pathogens and environmental stimuli.[38] Numerous studies have shown that both increased T_H1/IFN-γ responses,[39,40] increased Treg cells, and enhanced levels of neutrophils[37] at birth predict decreased risk of allergic disease and asthma.[41-44] Consistent with prenatal priming, farm children demonstrate accelerated progression from T_H2/eosinophil-skewed immunity at birth, which is associated with susceptibility to both infections and atopy, to a more balanced and competent immune state characterized by robust antimicrobial responses to pathogens, and enhanced Treg-cell function required for maintenance of immune homeostasis and control of excessive inflammation.[37] Further studies are required to evaluate the contribution of the maternal microbiome to the "farm effect."

Antibiotics

Antibiotics have profound and lasting effects on the gut microbiome.[45] Maternal treatment with antibiotics during pregnancy has been linked to increased eczema,[46] food allergy,[46-48] and asthma[49-51] in offspring. Evidence of a dose-response between maternal treatment with antibiotics and offspring asthma in human birth cohort studies[52] and experimental models[53] strengthens the case for causality. Antibiotic treatment during pregnancy may influence both the maternal and infant gut microbiome in mice,[54] so it is difficult to delineate the relative importance of the prenatal and postnatal periods. It is also impossible to exclude confounding by indication, in particular antibiotic treatment of maternal respiratory infections, which may relate to maternal asthma, and thus shared maternal/infant genetic risk. From large register-based studies, where it is also possible to evaluate associations with antibiotic exposure to the mother around the pregnancy period[55] and by applying siblings design,[56] evidence of antibiotic use in pregnancy as a causal link to later disease disappears. Instead, these studies suggest that a susceptibility to infections may be the factor inherited, because the mother's use of antibiotics outside the pregnancy period was just as important a risk factor for asthma in the child, and a higher risk of disease was also found in siblings unexposed to maternal antibiotics during pregnancy.

Diet

Although the relationship between maternal diet and offspring allergic disease remains controversial, recent work has added to the evidence that a maternal diet high in vegetables is associated with a reduced risk of offspring allergic disease in humans.[57] Diet has an important impact on gut microbiota composition and metabolic function.[8] Ecological studies have consistently shown associations between a preagrarian or hunter-gatherer diet and lifestyle, and increased diversity of the gut microbiota, and increased production of specific microbial metabolites, in particular, short-chain fatty acids (SCFAs).[1,2,9] In each of these studies, the hunter-gatherer communities had a diet high in plant-derived fibers,[58] which have a central role in shaping the composition and metabolic activity of the gut microbiome.[10] A maternal diet high

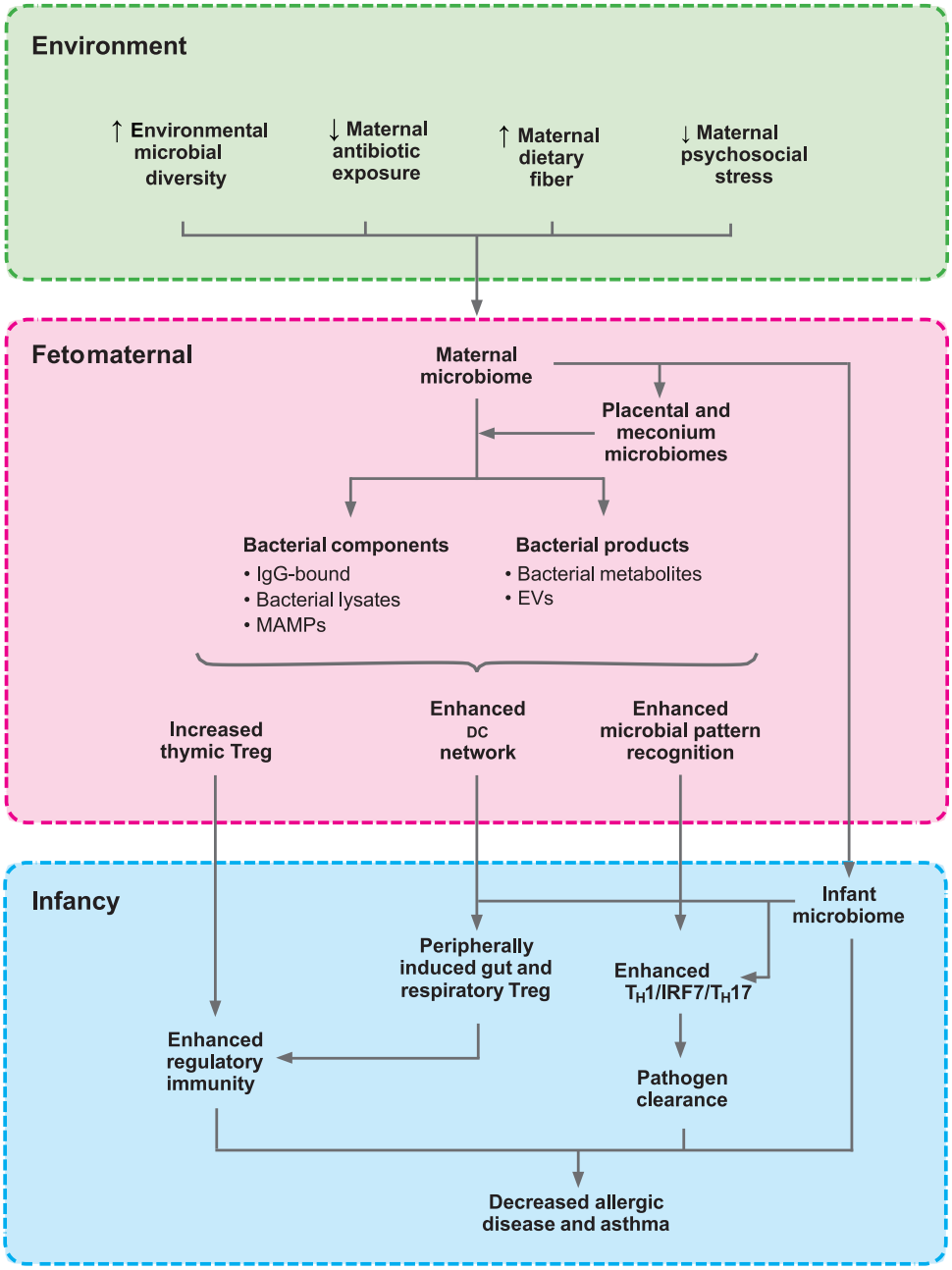

FIG 1. Putative pathways linking environmental factors, the maternal gut microbiome during pregnancy, early-life immune development, and protection against allergic disease and asthma. IRF7, *Interferon regulatory transcription factor 7; MAMPs,* microbe-associated molecular patterns.

in fiber is associated with increased diversity and richness of gut microbiota during pregnancy, with increases in specific taxa including *Holdemania*, *Roseburia*, and *Lachnospira* and *Coprococcus*.[59-61] Studies in mice have shown that a diet low in fiber leads to intergenerational depletion in the diversity of gut microbiota, which only partially recovers following reinstitution of a high-fiber diet.[62] This suggests that the low-fiber diet, which is now endemic in many parts of the world, is contributing to the loss of our "ancestral" gut microbes. Anaerobic bacteria in the gut ferment fiber to produce SCFAs, which have potent local and systemic immune effects[63] and can cross the placenta. In mice, a maternal diet high in fiber attenuates inflammatory airway responses to ovalbumin challenge in the offspring.[64] Although the underlying mechanisms are not fully characterized, SCFA induction of Treg cells may be relevant,[65] and human studies investigating maternal diet-by-microbiome pathways to enhanced infant immune competence are required.

Psychosocial stress and mental illness

Maternal psychosocial stress and mental illness during pregnancy are associated with increased risk of allergy and asthma in the offspring.[66-68] Stress-induced release of glucocorticoids has been suggested to mediate these effects,[69] but the impact of

psychological stress and anxiety on the maternal gut microbiome may also be relevant.[11,70-72] In support of this, animal studies have demonstrated that maternal psychosocial stress before and during pregnancy may alter both the mothers' and infants' intestinal microbiota, in part mediating the effects of maternal stress on immune development of the offspring.[73] Related studies are needed in humans.

POTENTIAL MECHANISMS

Maternal-fetal immune alignment

Preparing the fetal immune system *in utero* for the extrauterine environment is germane for survival, with maternally conferred immune protection against environmental pathogens representing a key defense mechanism. In this context, maternal transfer of humoral immunity, specifically of maternal IgG through an active mechanism enabled through neonatal Fc receptors, has been well established.[74] Through this process serum IgG levels of the term newborn are equal to or even exceed maternal levels. Of note, maternal IgG transfer of gut microbial components to the offspring during gestation also plays a crucial role in fetal innate immune development in mice.[17] Postpartum, transfer of maternal immunoglobulins continues through lactation, but these antibodies confer mucosal immunity only.[75]

In contrast, for cellular immunity it has been a long-held view that the fetus holds an immune privileged status, keeping the fetal and maternal immune cells separate, allowing physiologic development of the semiallogenic fetus without triggering inflammatory responses.[76] Immune tolerance toward the fetus is upheld by a series of highly effective mechanisms, with the placenta providing a barrier preventing immune rejection and/or trafficking of immune cells across the maternal-fetal interface.[77] The latter concept has been challenged by studies providing compelling evidence that traces of maternal cells can be found in fetal organs including the thymus[78] and vice versa.[79] This early exchange seems to shape the offspring's immune response and can in part explain the better acceptance of maternal versus paternal organ transplants.[80]

There is also evidence for alignment of maternal and fetal lymphocytes, specifically of Treg cells. This alignment is likely linked to soluble factors that cross the placenta from the mother to the fetus and effectively influence the size of the fetal Treg-cell pool[81,82] and metabolites of the maternal gut microbiome are likely candidates.[83]

Bacterial metabolites

The SCFAs butyrate, propionate, and acetate are by-products of dietary fiber fermentation by gut bacteria.[58] In mouse models, maternal SCFAs attenuate inflammatory airway response in offspring.[64] Although the underlying mechanism is unknown, SCFAs have potent immunomodulatory properties including induction of Treg cells from naive T cells via dendritic cells (DCs) either through epigenetic changes by inhibiting histone deacetylases[64,84,85] or through the activation of specific G-protein–coupled receptor (GPR)43.[86] Recently, acetate has been identified as an important factor in fetal thymic development, linked to the expression of the autoimmune regulator, which is known to contribute to Treg generation.[87] Succinate is another immune-active microbial metabolite that is increased in animals fed a high-fiber and high-fat diet.[88] Succinate binds GPR91, stimulating DCs and enhancing DC-mediated T-cell induction and cytokine production.[89] Further studies are needed to investigate the impact of these metabolites of the maternal microbiome on fetal immune development and offspring allergic disease.

Bacterial components

In mice, repeated administration of the bacterial lysate OM-85 during pregnancy attenuates aeroallergen-induced asthma-like responses in the offspring.[90] This finding is consistent with earlier work showing that maternal oral intake of *Acinetobacter lwoffii* F78, derived from farm (barn) dust, during pregnancy attenuated aeroallergen-induced inflammation in the offspring, and that this process was TLR-dependent.[91] The offspring of mothers treated with OM-85 during pregnancy demonstrate increased capacity for expansion and functional activation of Treg cells in the airway mucosa in response to aeroallergen challenge.[90] This enhanced regulatory capacity is underpinned by a dramatic expansion of bone marrow myeloid progenitors during late pregnancy, which in turn supply the establishment of tissue DC networks that mediate local immune surveillance and homeostasis.[90] Subsequent studies have shown that maternal OM-85 treatment is associated with transcriptional activation of cellular cholesterol biosynthesis pathways (mediated via the mevalonate pathway) and expansion of myeloid progenitors (myelopoiesis) and downstream conventional DCs displaying enhanced functional maturation within fetal bone marrow.[92] The establishment of DC networks in late gestation can be considered a form of immune training—a process in which innate immune cells exhibit a sustained state of enhanced functionality. Recently, it has also been demonstrated that treatment of pregnant mice with OM-85 during pregnancy protects offspring against mouse-adapted rhinovirus, reducing cellular inflammation and clinical severity of lung disease.[93] Studies are needed to determine whether maternal gut microbiota and their products promote fetal immune development via the same pathways.

Bacterial extracellular vesicles

Like other cells, gut bacteria produce extracellular vesicles (EVs), which are blebs of plasma membrane carrying a cargo of intracellular products such as proteins, nucleic acids, and lipids.[94] There is growing interest in the role of EVs in mediating the immune effects of gut bacteria. For example, the activation of TLR2 by *Bacteroides fragilis* has been shown to be mediated by the release of EVs.[95] In postnatal mouse studies, the administration of *Lactobacillus plantarum* EVs prevented the development of atopic dermatitis,[96] whereas the administration of EVs from *Bifidobacterium longum* prevented food allergy.[97] Given that EVs are small structures that can be actively transported from the mother to the fetus,[98] studies are needed to evaluate whether EVs produced by maternal gut microbiota impact fetal immune development.

In utero bacterial colonization

The presence of bacteria in sites including placenta, amniotic fluid, and meconium was reported in 2011,[99,100] and has since been the subject of intense debate. Subsequent studies have identified bacteria in placenta,[101-105] amniotic fluid,[106-111] and meconium,[103,104,112-114] and 1 study found that maternal consumption

of probiotics and contact with furry pets during pregnancy may each influence the microbiota present in meconium.[115]

Ascension and translocation through the choriodecidual barriers is one potential mechanism of *in utero* bacterial colonization.[102,108,116,117] Alternatively, microbes derived from the maternal intestine and oral cavity may be translocated by hematogenous spread to the placenta during implantation,[17,102] due to higher intercellular junctional permeability and/or DC transport.[118] Translocation of bacteria to the maternal circulation, via the epithelial gaps of the intestine and the oral mucosa, is enhanced during pregnancy, and this enables the transfer of low numbers of bacteria to possibly seed the placenta.[102,118-120]

Although some studies suggest that the presence of bacterial DNA in pregnancy tissues may relate to contamination of samples or reagents,[104,105,121] it remains a possibility that a fetoplacental microbiome plays a role in fetal immune development. The meconium microbiota was found to reflect microbial composition of the amniotic fluid,[122] suggesting that the fetal intestine could become colonized via ingestion of small quantities of amniotic fluid during the antenatal period and thus the meconium microbiome could be a proxy for the fetoplacental microbiome. Of relevance, a study from the Canadian Healthy Infant Longitudinal Development (CHILD) cohort suggests that the metabolic diversity of meconium collected at birth predicts both the infant's postnatal gut microbiota and the risk of allergic sensitization at 1 year.[123] Moreover, a recent study found live bacterial strains including *Staphylococcus* and *Lactobacillus* in fetal tissue, which induced *in vitro* activation of memory T cells in fetal mesenteric lymph nodes, supporting a role for microbial exposure in fetal immune programming.[124]

Vertical transmission of gut microbiota at birth

In infants delivered vaginally, the maternal gut microbiome is the largest donor of the infant-acquired strains[125,126] whereas the maternal vaginal transfer appears of minor importance.[127] Strain-level analyses of mother-infant pairs revealed that 50% of the microbial species in the infant gut on the day of delivery were transmitted from the mother's microbiome, and this fraction was relatively stable over the next 4 months.[125] The largest contribution at birth was from the mothers' gut, accounting for 22%, followed by the vagina (16%), the oral cavity (7%), and the skin (5%).[125] Moreover, the maternal gut microbial strains were more persistent in the infant's gut and more stably detected than those obtained from other maternal or environmental sources.[125,126,128]

It is therefore unsurprising that mode of birth profoundly influences the infant's gut microbiome.[128-133] Although the link between mode of birth and allergic disease remains controversial, a recent study found that increased risk of asthma following cesarean delivery was apparent in only those children who retained a "cesarean section signature" in the gut microbiome to age 1 year.[134] Strain-level analyses directly implicate the interruption of maternal-infant transmission in this effect.[125,126,133,135] Interventions proposed to offset the alteration in gut microbiota induced by cesarean section include dietary supplementation with a mix of probiotics during pregnancy and to the infants,[136] and postnatally, orally delivered fecal microbiota transplantation from their mothers.[137] Both techniques appear to ameliorate birth mode–associated differences in infant intestinal microbiota, but there is currently no evidence that either reduces the baby's risk of allergic disease or asthma.

DIRECT LINKS BETWEEN MATERNAL GUT MICROBIOTA AND OFFSPRING ALLERGY IN HUMANS

As far as we are aware, only 2 published studies have directly investigated the relationship between the maternal gut microbiota and offspring allergic disease in humans. A small study (n = 60) from 2012 used culture-based techniques to show that higher total aerobes and *Enterococci* in maternal fecal samples predicted increased risk of infant wheeze.[19] More recently, we used 16S gene amplicon sequencing to demonstrate that maternal carriage of *Prevotella copri* during pregnancy predicts decreased IgE-mediated food allergy in the offspring.[18] Importantly, maternal rather than infant carriage drove this association. *Prevotella* carriage is virtually ubiquitous in preagrarian, hunter-gatherer populations,[1,2,9] and the genus is among the so-called ancestral microbes present in the human gut at least as far back as the Neanderthal period,[7,138] and which are now disappearing in Westernized populations.[1-7] *P copri* is the predominant *Prevotella* species within the human gut microbiome. Our study showed that reduced household size and the recent exposure to antibiotics were associated with decreased maternal carriage of *P copri*.[18] The link between household size and *P copri* carriage is consistent with previous studies,[139] and intriguing given the well-known, yet still unexplained, association between larger household size and decreased allergic disease.[140] *P copri* is an important producer of succinate,[88] which can stimulate function and migration of DCs.[89] Previous studies have shown that the metabolic products of the gut microbiome stimulate bone marrow hemopoiesis of DC precursors, which then migrate to peripheral tissues including the lung, attenuating the features of allergic airways disease.[141] Considering the mounting evidence regarding the role of DC hemopoiesis in late pregnancy in prevention of allergic disease,[90-92] we hypothesize that maternal carriage of *P copri*, via production of succinate, promotes the establishment of fetal DC networks, conveying protection against allergic disease and asthma (Fig 2).

PROBIOTIC SUPPLEMENTATION DURING PREGNANCY

Recent meta-analyses have found no consistent beneficial effects from probiotic supplementation (either prenatal or postnatal or both) for prevention of eczema[142] or asthma.[143] These inconsistencies may be due to differences in the study populations, the probiotic strains, using a single strain rather than a complex mixture, and administration protocols. Although several trials found that combined maternal and infant probiotic supplementation with various strains of *Lactobacillus* reduced the risk of allergy,[144] others found no benefit[145,146]; and the only trial of *Lactobacillus* in pregnancy alone was negative.[147] Potential benefits may be modified by mode of delivery. One trial randomized high-risk mothers to receive either a probiotic mixture (*L rhamnosus* GG ATCC 53103 5, *L rhamnosus* LC705 DSM 7061, *Bifidobacterium breve* Bb99 DSM 13692, and *Propionibacterium freudenreichii* ssp. shermanii JS DSM 7076) or placebo twice daily starting from 36 weeks of gestation.[148] Their infants then received the same probiotic combination mixed with galacto-

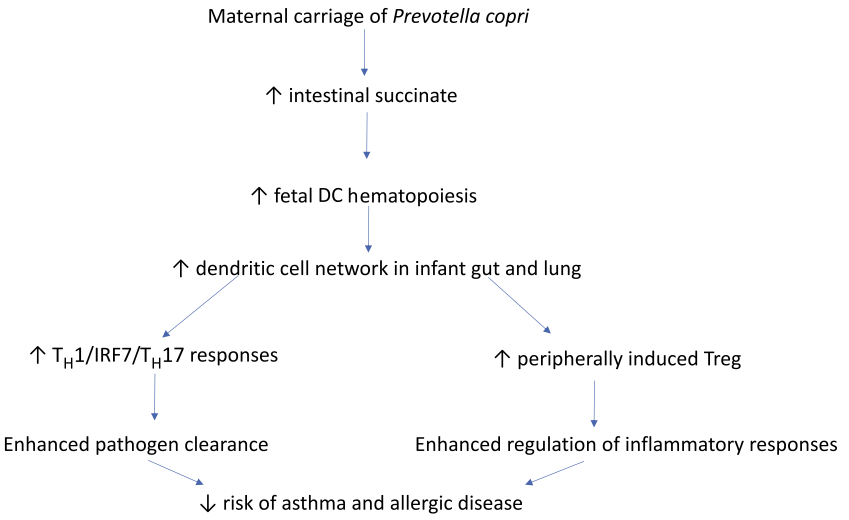

FIG 2. Hypothesized pathway by which maternal carriage of *Prevotella copri* trains fetal immunity, conveying protection against subsequent allergic disease and asthma. *IRF7,* Interferon regulatory transcription factor 7.

oligosaccharides once daily from birth until 6 months. Treatment with either probiotic mixture was associated with a reduced risk of allergen sensitization and eczema among children born via cesarean section but not among those born vaginally. Another trial found that maternal ingestion of probiotic milk containing *Lactobacillus acidophilus* LA-5, *Bifidobacterium lactis* Bb12, or *L rhamnosus* in pregnancy was associated with a slightly reduced relative risk of atopic eczema at 6 months and allergic rhinitis between 18 and 36 months compared with no consumption during pregnancy.[149]

Importantly, to date there have been no trials of supplementation with Gram-negative organisms, which may be relevant given that oral administration of the Gram-negative *Acinetobacter lwoffii* F78 in mice resulted in decreased allergy in the offspring.[91] This benefit was TLR-dependent, suggesting that LPS in the cell membrane of Gram-negative organisms may be important. Another consideration is that, in general, probiotic strains that do not colonize the bowel are specifically chosen; that is, they do not live permanently and multiply in the gastrointestinal tract. It is possible that active growth and multiplication is required to produce the metabolites that influence fetal and neonatal immune development, with subsequent reduction in offspring allergic disease.

FUTURE DIRECTIONS

Correcting the absence of deep whole metagenome sequencing data from human prebirth cohorts incorporating robust allergy and asthma outcomes is a priority. In contrast to 16S rRNA gene amplicon, deep metagenomic sequencing provides sufficient resolution at the species and strain level to infer the functional capacity and nutrient interactions of a given bacterium.[150] Estimation of absolute abundances of microbiota may also provide more accurate information than currently used measures of relative abundance.[151] In principle, the integration of metagenomics, downstream measures of microbiome activity including metabolomics, transcriptomics, proteomics, and lipidomics with various immune measures has significant promise. However, multiomic data generated using separate assays over the course of prenatal and postnatal life are both multimodal and temporal in nature. As such, their analyses and interpretation pose formidable challenges, and methodological frameworks built specifically for modeling time-varying multiomic data are required.[152] The compositional nature of microbiome data also has important implications.[153] This is generally addressed through various forms of preprocessing steps, the choice of which can substantially impact the results,[154] making quantitative metagenomics a preferable strategy, whenever possible.

Robust consideration of confounding bias and mediation pathways also poses an important, but neglected, challenge for the field. Microbiome data are highly dimensioned, and the determinants of specific components/metrics are largely unknown; thus, *a priori* identification of common causes of exposures and outcomes (confounders) is largely speculative. One approach to this dilemma is to apply the so-called disjunctive cause criterion,[155] which has an elegant proof and removes the need for previous knowledge of common causes. Others argue that a strictly *a priori*–defined approach to estimating causal effects using highly dimensioned data risks missing important signals and that one should therefore conduct multiple analyses aimed to build up layers of causal features and exclude noncausal explanations such as bias, chance, or reverse causation.[156]

Whichever approach is taken to preprocessing and analysis of human multiomics data, it is crucial to formulate clear research questions, and to then choose methods that are appropriate. This is a highly complex task requiring clinical, biological, bioinformatic, biostatistical, and epidemiological input. Reporting from human studies should include effect sizes both with and without adjustment along with a clear description of the causal framework that underlies the model(s) that has been applied, and the sensitivity of the findings to key assumptions. Importantly, the risk of false discoveries in highly dimensioned data must be controlled for and replication is crucial.

Progressing from correlation in human microbiome studies to causation and effective interventions requires mechanistic insights.[157] Preclinical studies have generally involved the transplantation of bacteria from humans with and without disease into germ-free mice and comparative analysis of pathological

outcomes. Although this approach has merit, it is of concern that the vast majority of published studies using this approach have found that transplantation of a given human-derived microbe results in altered expression of the relevant disease phenotype in mice, which is implausible, and a more rigorous approach to inferring causality is required.[158]

CONCLUSIONS

The maternal gut microbiome plays a fundamental role in priming the infant's developing immune system to acquire immune competence and regulation following exposure to the extrauterine environment. Evidence from a diverse range of studies suggests that loss of our ancestral gut microbes and altered metabolic activity of the maternal microbiome may be contributing to the high rate of allergic disease and asthma in the modern environment. Although rapid progress in omics technology has created tantalizing opportunities to progress current knowledge regarding interplay between the microbiome and early-life immunity, analysis and interpretation of such complex data brings formidable challenges. In this context, transdisciplinary collaboration is needed to generate strong and clearly defined research questions, fit-for-purpose analysis plans, and iterative human and animal research to identify mechanisms and confirm causality.

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Received: 8 September 2017 | Revised: 25 March 2018 | Accepted: 29 March 2018

DOI: 10.1111/cea.13181

ORIGINAL ARTICLE

Asthma and Rhinitis

WILEY

Novel genes and insights in complete asthma remission: A genome-wide association study on clinical and complete asthma remission

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Summary

Background: Asthma is a chronic respiratory disease without a cure, although there exists spontaneous remission. Genome-wide association (GWA) studies have pinpointed genes associated with asthma development, but did not investigate asthma remission.

Objective: We performed a GWA study to develop insights in asthma remission.

Methods: Clinical remission (ClinR) was defined by the absence of asthma treatment and wheezing in the last year and asthma attacks in the last 3 years and complete remission (ComR) similarly but additionally with normal lung function and absence of bronchial hyperresponsiveness (BHR). A GWA study on both ClinR and ComR was performed in 790 asthmatics with initial doctor diagnosis of asthma and BHR and long-term follow-up. We assessed replication of the 25 top single nucleotide polymorphisms (SNPs) in 2 independent cohorts (total n = 456), followed by

expression quantitative loci (eQTL) analyses of the 4 replicated SNPs in lung tissue and epithelium.

Results: Of the 790 asthmatics, 178 (23%) had ClinR and 55 ComR (7%) after median follow-up of 15.5 (range 3.3-47.8) years. In ClinR, 1 of the 25 SNPs, rs2740102, replicated in a meta-analysis of the replication cohorts, which was an eQTL for *POLI* in lung tissue. In ComR, 3 SNPs replicated in a meta-analysis of the replication cohorts. The top-hit, rs6581895, almost reached genome-wide significance (*P*-value 4.68×10^{-7}) and was an eQTL for *FRS2* and *CCT* in lung tissue. Rs1420101 was a cis-eQTL in lung tissue for *IL1RL1* and *IL18R1* and a trans-eQTL for *IL13*.

Conclusions and Clinical Relevance: By defining a strict remission phenotype, we identified 3 SNPs to be associated with complete asthma remission, where 2 SNPs have plausible biological relevance in *FRS2*, *CCT*, *IL1RL1*, *IL18R1* and *IL13*.

1 | INTRODUCTION

Asthma is a highly prevalent chronic airway disease that affects over 300 million subjects worldwide. Asthma symptoms usually start in childhood, but not all preschool wheezers will continue to have asthma symptoms in childhood. In a birth cohort of asthmatic children diagnosed before the age of 6 and followed till 12 years of age,[1] asthma remission was present in 48% of the children when defined as the absence of any asthma events (hospitalization and/or physician visit) between the ages 6 and 11 years.

In contrast, asthma remission is less common in adults. We previously defined 2 types of remission,[2] one being "clinical remission," where asthmatics lose their symptoms for at least 12 months without using asthma treatment. This type of remission occurred in 52% of adult asthmatics. In contrast, remission occurred only in 22% of asthmatics when defined as "complete remission." The latter type had clinical remission and additionally met the more stringent criteria of normal lung function and absence of bronchial hyperresponsiveness (BHR), a hallmark of asthma.[2] In an observational study in adults with and without complete asthma remission, we found that those with complete remission lacked inflammatory infiltration of the airway mucosa, suggesting the inflammatory response had faded away.[3] This was supported by the observation that individuals with complete asthma remission did not develop sputum eosinophilia after inhaling adenosine monophosphate,[4] a trigger previously shown to attract eosinophils to sputum in asthmatics, independently of the use of inhaled corticosteroids.[5]

Genome-wide association (GWA) studies have provided insights into the origins of asthma, and multiple genes have been associated with asthma development and subsequently replicated, such as the *ORMDL3* region, *IL1RL1*, *IL33* and *TSLP*.[6] This has led to some promising novel asthma treatments like anti-TSLP.[7] However, despite better treatments, there is still no cure for asthma. We hypothesized that genetics also plays a role in asthma remission and that the genes associated with asthma remission may differ from those associated with the development of asthma. Therefore, we performed a GWA study on asthma remission to provide us with clues to develop a cure for asthma. We investigated a Dutch identification cohort of 790 asthmatics initially characterized by the presence of a doctor diagnosis of asthma and BHR and assessed their remission status after a median 15.5 years of follow-up. We verified our findings in 2 European cohorts using the same phenotypic criteria and finally we performed expression quantitative loci (eQTL) analyses to determine allelic effects on gene expression in lung tissue and epithelial cells.

2 | METHODS

2.1 | Study population

We included 790 subjects from a long-term follow-up cohort with a doctor diagnosis of asthma and a positive BHR test at baseline. All participants who were younger than 12 years at baseline had an additional follow-up visit during early adulthood in which the persistence of asthma was confirmed by symptoms and objective measures during a clinic visit. All subjects had at least one follow-up measurement during adulthood (>18 years) in which their asthma status was evaluated by questionnaires and in most patients additionally with spirometry and a BHR test. All subjects participated in asthma studies performed at the University Medical Center Groningen[2,8-15] and provided written informed consent. All studies were approved by the medical ethical committee of the University Medical Center Groningen (ethics approval numbers METc 2009/007, METc 2010/378 and METc 2012/173).

2.2 | Definition of asthma remission

Clinical remission (ClinR) at follow-up visit was defined as the absence of asthma symptoms (no wheezing without having a cold in the last year and no asthma attacks in the last 3 years) and no use

of asthma medication in the last year but individuals could still have a low lung function or the presence of BHR. Complete remission (ComR) was defined as the absence of asthma symptoms and no use of asthma medication, a normal lung function (FEV_1%predicted pre-bronchodilator >80% or FEV_1%predicted post-bronchodilator >90%) and no BHR (no 20% decrease in FEV_1 at the maximal dose of histamine, methacholine or AMP according to the protocol[3-5,16,17]). Persistent asthma (PersA) was defined as the presence of asthma symptoms and/or the use of asthma medication. If more than one follow-up visit was available, the most recent follow-up visit with information on BHR and spirometry was used to determine asthma remission status.

2.3 | Genotyping and statistical analyses

DNA quality control is described in the Supplemental Methods. After quality control, 156 954 single nucleotide polymorphisms (SNPs) were present in all 790 subjects after combining the different genotype platforms (Illumina 317 Chip, the Illumina 370 Duo Chip, or the Omni Express Exome chip (Illumina Inc., San Diego, CA) and subsequently used in analyses. These 156 954 SNPs tagged 36%-60% of all SNPs in the genome (Table S1). All statistical analyses were performed using PLINK v1.07. Logistic regression analyses investigated the association between SNPs and asthma remission with adjustment for sex without additional covariates given the relatively low number of participants. Due to the low number of subjects with ClinR (n = 178) and ComR (n = 55), not all SNPs were tested in an additive genetic model. In the analysis on ClinR, SNPs with a minor allele frequency (MAF) below 0.25 were tested in a dominant model and SNPs with a MAF $\geq$ 0.25 were tested in an additive model. For ComR, SNPs with a MAF below 0.40 were tested in a dominant model and SNPs with a MAF $\geq$ 0.40 were tested in an additive model. With these cut-off values, the minimal expected number of subjects in remission having the homozygote mutant genotype in the additive models was approximately 10. Two separate analyses were performed: (1) association between SNPs and ClinR vs PersA, (2) association between SNPs and ComR vs PersA.

For each analysis, the top 25 SNPs (after excluding SNPs in LD and regardless of their *P*-value) were analysed in 2 replication cohorts. The results of the replication cohorts were meta-analysed using METAL.[18] We assessed the direction of the effect in the identification cohort, that is a negative effect implying a lower chance of asthma remission, and a positive effect implying higher a chance of asthma remission. Significant replication was achieved if the direction of the effect in this meta-analysis was the same as in the identification cohort, and if the 1-sided *P*-value was <.05 (expected direction based on the identification analysis).

2.4 | Replication cohorts

Replication was performed in the EGEA-study[19] and the SAPALDIA cohort-study.[20-22] Extensive information on these cohorts is presented in the Supporting information. All subjects with doctor-diagnosed asthma irrespective of age of onset and a positive BHR test at the baseline visit (early 1990s) were included in the current analysis. Remission status was determined at the most recent follow-up visit according to the same definitions as used in the identification cohort.

2.5 | Gene expression analysis in lung tissue and lung epithelial cells

The 4 significantly replicated SNPs were assessed for their association with cis- and trans-acting gene expression (mRNA) in lung tissue (n = 1087).[23] Subsequently, significant eQTLs identified in lung tissue were analysed in an epithelial-specific eQTL database (n = 85) (see Supporting information).

3 | RESULTS

3.1 | Subject characteristics

Subject characteristics of the identification cohort, according to their most recent asthma status, are shown in Table 1. The median [range] follow-up was 15.5 [3.3; 47.8] years. Of the 790 included subjects, 178 (23%) had ClinR. Of these, 55 were in ComR (7% of total cohort). In 41 subjects, a BHR-test was not performed and these patients were assigned to the ClinR group. Subjects in remission were more often male, had a longer duration of follow-up, were younger at baseline and had a better lung function at baseline compared to subjects with PersA. The age of asthma onset was comparable between ClinR and PersA, while subjects in ComR had a younger age at the start of their asthma compared to PersA. Table 1 also shows the characteristics in the replication cohorts. In EGEA (n = 351), 23% of the subjects were in ClinR and 2% in ComR. In SAPALDIA (n = 105), these percentages were 51% and 13%, respectively.

3.2 | Clinical asthma remission

The results of the identification GWAS on ClinR are shown in Figure 1. There existed no inflation of data based on the lambda of 1.00 and QQ-plot (Figure S5). The results of the replication analysis (Table 2) show that 1 of the 25 SNPs tested, rs7240102, met the criteria for significant replication (see Figure S6 for regional association plot).

3.3 | Complete asthma remission

The results of the identification GWAS on ComR are shown in Figure 2. The lambda of 1.01 and QQ-plot (Figure S7) gave no indication of inflation. The results of the replication analysis on ComR are presented in Table 3. Three of the 25 SNPs tested met the criteria for significant replication (see Figure S6 for regional association plots): rs6581895 (intergenic between *YEATS4* and *FRS2*), rs12405429 (intergenic between *FAM89A* and *TRIM67*), and

TABLE 1 Characteristics of patients according to their asthma status

	Identification Cohort			EGEA			SAPALDIA		
	Persistent asthma (n = 612)	Clinical remission (n = 178)	Complete remission (n = 55)	Persistent asthma (n = 270)	Clinical remission (n = 81)	Complete remission (n = 7)	Persistent asthma (n = 51)	Clinical remission (n = 54)	Complete remission (n = 14)
Male sex, n (%)	284 (46)	**105 (59)**	**41 (75)**	165 (61)	46 (57)	4 (57)	24 (47)	17 (32)	4 (29)
Age at remission status, years	49.8 (41.2; 59.2)	50.3 (42.0; 59.4)	50.5 (40.9; 56.7)	37.9 (29.6; 60.5)	30.3 (24.8; 58.6)	36.1 (29.8; 55.5)	49.1 (43.1; 56.5)	53.3 (44.9; 60.0)	52.0 (44.9; 59.6)
Duration of follow -up, years	14.4 (10.3; 39.2)	**28.7 (11.7; 40.6)**	**32.9 (19.5; 40.4)**	18.2 (14.8; 19.0)	11.0 (10.7; 12.2)	17.3 (12.1; 18.8)	10.9 (10.8; 11.0)	10.9 (10.8; 11.0)	10.9 (10.8; 11.0)
Characteristics at first survey									
Age, years	29.7 (20.0; 38.9)	**24.0 (14.0; 37.0)**	**23.4 (12.3; 37.8)**	20.7 (11.5; 42.3)	18.8 (13.8; 46.1)	36.1 (29.8; 55.5)	38.4 (32.6; 45.6)	42.4 (33.9; 49.3)	41.2 (33.9; 48.8)
FEV_1%pred pre bronchodilator	78.4 (62.6; 90.1)	**83.4 (73.6; 93.1)**	**85.8 (75.0; 95.3)**	89.1 (74.9; 100.3)	95.6 (80.1; 103.5)	90.4 (82.0; 100.7)	90.4 (82.2; 98.0)	97.7 (91.2; 106.3)	95.4 (88.6; 104.6)
FEV_1%pred post bronchodilator	91.1 (79.7; 100.9)	**95.5 (87.1; 103.6)**	**99.1 (91.7; 107.1)**						
FEV1%IVC pre bronchodilator	71.0 (60.0; 78.0)	**75.0 (66.0; 81.3)**	**77.0 (66.0; 83.0)**						
FEV1%FVC pre bronchodilator				80.0 (69.0; 86.0)	83.0 (81.0; 91.0)	83.0 (76.0; 86.0)	73.3 (69.0; 79.1)	78.0 (75.1; 84.4)	76.2 (72.7; 82.4)
FEV1%IVC post bronchodilator	77.0 (69.0; 83.0)	**80.0 (77.0; 86.0)**	**82.0 (78.0; 86.0)**						
FEV1 reversibility % pred	11.8 (7.2; 19.3)	**9.2 (4.5; 16.4)**	11.4 (5.0; 18.2)						
Age start asthma, years	5.0 (1.0; 15.0)	4.0 (1.0; 12.3)	**2.0 (0.0; 8.0)**	7.0 (3.0; 19.0)	11.0 (8.0; 35.0)	7.0 (3.0; 18.0)	21 (6.0; 30.0)	5.5 (2.0; 18.0)	16.0 (5.0; 28.0)
Start asthma before age 16, n (%)	437 (74)	130 (80)	42 (86)	195 (72)	59 (73)	5 (71)	20 (44)	25 (56)	10 (22)

Data are presented as median (25th percentile—75th percentile) unless indicated otherwise. Bold values signify significant differences from persistent asthma with $P < 0.05$ in the identification cohort.

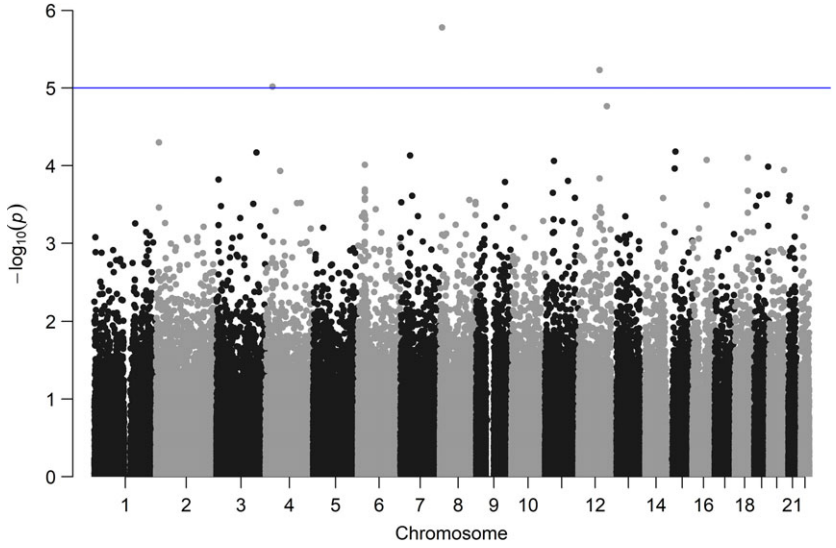

FIGURE 1 Manhattan plot of Clinical remission in the identification cohort

rs1420101 (located in *IL1RL1*). In the meta-analysis of the identification and replication cohorts, the top-hit rs6581895 had a *P*-value of 4.68×10^{-7}, very close to the Bonferroni-corrected genome-wide significant *P*-value of 3.1×10^{-7}.

3.4 | Gene expression analysis in lung tissue

To investigate whether replicated SNPs had allelic effects on gene expression in lung tissue, we assessed the association between genotype and lung tissue gene expression. For ClinR, the replicated SNP rs7240102 was associated with gene expression levels of *Polymerase (DNA) iota (POLI)*. Two of the 3 significantly replicated SNPs that were associated with ComR were identified as e-QTL measured by 9 gene expression probe sets (Table 4, Figure 3): rs6581895 was associated with expression of *Fibroblast growth factor 2 (FRS2)*, *Chaperonin containing TCP1 subunit 2 (CCT2)*, and rs1420101 located in *Interleukin 1 receptor 1 (IL1RL1)* was associated with *IL1RL1* and *Interleukin 18 Receptor 1 (IL18R1)*-expression (Table S2). In addition, rs1420101 was a trans-eQTL for *Interleukin 13* (*IL13*) expression.

3.5 | Gene expression analysis in lung epithelial cells

The significant eQTLs identified in the lung tissue analysis were further explored in an epithelial specific eQTL database. None of the SNPs showed a significant association (Table S3).

4 | DISCUSSION

The goal of our study was to identify genetic markers associated with asthma remission by performing a genome-wide association study. This may provide insight into mechanisms underlying asthma remission and possible provide us with clues to develop a cure for asthma. We applied a restrictive asthma remission phenotype and defined complete remission by cessation of symptoms and asthma medication in addition to good lung health (normal lung function and no longer BHR present). In this way, we identified 3 consistently associated genetic loci across all 3 cohorts with in total 76 individuals in complete asthma remission. One SNP reached almost genome-wide significance in relation to complete asthma remission and also showed an association with the expression of *FRS2* and *CCT2*. The replicated SNP rs7240102 with the loose asthma remission definition, clinical remission, was associated with gene expression levels of *POLI*. Thus, our study shows that it is possible to identify gene polymorphisms that are consistently associated with a strict phenotype definition of complete asthma remission across multiple cohorts, even though none of the SNPs reached genome-wide significance in our small number of individuals. Given the functional relevance in expression (eQTL) in lung tissue, the presented data suggest that *FRS2*, *CCT2*, *IL1RL1*, *IL18R1 and IL13* are candidate genes for further functional studies into their biological role in complete asthma remission.

This is the first genome-wide study on asthma remission, so no comparator data are available. Several of the genes identified are yet not known to be involved in asthma or inflammation. *FRS2* (*Fibroblast growth factor receptor substrate* 2) belongs to the FRS2 family of adaptor/scaffold proteins that has 2 members, FRS2alpha and FRS2-beta.[24] Both proteins contain N-terminal myristoylation sites for localization on the plasma membrane, a PTB domain for binding to limited species of receptor tyrosine kinases, including the FGF receptor, and a long C-terminal tail with 4 binding sites, 2 for the adaptor protein Frb2 and 2 for the tyrosine phosphatase Shp2.[24] Activation of these receptor tyrosine kinases allows FRS2 proteins to become phosphorylated at tyrosine residues and then bring about a cascade of events including activation of the Ras/ERK, PI3kinase and MAPK pathway.[25] This may among others affect neurite outgrow.[26] FRS can additionally inhibit EGF signalling, resulting in inhibition of EGF-induced cell proliferation and cell transformation. An interesting finding is that FRS2 alpha is not only affecting FGF signalling pathways, but is also a critical regulator of VEGF receptor signalling which may

TABLE 2 Results of top 25 SNPs of GWAs on Clinical asthma remission. Gene annotation was performed using SCAN (www.scandb.org)

					Identification cohort Cases: 178 Controls: 612		EGEA Cases: 81 Controls: 270		SAPALDIA Cases: 54 Controls: 51		META-analysis EGEA+SAPALDIA			META-analysis identification+EGEA+SA-PALDIA			Gene—annotation	
SNP	CHR	TA	TEST	MAF	OR	2-sided *P*	OR	1-sided *P*	OR	1-sided *P*	OR	1-sided *P*	Dir	OR	2-sided *P*	Dir	Gene	Left gene/Right gene
rs565169	8	C	DOM	0.15	2.37	1.7×10^{-6}	1.06	4.2×10^{-1}	1.50	2.1×10^{-1}	1.15	2.9×10^{-1}	++	1.84	2.7×10^{-5}	+++	NA	CLDN23/MFHAS1
rs1471239	12	G	ADD	0.37	1.78	5.9×10^{-6}	0.98	5.5×10^{-1}	0.96	5.6×10^{-1}	0.97	5.7×10^{-1}	−−	1.40	6.5×10^{-4}	+−−	NA	LOC100128335/SLC6A15
rs6850690	4	G	ADD	0.27	1.79	9.6×10^{-6}	1.31	8.0×10^{-2}	0.81	7.5×10^{-1}	1.15	2.0×10^{-1}	+−	1.51	7.0×10^{-5}	++−	NA	LOC391642/LOC645641
rs7137598	12	G	DOM	0.13	2.23	1.7×10^{-5}	0.81	7.6×10^{-1}	0.84	6.5×10^{-1}	0.82	7.9×10^{-1}	−−	1.56	3.0×10^{-3}	+−−	TBX5	RBM19/LOC255480
rs4669688	2	A	ADD	0.38	0.58	5.0×10^{-5}	0.87	2.2×10^{-1}	1.13	6.6×10^{-1}	0.94	3.4×10^{-1}	−+	0.71	7.9×10^{-4}	−−+	NA	KCNF1/LOC645054
rs8041207	15	A	ADD	0.43	1.66	6.6×10^{-5}	1.02	4.6×10^{-1}	1.01	4.9×10^{-1}	1.02	4.6×10^{-1}	++	1.36	1.6×10^{-3}	+++	NA	GOLGA8B/GJD2
rs1515735	3	A	DOM	0.15	2.06	6.8×10^{-5}	0.73	8.6×10^{-1}	0.56	8.8×10^{-1}	0.68	9.4×10^{-1}	−−	1.42	1.7×10^{-2}	+−−	NA	OTOL1/LOC100132484
rs12701705	7	G	ADD	0.46	1.66	7.4×10^{-5}	1.23	1.2×10^{-1}	0.97	5.4×10^{-1}	1.15	1.8×10^{-1}	+−	1.42	3.1×10^{-4}	++−	POU6F2	VPS41/C7orf36
rs7240102	**18**	**G**	**DOM**	**0.24**	**1.99**	$\mathbf{7.9 \times 10^{-5}}$	**1.54**	$\mathbf{4.6 \times 10^{-2}}$	**1.19**	$\mathbf{3.3 \times 10^{-1}}$	**1.43**	$\mathbf{4.9 \times 10^{-2}}$	**++**	**1.75**	$\mathbf{3.9 \times 10^{-5}}$	**+++**	**NA**	**LOC100130003/C18orf26**
rs2967137	16	G	ADD	0.41	0.61	8.4×10^{-5}	1.02	5.4×10^{-1}	1.86	9.8×10^{-1}	1.19	8.7×10^{-1}	++	0.80	2.1×10^{-2}	−++	NA	LOC388282/CNGB1
rs1518746	11	A	ADD	0.43	0.61	8.7×10^{-5}	1.34	9.5×10^{-1}	0.85	2.9×10^{-1}	1.18	8.7×10^{-1}	+−	0.80	2.2×10^{-2}	−+−	NA	C11orf74/LOC100129825
rs203888	6	A	ADD	0.27	1.68	9.7×10^{-5}	0.92	6.4×10^{-1}	0.97	5.3×10^{-1}	0.94	6.4×10^{-1}	−−	1.37	3.2×10^{-3}	+−−	NA	OR2B7P/OR1F12
rs10403164	19	A	ADD	0.49	0.61	1.0×10^{-4}	0.99	4.7×10^{-1}	1.10	6.3×10^{-1}	1.02	5.4×10^{-1}	−+	0.75	3.1×10^{-3}	−−+	HSPBP1	SAPS1/HSPBP1
rs2219507	15	A	DOM	0.22	1.95	1.1×10^{-4}	0.67	9.4×10^{-1}	0.54	9.3×10^{-1}	0.63	9.8×10^{-1}	−−	1.27	7.8×10^{-2}	+−−	OTUD7A	LOC100128855/DEPDC1P1
rs4925184	20	G	DOM	0.05	2.60	1.1×10^{-4}	1.12	3.8×10^{-1}	3.02	6.2×10^{-2}	1.37	1.7×10^{-1}	++	2.05	2.6×10^{-4}	+++	GATA5	C20orf151/GATA5
rs7679186	4	C	ADD	0.30	0.58	1.2×10^{-4}	0.91	3.2×10^{-1}	0.81	2.4×10^{-1}	0.87	2.1×10^{-1}	−−	0.68	5.0×10^{-4}	−−−	NA	LOC255130/SRIL
rs12828146	12	G	DOM	0.07	2.33	1.5×10^{-4}	0.60	8.5×10^{-1}	0.63	7.6×10^{-1}	0.61	8.9×10^{-1}	−−	1.70	6.5×10^{-3}	+−−	NA	LOC100128335/SLC6A15
rs1488793	3	A	ADD	0.33	1.61	1.5×10^{-4}	0.86	7.9×10^{-1}	0.91	6.2×10^{-1}	0.87	8.0×10^{-1}	−−	1.28	1.2×10^{-2}	+−−	NA	GRM7/LMCD1
rs1579874	11	A	DOM	0.06	2.58	1.6×10^{-4}	0.97	5.3×10^{-1}	0.51	8.0×10^{-1}	0.84	6.8×10^{-1}	−−	1.83	3.8×10^{-3}	+−−	NA	LOC100128230/CCDC67
rs1411605	9	A	DOM	0.16	1.97	1.6×10^{-4}	0.93	6.0×10^{-1}	1.36	2.6×10^{-1}	1.03	4.6×10^{-1}	−+	1.55	2.1×10^{-3}	+−+	ZFP37	LOC169834/LOC100128385
rs10835893	11	A	ADD	0.28	0.58	2.2×10^{-4}	0.79	1.3×10^{-1}	1.60	9.4×10^{-1}	0.98	4.6×10^{-1}	−+	0.73	4.2×10^{-3}	−−+	NA	RCN1/WT1
rs3810114	19	A	DOM	0.17	1.92	2.3×10^{-4}	1.34	1.4×10^{-1}	0.67	8.2×10^{-1}	1.10	3.4×10^{-1}	+−	1.57	1.5×10^{-3}	++−	NA	SIGLEC12/SIGLECP11
rs2825209	21	A	DOM	0.18	1.91	2.4×10^{-4}	0.93	6.2×10^{-1}	3.28	7.2×10^{-3}	1.16	2.3×10^{-1}	−+	1.53	1.2×10^{-3}	+−+	NA	PRSS7/PPIAL3
rs2043293	19	C	DOM	0.19	1.90	2.4×10^{-4}	1.06	4.1×10^{-1}	1.07	4.4×10^{-1}	1.06	3.9×10^{-1}	++	1.52	2.4×10^{-3}	+++	GMIP	LPAR2/ATP13A1
rs1554495	7	A	ADD	0.46	1.58	2.5×10^{-4}	0.97	5.6×10^{-1}	1.01	4.9×10^{-1}	0.98	5.5×10^{-1}	−+	1.31	5.6×10^{-3}	+−+	PKD1L1	FLJ21075/HUS1

SNP, single nucleotide polymorphism; CHR, chromosome; TA, tested allele; TEST: DOM, dominant model; ADD, additive model; MAF, minor allele frequency; OR, Odds Ratio; Dir, direction of the effect; "+/−" indicates positive/negative association between the tested allele and remission. In bold; significantly replicated SNPs.

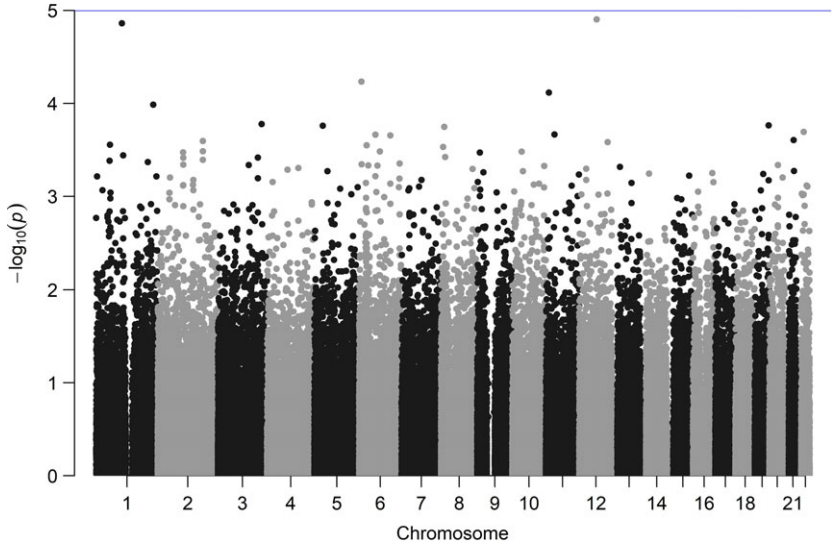

FIGURE 2 Manhattan plot of Complete remission in the identification cohort

affect angiogenesis as well as actin reorganization.[25] If the latter is impaired, this might contribute to a reduced load and resolution of inflammation.[25] Finally, changes in this receptor may affect the function of multiple FGFs, some previously associated with lung development or disease, including FGF10[27] and FGF7.[28]

The protein encoded by *CCT2 (Chaperonin Containing TCP1, Subunit 2)* is a molecular chaperone that is a member of the chaperonin containing TCP1 complex (CCT), also known as the TCP1 ring complex (TRiC). This complex consists of 2 identical stacked rings, each containing 8 different proteins. CCT2 assists the folding of polypeptides that enter the central cavity of the complex in an ATP-dependent manner, including actin and tubulin. This is of importance as tissue responses to injury fundamentally involve reorganization of the actin cytoskeleton of participating cells, including epithelial cells, fibroblasts, endothelial cells and macrophages. Mutation analysis in plants has shown that this may affect cell growth.[29] CCT2 has additionally been associated with primary cilia, non-motile microtubule-based organelles that function as central antennae that sense a wide variety of signals during development and tissue homeostasis, including involvement in the maintenance of cell proliferation.[30]

We found that the A-allele of rs1420101, a SNP located in *IL1RL1*, is associated with a lower chance for complete asthma remission in our adult asthma patients. This seems remarkable, as this rs1420101 A-allele has been identified previously as a risk for asthma in GWAS.[31] The A-allele of this *IL1RL1* variant was associated with lower *IL1RL1* and *IL18R1* lung tissue expression[32] and higher *IL13* lung tissue expression in our study. Moreover, it was also associated with higher numbers of eosinophils in childhood asthma and with a higher risk for asthma in a previous study.[33] This suggests that higher *IL1RL1* expression might eventually, and perhaps in association with other factors, contribute to remission of asthma by negatively regulating Type 2 inflammation as reflected by lower lung *IL13* mRNA levels. *IL1RL1* encodes 3 splice variants, and the synonymous asthma SNP rs1420101 is located in exon 5E, which is only retained in the transcript encoding the truncated IL-1RL1-c, also called ST2V.[34] This might indicate an effect of rs1420101 on this specific transcript. However, this variant is not supported by refseq annotation and the function of this transcript is yet unknown.

We found 1 SNP to be associated with the more loose definition of asthma remission, clinical remission. This SNP associated with *POLI* (*Polymerase iota*) expression in lung tissue. Human DNA polymerase iota (Polι) belongs to the Y family of specialized DNA polymerases and is one of the most error-prone enzymes involved in DNA synthesis.[35] The functions of Polι in human cells remain largely unknown, and it is not clear which might be the role of this gene in clinical asthma remission.

There are strengths and weaknesses to the current study. A strength of our approach is that both active asthma and asthma remission was defined using objective characteristics of the disease, that is bronchial hyperresponsivess, airway obstruction, in combination with the presence or absence of patient reported symptoms and medication use. Due to the use of different genotyping arrays, we investigated "only" 156,954 SNPs that tagged 36%-60% of all SNPs in the genome. The term "genome-wide" is thus not really applicable to our study. Our study has a rich characterization of the asthmatics collected over a follow-up period of 3-47 years. Because of the detailed characterization, which included among others hyperresponsiveness both at baseline and follow-up visits, a strict asthma remission definition could be used. This reduced the number of asthmatics with complete remission to 55 in the identification cohort and 7 and 14 in the replication cohorts, EGEA and SALPALDIA, respectively. This might theoretically lead to false-negative or spurious findings, but the fact that all 3 cohorts had the same direction and magnitude of effect of the identified SNPs in relation to complete asthma remission and that these genes had biologically plausible eQTLs may suggest that the study has identified promising new leads for further research on the mechanisms of asthma remission.

TABLE 3 Results of top 25 SNPs of GWAs on Complete asthma remission. Gene annotation was performed using SCAN (www.scandb.org)

					Identification cohort Cases: 55 Controls: 612		EGEA Cases: 7 Controls: 270		SAPALDIA Cases: 14 Controls: 51		META-analysis EGEA+SAPALDIA			META-analysis identification+EGEA+SA-PALDIA			GENE—ANNOTATION	
SNP	CHR	TA	TEST	MAF	OR	2-sided *P*	OR	1-sided *P*	OR	1-sided *P*	OR	1-sided *P*	Dir	OR	2-sided *P*	Dir	Gene	Left gene/Right gene
rs6581895	**12**	**G**	**DOM**	**0.24**	**3.83**	**1.3×10^{-5}**	**3.74**	**6.0×10^{-2}**	**3.75**	**2.5×10^{-2}**	**3.75**	**6.0×10^{-3}**	**++**	**3.81**	**4.7×10^{-7}**	**+++**	**NA**	**YEATS4/FRS2**
rs7552867	1	G	DOM	0.18	3.57	1.4×10^{-5}	1.05	4.8×10^{-1}	0.89	5.7×10^{-1}	0.94	5.5×10^{-1}	+–	2.55	2.2×10^{-4}	++–	NA	LOC642337/LOC100130867
rs9379986	6	C	DOM	0.38	0.31	5.9×10^{-5}	2.10	8.1×10^{-1}	0.82	3.8×10^{-1}	1.15	6.1×10^{-1}	+–	0.43	8.2×10^{-4}	–+–	NA	LOC389365/OFCC1
rs1866714	11	G	DOM	0.33	4.74	7.7×10^{-5}	3.38	1.3×10^{-1}	1.85	2.0×10^{-1}	2.23	9.5×10^{-2}	++	3.80	5.5×10^{-5}	+++	TEAD1	PARVA/RASSF10
rs12405429	**1**	**G**	**DOM**	**0.15**	**3.10**	**1.0×10^{-4}**	**2.51**	**1.2×10^{-1}**	**2.65**	**9.2×10^{-2}**	**2.58**	**3.8×10^{-2}**	**++**	**2.97**	**2.1×10^{-5}**	**+++**	**NA**	**FAM89A/TRIM67**
rs7628387	3	C	DOM	0.14	2.99	1.7×10^{-4}	1.45	3.3×10^{-1}	NA	NA	1.45	3.3×10^{-1}	+?	2.77	2.1×10^{-4}	++?	NA	LOC730168/TBL1XR1
rs10403164	19	A	ADD	0.49	0.44	1.7×10^{-4}	0.38	7.2×10^{-2}	0.85	3.7×10^{-1}	0.63	1.3×10^{-1}	––	0.48	1.2×10^{-4}	–––	HSPBP1	SAPS1/HSPBP1
rs299596	5	A	DOM	0.33	0.31	1.8×10^{-4}	1.64	7.2×10^{-1}	1.58	7.6×10^{-1}	1.60	8.2×10^{-1}	++	0.48	6.4×10^{-3}	–++	C1QTNF3	AMACR/LOC643373
rs919590	8	A	ADD	0.41	0.42	1.8×10^{-4}	0.85	3.9×10^{-1}	1.07	5.6×10^{-1}	0.97	4.7×10^{-1}	–+	0.53	1.3×10^{-3}	––+	DLC1	C8orf79/C8orf48
rs713702	22	G	DOM	0.26	3.12	2.0×10^{-4}	0.45	8.3×10^{-1}	1.23	3.7×10^{-1}	0.87	6.1×10^{-1}	–+	2.19	2.6×10^{-3}	+–+	NA	LOC100129936/LOC100130624
rs3751031	11	G	DOM	0.07	3.33	2.2×10^{-4}	1.93	2.2×10^{-1}	1.34	3.7×10^{-1}	1.62	2.2×10^{-1}	++	2.85	2.8×10^{-4}	+++	CD44	PDHX/SLC1A2
rs6915644	6	A	ADD	0.44	2.16	2.2×10^{-4}	0.35	9.5×10^{-1}	0.49	9.3×10^{-1}	0.43	9.8×10^{-1}	––	1.52	2.3×10^{-2}	+––	NA	LOC100130393/LOC727977
rs1353203	6	A	DOM	0.37	4.29	2.2×10^{-4}	0.94	5.3×10^{-1}	2.32	1.2×10^{-1}	1.52	2.1×10^{-1}	–+	2.96	5.9×10^{-4}	+–+	NA	HDDC2/LOC643623
rs1041868	21	A	DOM	0.16	2.88	2.5×10^{-4}	NA	NA	1.22	3.9×10^{-1}	1.22	3.9×10^{-1}	?+	2.53	4.9×10^{-4}	+?+	IFNAR1	IL10RB/IFNGR2
rs10497579	2	A	ADD	0.44	2.18	2.5×10^{-4}	0.57	8.2×10^{-1}	0.61	8.4×10^{-1}	0.60	9.1×10^{-1}	––	1.60	1.1×10^{-2}	+––	NA	LOC729026/LOC100127923
rs7137598	12	G	DOM	0.13	2.96	2.6×10^{-4}	1.06	4.8×10^{-1}	0.50	8.0×10^{-1}	0.72	7.1×10^{-1}	+–	2.23	2.5×10^{-3}	++–	TBX5	RBM19/LOC255480
rs12137498	1	A	DOM	0.11	2.94	2.8×10^{-4}	1.57	3.0×10^{-1}	0.75	6.5×10^{-1}	1.03	4.8×10^{-1}	+–	2.34	1.2×10^{-3}	++–	NA	LOC127406/PPAP2B
rs3094694	6	G	DOM	0.22	2.89	2.8×10^{-4}	1.07	4.7×10^{-1}	0.84	6.0×10^{-1}	0.92	5.6×10^{-1}	+–	2.21	1.9×10^{-3}	++–	NA	LOC646520/RANP1
rs565169	8	C	DOM	0.15	2.87	3.0×10^{-4}	0.42	7.9×10^{-1}	2.17	1.4×10^{-1}	1.32	3.2×10^{-1}	–+	2.47	5.5×10^{-4}	+–+	NA	CLDN23/MFHAS1
rs6909889	6	A	DOM	0.19	2.85	3.3×10^{-4}	0.91	5.5×10^{-1}	2.11	1.4×10^{-1}	1.51	2.2×10^{-1}	–+	2.46	4.3×10^{-4}	+–+	NA	TPBG/UBE2CBP
rs624359	10	A	DOM	0.25	2.96	3.3×10^{-4}	0.94	5.3×10^{-1}	1.11	4.4×10^{-1}	1.04	4.7×10^{-1}	–+	2.21	2.0×10^{-3}	+–+	ZNF33A	ZNF25/ZNF33A
rs1420101	**2**	**A**	**ADD**	**0.41**	**0.44**	**3.4×10^{-4}**	**0.39**	**7.1×10^{-2}**	**0.60**	**1.3×10^{-1}**	**0.52**	**3.9×10^{-2}**	**––**	**0.46**	**6.9×10^{-5}**	**–––**	**IL1RL1**	**LOC100129822/IL18R1**
rs2784609	9	C	DOM	0.11	2.91	3.4×10^{-4}	0.72	6.2×10^{-1}	1.96	1.7×10^{-1}	1.45	2.7×10^{-1}	–+	2.53	4.9×10^{-4}	+–+	PTPRD	RN7SLP2/LOC646087
rs650985	1	G	DOM	0.07	3.25	3.6×10^{-4}	2.84	1.1×10^{-1}	1.05	4.9×10^{-1}	2.01	1.6×10^{-1}	++	2.98	2.6×10^{-4}	+++	GSTM4	LOC441896/GSTM2
rs11203826	8	A	DOM	0.21	2.82	3.8×10^{-4}	0.92	5.4×10^{-1}	1.34	3.4×10^{-1}	1.15	4.0×10^{-1}	–+	2.30	1.2×10^{-3}	+–+	EFHA2	FGF20/LOC100130392

SNP, single nucleotide polymorphism; CHR, chromosome; TA, tested allele; TEST: DOM, dominant model; ADD, additive model; MAF, minor allele frequency; OR, Odds Ratio; Dir, direction of the effect; "+/−" indicates positive/negative association between the tested allele and remission. In bold; significantly replicated SNPs.

TABLE 4 Significant associations between SNPs and gene expression in lung tissue

SNP	CHR	TA	Cis/Trans	Number of tested probes	Probe	Gene	B	SE	*P*
SNP associated with ClinR									
rs7240102	18	G	Cis	27	100130406_TGI_at	*POLI*	0.041	.010	5.86×10^{-5}
					100313174_TGI_at	*POLI*	0.051	.012	2.23×10^{-5}
					100157415_TGI_at	*POLI*	0.055	.012	2.11×10^{-6}
SNP associated with ComR									
rs6581895	12	G	Cis	53	100133437_TGI_at	*FRS2*	−0.043	.013	8.88×10^{-4}
					100306086_TGI_at	*FRS2*	0.029	.009	8.77×10^{-4}
					100156179_TGI_at	*CCT2*	−0.029	.006	4.65×10^{-6}
rs1420101	2	A	Cis	42	100302151_TGI_at	*IL1RL1*	−0.061	.011	1.10×10^{-7}
					100302783_TGI_at	*IL1RL1*	−0.860	.049	5.10×10^{-69}
					100148210_TGI_at	*IL1RL1*	−0.741	.043	2.82×10^{-65}
					100148162_TGI_at	*IL1RL1*	−0.578	.035	9.61×10^{-63}
					100312840_TGI_at	*IL1RL1*	−0.548	.033	1.07×10^{-63}
					100136977_TGI_at	*IL18R1*	−0.221	.022	9.88×10^{-23}
			Trans	49 236	100125222_TGI_at	*IL13*	0.100	.020	3.69×10^{-7}

SNP, single nucleotide polymorphism; CHR, chromosome; TA, tested allele; Cis: association with gene-expression of a gene within a 1 Mb distance of the SNP; Trans: association with gene-expression of a gene anywhere in the genome; B, Beta; SE, standard error; *P*: *P*-value.

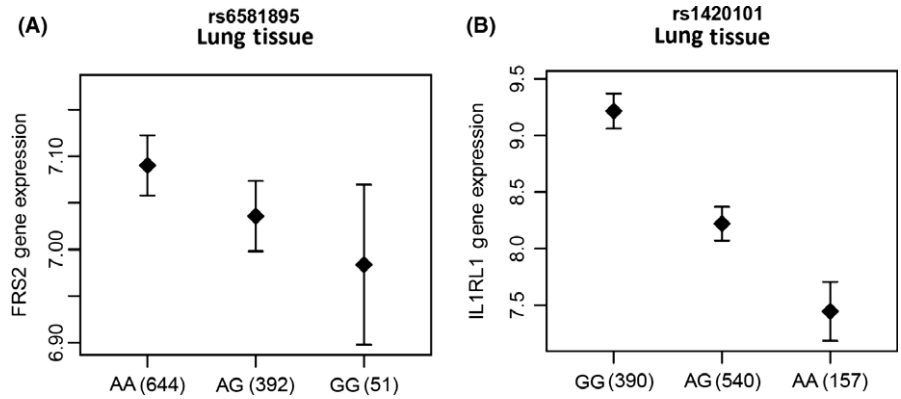

FIGURE 3 Lung eQTL associated with complete asthma remission. A, Association between SNP rs6581895 and gene expression of *FRS2* (probe set: 100133437_TGI_at), (B) association between SNP rs1420101 and gene expression of *IL1RL1* (probe set: 100302783_ TGI_at)[32]

Of interest, none of the replicated SNPs was an eQTL in epithelial cells, while these cells are thought to be fundamental to asthma development.[36] This suggests that either larger groups of patients should be studied to ensure that epithelial cells do not contribute to asthma remission, or that different inflammatory or resident cells in the lung may be important for asthma remission compared to those for asthma inception. The prevalence of asthma remission in the SAPALDIA study is higher than in the other 2 cohorts. This may be the result of the fact that the SAPALDIA study is a general population-based cohort while the other 2 cohorts are hospital-based studies that generally attract patients with more severe asthma.

In conclusion, we found suggestive evidence that a set of genes may contribute to asthma remission in a subset of asthmatics, using a strict definition of complete asthma remission. The study points to the value of deep phenotyping for the identification of novel drug targets through genetic epidemiology complemented with functional characterization of associated SNPs. We invite the scientific community to contribute to further characterization of asthmatics in a similar way to increase the numbers of asthmatics with complete remission to be studied with a genome-wide screen, thus allowing gene-gene interaction and epigenetic assessments as well, or even better to do so with whole genome sequencing as this may additionally elucidate rare variants and pinpoint genes that associate with such a rare, but extremely important phenotype of asthma: full remission!

ACKNOWLEDGEMENT

We thank Dr. Victor Guryev (European Institute of the Biology of Aging, University Medical Center Groningen) for his assistance in calculating the percentage of tagged SNPs in the GoNL database (www.nlgenome.nl).

CONFLICT OF INTEREST

Dr. Siroux reports personal fees from TEVA, AstraZeneca, and Novartis, outside the submitted work. Dr. Sin reports grants and personal fees from AstraZeneca, Sanofi-Aventis, Regeneron, Merck, Boehringer Ingelheim, and Novartis, outside the submitted work. Dr. van den Berge reports research grants paid to University from GlaxoSmithKline, TEVA, Chiesi, and AstraZeneca, outside the submitted work. Dr. Koppelman reports research grants paid to the University from TEVA and the TETRI foundation, outside the submitted work. Dr. Postma reports grants and fees for consultancies paid to the University from Astra Zeneca, Chiesi, Genentec, Boehringer Ingelheim, Chiesi, GSK, Takeda, TEVA and Roche, outside the submitted work. Dr. Vonk, Dr. Nieuwenhuis, Dr. Dijk, Dr. Boudier, Dr. Bouzigon, Dr. Probst-Hensch, Dr. Imboden, Dr. Keidel, Dr. Bossé, Dr. Hao and Dr. Faiz report no conflicts of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

How to cite this article: Vonk JM, Nieuwenhuis MAE, Dijk FN, et al. Novel genes and insights in complete asthma remission: A genome-wide association study on clinical and complete asthma remission. *Clin Exp Allergy*. 2018;48:1286–1296. https://doi.org/10.1111/cea.13181

ORIGINAL ARTICLE

Heterogeneity of Airway Smooth Muscle Remodeling in Asthma

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Abstract

Rationale: Ventilatory defects in asthma are heterogeneous and may represent the distribution of airway smooth muscle (ASM) remodeling.

Objectives: To determine the distribution of ASM remodeling in mild–severe asthma.

Methods: The ASM area was measured in nine airway levels in three bronchial pathways in cases of nonfatal ($n = 30$) and fatal asthma ($n = 20$) and compared with control cases without asthma ($n = 30$). Correlations of ASM area within and between bronchial pathways were calculated. Asthma cases with 12 large and 12 small airways available ($n = 42$) were classified on the basis of the presence or absence of ASM remodeling (more than two SD of mean ASM area of control cases, $n = 86$) in the large or small airway or both.

Measurements and Main Results: ASM remodeling varied widely within and between cases of nonfatal asthma and was more widespread and confluent and more marked in fatal cases. There were weak correlations of ASM between levels within the same or separate bronchial pathways; however, predictable patterns of remodeling were not observed. Using mean data, 44% of all asthma cases were classified as having no ASM remodeling in either the large or small airway despite a three- to 10-fold increase in the number of airways with ASM remodeling and 81% of asthma cases having ASM remodeling in at least one large and small airway.

Conclusions: ASM remodeling is related to asthma severity but is heterogeneous within and between individuals and may contribute to the heterogeneous functional defects observed in asthma. These findings support the need for patient-specific targeting of ASM remodeling.

The variable and excessive airway narrowing that characterizes asthma can be demonstrated by the inhalation of agents that cause airway smooth muscle (ASM) shortening (1). The thickness of the ASM layer is related to the degree of narrowing in isolated airway segments from patients with asthma (2) and to the clinical severity of asthma (3). Therefore, ASM represents an important target for the treatment of asthma.

It has been suggested that phenotypes of asthma exist, defined by the distribution of ASM remodeling in the large and/or small airways (4, 5), and localized functional abnormalities in patients with asthma suggest regions of localized remodeling. Heterogeneity of airway narrowing occurs with histamine challenge (6), and

(Received in original form November 5, 2021, accepted in final form November 18, 2022)

Supported by the National Health and Medical Research Council of Australia (1141552 and 1180854).

Author Contributions: A.L.J. collected the cases for the Perth fatal asthma study, devised the project, coordinated the tissue collections, analyzed the data, and wrote the manuscript. G.M.D. analyzed the data and assisted with the preparation of the manuscript. F.H.Y.G. collected cases for the Prairie Provinces fatal asthma study and assisted with the preparation of the manuscript. T.M. collected the cases for the Sao Paulo fatal asthma study and assisted with the preparation of the manuscript. M.J.A. collected cases for the Melbourne fatal asthma study, assisted with data analysis, and assisted with the preparation of the manuscript. A.C. assisted with the preparation of the manuscript. P.B.N. devised the project and assisted with the preparation of the manuscript. J.G.E. assisted in the collection of the cases from the Perth fatal asthma study, made airway measurements, analyzed the data, and assisted with the preparation of the manuscript.

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This article has a related editorial.

This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org.

Am J Respir Crit Care Med Vol 207, Iss 4, pp 452–460, Feb 15, 2023

Originally Published in Press as DOI: 10.1164/rccm.202111-2634OC on November 18, 2022
Internet address: www.atsjournals.org

At a Glance Commentary

Scientific Knowledge on the Subject: Although the role of excess airway smooth muscle (ASM) is recognized in asthma, and mean data show that it is increased in relation to asthma severity, little is known about the distribution of the increased smooth muscle throughout the small and large airways of people with mild and severe asthma. The presence of regional heterogeneity of ventilation in patients with asthma suggests a possible structural basis that may act locally or influence the behavior of the distal airways or lungs.

What This Study Adds to the Field: This study shows that using mean data to compare cases of asthma with control subjects greatly underestimates the extent and potential functional impact of ASM remodeling in asthma. ASM remodeling is heterogeneous within and between cases of mild to moderate asthma but more confluent and more severe in fatal (severe) cases of asthma. This may explain the benefits of bronchial thermoplasty in severe cases. The distribution of ASM remodeling points to a need for specific identification of remodeling and targeting and monitoring of current and new treatments for ASM remodeling.

intraindividual heterogeneity of airway tone is greater in patients with asthma (7). Functional magnetic resonance imaging (MRI) in patients with asthma shows reasonably stable areas of reduced ventilation (8). Although deficits in ventilation may correspond to regions of ASM remodeling, it is recognized that remodeling in large airways may give rise to abnormal airway behavior in structurally normal small airways (9).

Currently, direct treatment of ASM in asthma is confined to selected patients using bronchial thermoplasty (10, 11), and side effects and duration of treatment can be reduced if treatment is directed to areas of ikely focal ASM remodeling (12, 13). The prospect of imaging the ASM *in vivo* using optical coherence tomography (14–16) to direct treatment and measure the effects of new treatments raises practical problems of protocols for assessing the bronchial tree during bronchoscopy (i.e., not all airways can be assessed). Whether ASM remodeling is related to airway size, bronchial pathways, or anatomic position in the lung or occurs in discrete, randomly distributed regions of the lung is largely unknown. We mapped the distribution of ASM remodeling in individuals with mild–severe asthma and the effect of random sampling on the classification of ASM remodeling phenotypes. The hypothesis for this study was that ASM remodeling in asthma would be evident in specific patterns related to airway size and bronchial pathways and that specific phenotypes of ASM remodeling could be distinguished.

Methods

Subjects

Postmortem airway samples used in this study were obtained from six centers contributing to the Airway Disease Biobank (17). Subjects were defined as: cases of fatal asthma, in which the primary cause of death was asthma; cases of nonfatal asthma, in which the cause of death was nonrespiratory and there was a history of asthma; and control cases in which the cause of death was nonrespiratory and there was no history of asthma or evidence of other respiratory disease. Age at the time of death, sex, and when available, smoking history, clinical severity (on the basis of admissions to hospital for asthma, time from work or school, frequency of asthma symptoms, and use of oral corticosteroids), duration of asthma, age of onset of asthma, and current treatment requirements were recorded. Ethics committee approval was granted from all participating centers and the Sir Charles Gairdner Hospital Human Research Ethics Committee (HREC No: 2015-053).

Tissue Preparation

In left lungs from the Prairie Provinces study (18, 19), fixed in inflation via the main bronchus and pulmonary artery with glutaraldehyde, nine equidistant cross-sections of airways were cut along three bronchial pathways from the origins of the upper lobe, lower lobe posterior segment, and lower lobe anterior segment to the lung periphery (Figure 1A). Additional control cases and cases from other centers were sampled in a systematic stratified manner after fixation in inflation via the trachea with formaldehyde (20) or chosen at random at the discretion of a pathologist and fixed by immersion. Tissue blocks of large and small airways were embedded in paraffin wax, sectioned at 5 μm, and stained with hematoxylin and eosin or with the Masson's or Gomori trichrome techniques.

ASM Measurements

On airways cut in cross-section, the area of the ASM layer was measured using planimetry on stereological software (newCAST version 4.2.1; Visiopharm A/S) or by point count (*see* online supplement). The basement membrane perimeter (Pbm) was used as a marker of airway size, with airways classified as small (Pbm $<$ 6 mm) or large (Pbm $>$ 6 mm) (17). The average thickness of the ASM layer was calculated as ASM area/Pbm^2 and log-transformed for comparisons between groups.

Analysis

To visualize the distribution of ASM remodeling in large and small airways, each of the nine levels of the three bronchial pathways from the Prairie Provinces Study asthma cases (Table 1) was assigned a color on the basis of its ASM area/Pbm^2 value relative to the mean value for the same level in the control group, from pale blue ($\leqslant$3 SD) to pale yellow ($\geqslant$3 SD), log-normal *z*-score equivalent (Figure 1B). For each case, the three bronchial pathways were laid horizontally to form a heat map (Figure 1C) of ASM remodeling. The effects of the pathway on remodeling were examined using Cohen's *d* effect size. For clarity, the distribution of remodeling was also presented in binary form as either less than or more than the upper 95% confidence interval of the control mean value (Figure 2B). The percent of large and small airways with ASM area/Pbm^2 above the upper 95% confidence interval of the control mean value was calculated for each case, and differences between group means were compared using one-way ANOVA with *post hoc* Tukey's test. To examine the regional distribution of remodeling along bronchial pathways or at specific airway levels (peripheral vs. central), the Pearson correlation coefficients of ASM area/Pbm^2 between adjacent levels from the same

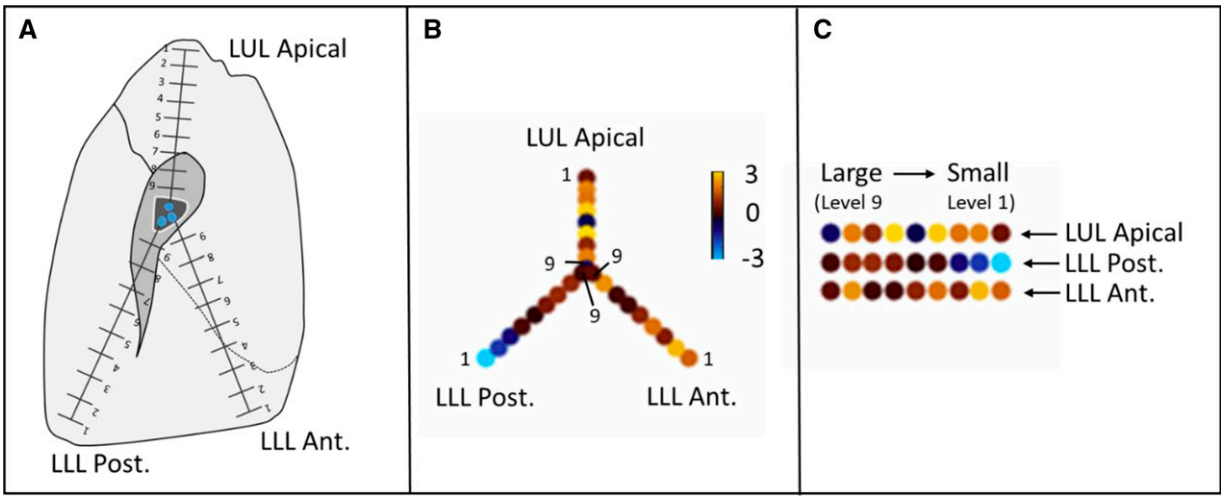

Figure 1. (*A*) Medial view of left lung showing sampling strategy of nine levels of airway (level nine being most central and level one being most peripheral) in three bronchial pathways used in the Prairie Provinces Study (18, 19). (*B*) In each case, the area of airway smooth muscle measured on a section from each level was assigned a color on the basis of its value relative to the mean and SD derived from control cases without asthma (Table 1). Pale blue and pale yellow represent airway smooth muscle areas three SDs below or above the control mean value, respectively. (*C*) The three bronchial pathways were aligned to form a heat map for visual comparisons between the same levels in different bronchial pathways and between adjacent levels within the same bronchial pathways. The example represents a single case of nonfatal asthma (from Figure 2). LLL Ant. = anterior segment of the left lower lobe; LLL Post. = posterior segment of the left lower lobe; LUL Apical = apical segment of the left upper lobe.

branching pathway and between the same levels (one through nine) in different branching pathways were calculated.

The effect of sampling frequency and definition of remodeling on the classification of cases into remodeling categories was assessed in cases of asthma ($n = 42$) with both 12 large and 12 small airways available for measurement and compared with mean and SD of the ASM area/Pbm^2, established in 86 control cases (Table 2) from large ($n = 1{,}237$) and small ($n = 688$) airways. Cases of asthma were then classified on the basis of the presence of ASM remodeling in the large and/or small airways as large and small airways (LS), large airways only (LO), small airways only (SO), or cases with ASM remodeling in neither large nor small airways (NI). ASM remodeling was defined in separate analyses as present if either: *1*) the ASM area/Pbm^2 of any large or small airway was greater than two SD above the control mean ASM area/Pbm^2; or *2*) the mean ASM area/Pbm^2 of the sampled large or small airways in each case was greater than two SD above the control mean values. The percentage of cases classified into each category was calculated, initially on the basis of the ASM area/Pbm^2 of only one large airway and one small airway (chosen at random without replacement), then successively recalculated after the addition of the ASM area/Pbm^2 of another large and another small airway, continuing until the ASM area/Pbm^2 of a total of 12 large and 12 small airways for each case had been included. This process was repeated 1,000 times to estimate the confidence of the estimates of classification.

Case means were compared between case groups (control, nonfatal asthma, and fatal asthma) and airway size groups using one-way ANOVA or Student's *t* test and appropriate *post hoc* tests. Nonparametric tests were used to compare subject groups in which data were not normally distributed or could not be normalized.

Table 1. Subject Characteristics Pathway and Level Analysis for Figures 1–3

Subject Characteristics	No Asthma ($n = 30$)	Nonfatal Asthma ($n = 30$)	Fatal Asthma ($n = 20$)
Sex (M/F), *n*	19/11	14/16	10/10
Age, yr	38 ± 10	36 ± 11	32 ± 13
Height (cm),* median (IQR); *n*	175 (172–190);15	173 (166–186); 18	167 (165–186); 11
Weight (kg),* median (IQR); *n*	75 (69–91)†; 14	101 (84–123); 19	76 (60–85)†; 11
Ever smoked,* n (%)	25 (72)	20 (55)	14 (64)
Age at onset of asthma,* *n* (yr)	—	19 (19 ± 14)	14 (14 ± 17)
Duration of asthma,* *n* (yr)	—	19 (18 ± 11)	14 (17 ± 9)
Asthma severity,* mild–moderate/severe, *n*	—	12/9	2/10†
Corticosteroid oral or inhaled,* *n* (%)	—	17 (59)	14 (79)†

Definition of abbreviation: IQR = interquartile range.
*Data not available for all cases.
†$P < 0.05$ compared with nonfatal asthma cases.

Figure 2. (*A*) Heat maps for all control cases and cases of asthma, derived as per Figure 1, of airway smooth muscle area/Pbm^2 for each of the cases, grouped as NA, cases of NFA, and cases of FA. Color scale shows variation from pale blue (equivalent to −3 or less standard deviations below the control value) through black (equivalent to the mean value of the control cases) to pale yellow (equivalent to three or more standard deviations above the control value). Red vertical bars represent medium or greater differences between pairs of pathways (Cohen's $d > 5$). Note: Using this scale, approximately 2% of airways are truncated. (*B*) Airways as for *A*, but shown in binary form as not remodeled (below the 95% confidence interval [blue]), or remodeled (above the 95% confidence interval), of the control value (brown). The percentage of remodeled airways is shown in Table 3. FA = fatal asthma; NA = control cases without asthma; NFA = nonfatal asthma.

Statistical analyses were undertaken using SigmaStat version 14.0 (Systat Software, Inc.) and Matlab R2020b (Mathworks).

Results

The subject characteristics are shown in Tables 1 and 2. On the basis of the available clinical data, the cases of fatal asthma were more severe and more often used oral and/or inhaled corticosteroids. Figure 2 shows the distribution of the remodeled airway sections, at nine levels (large to small airways, left to right), along the three bronchial pathways in each control and each asthma case. As expected, the control cases showed a distribution of colors around the mean value (black). There was considerable interindividual variability between and within individuals and between sections. In each group, there were medium or greater effects of the pathway (Cohen's d) observed in less than 20% of cases (Figure 2A).

In the nonfatal and fatal cases of asthma, there were more airways with ASM remodeling both in relation to case group and airway size (Figure 2B and Table 3). The airways sampled at the nine levels were of a similar size on the basis of Pbm (*see* Table E1 in the online supplement), and the mean area of the ASM was increased at most levels in cases of asthma (*see* Table E2). There were no apparent trends between levels or between bronchial pathways. There was considerable interindividual variability

Table 2. Subject Characteristics for Effect of Sampling Analysis (Figure 4)

Subject Characteristics	Control Cases ($n = 86$)	Nonfatal Asthma ($n = 21$)	Fatal Asthma ($n = 21$)
Sex (M/F), *n*	56/30	10/11	13/8
Age, yr (range)	35 (19–48)	29 (21–37)	31 (20–46)
Height,* cm (range); *n*	173 (162–180); 54	173 (164–177); 12	170 (165–180); 15
Weight,* kg (range); *n*	70 (58–81); 48	86 (76–141)†; 13	72 (64–79); 14
Ever smoked,* *n* (%)	40 (67)	16 (50)	17 (52)
Age at onset of asthma (yr),* median (IQR); *n*	—	9.5 (2–19.5); 16	12 (2.5–39); 17
Duration of asthma (yr),* median (IQR); *n*	—	12 (8–28); 15	16 (7–20); 17
Asthma severity (*n*),* mild–moderate/severe	—	10/6	5/11
Corticosteroid: oral or inhaled,* *n* (%)	—	13 (38)	16 (88)

For definition of abbreviation, *see* Table 1.
*Data not available for all cases.
†$P < 0.05$ compared with control cases.

Table 3. Percent of Airways with Smooth Muscle Remodeling*

	No Asthma (*N* = 30)	Nonfatal (*N* = 30)	Fatal Asthma (*N* = 20)
Large airways, *n*	5.5 ± 9; 568	18 ± 17†; 542	56 ± 25†‡; 348
Small airways, *n*	4.7 ± 8.7; 237	22 ± 24†; 256	50 ± 23†‡; 189

*Remodeling is defined as airway smooth muscle area/(perimeter of basement membrane)^2 above the upper 95% confidence interval of the control mean value. Percent of cases shown as mean ± SD.
†$P < 0.05$ compared with control cases.
‡$P < 0.05$ compared with nonfatal asthma cases.

in the nonfatal cases, but this was reduced in the fatal asthma cases (Figure 2).

The correlations of ASM area/Pbm^2 in cases of asthma between the same levels (one through nine) in different bronchial pathways and between adjacent levels within three bronchial pathways in the same lobes are shown in Figure 3 and Tables E3 and E4. Significant correlations were observed in the midsized airways, particularly at levels five and six, and within branching pathways there were no clear trends between adjacent levels.

The effect of sampling between 1 and 12 airways using any large or small airway with ASM area/Pbm^2 greater than two SDs above the mean control value to classify cases is shown in Figures 4A–4C (and Tables E5–E10). For all asthma cases (Figure 4A), categories of remodeling were largely established once eight or more large and small airways were sampled. After sampling 12 large and 12 small airways, 81% of cases were classified as LS, 5% as LO, 10% as SO, and 4% as NI. For nonfatal cases of asthma (Figure 4B), a similar pattern was seen, and 62%, 11%, 19%, and 8% were finally classified as LS, LO, SO, or NI, respectively. For fatal cases of asthma (Figure 4C), 99% of cases were finally classified as LS, less than 1% as LO or SO, and none as NI.

The analyses using the mean value of ASM thickness greater than two SDs of the mean control value to define ASM remodeling are shown in Figures 4D–4F. For all cases of asthma (Figure 4D), the proportion of cases classified as NI cases was 41%, with a slight increase up to 44% as more airways were sampled. There was a steady increase in cases classified as LS from 16% to 22% and a steady decrease in cases classified as LO to 22% and SO to 12%. For nonfatal cases of asthma (Figure 4E), cases classified as NI increased to 73%, LS increased to 8%, and LO and SO decreased to 2% and 17%, respectively. For fatal cases of asthma (Figure 4F), cases classified as LS increased to 37% with a corresponding decrease in NI to 15% and little change overall in LO and SO to 40% and 7%, respectively. This suggests that as more airways are sampled, it is increasingly likely that markedly remodeled airways (particularly large airways) are contributing to an increase in the mean value of ASM area/Pbm^2.

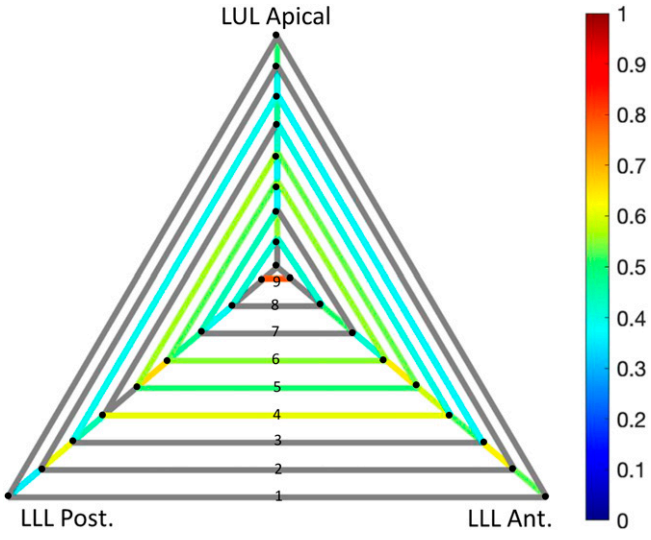

Figure 3. Correlations of airway smooth muscle area/Pbm^2 between levels (*see* Figure 1) within and between three branching pathways of the left lung in 50 cases of asthma (as seen in Figure 2). Levels one are the small, peripheral airways, and levels nine are the proximal bronchi of the LUL Apical, LLL Post., and LLL Ant. The three radial arms show the correlations between adjacent airway levels (one and two, two and three, three and four, … , eight and nine) within the same bronchial pathways. The concentric triangles show the correlations between each of the airway levels (one through nine) in different branching pathways. The color scale represents the *r* values of the pairwise comparisons. Gray segments are not statistically significant ($P > 0.05$, after Bonferroni correction). The *r* values and *P* values for all comparisons are shown in Tables E3 and E4. For definition of abbreviations, *see* Figure 1.

Discussion

We found that compared with control cases, cases of nonfatal asthma (mostly clinically mild–moderate) had more large and small airways with ASM remodeling, even if their mean ASM thickness was not increased. Remodeling of the ASM was more widespread and more severe in cases of fatal asthma. The area of ASM in cases with and without asthma varied between levels of the bronchial tree within and between individuals. Using mean data for the area of ASM, categories of remodeling were evident on the basis of the distribution of remodeling in the large and small airways. In cases of nonfatal asthma, approximately one in five large and small airways, and in cases of fatal asthma, approximately one in two large and one in 1.8 small airways, ASM remodeling was observed. There were weak correlations of ASM area between airways of similar size in different lobes of the lung and between different levels within lobes; however, other than those related to asthma severity, no predictable patterns of remodeling within or between lobes were observed. These findings extend previous studies by showing that although the frequency of remodeled airways is related to disease severity, a significant degree of heterogeneity exists, particularly in patients with mild to moderate asthma.

Previously, ASM remodeling was defined as the mean area of ASM only greater than one SD of the control mean

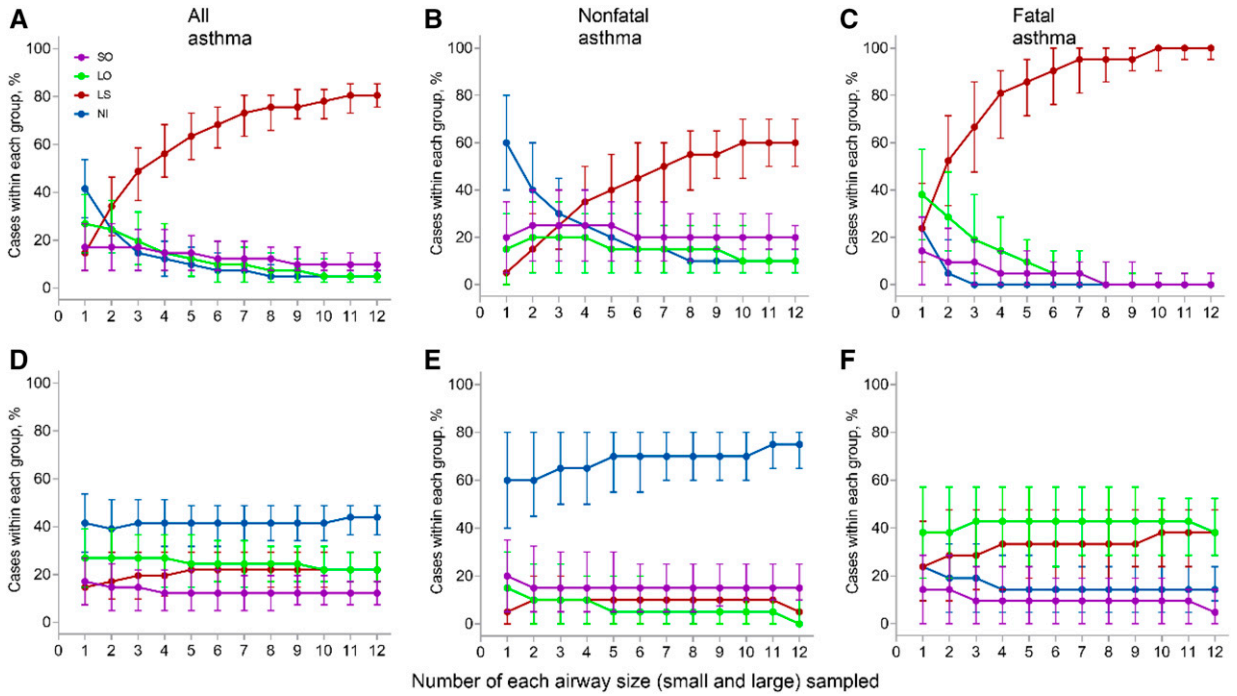

Figure 4. The effect of the number of large and small airways sampled (from 1 to 12) on the classification of cases into remodeling groups on the basis of airway smooth muscle (ASM) remodeling being present in both the large and small airways, the large airways only, the small airways only, or in neither the large or small airways. ASM remodeling was defined in two ways: *1*) (*A–C*) if the area of ASM in any large or small airway was greater than two SDs above the mean value for the control cases (Table 2); or *2*) (*D–F*) if the mean value for the large or small airway was greater than two SDs above the mean value for the control cases. Results are shown (percentages of each remodeling group, median ± 95% confidence interval) for all cases of (*A* and *D*) asthma, (*B* and *E*) nonfatal cases of asthma, and (*C* and *F*) fatal cases of asthma. Data for the graphs are shown in Tables E5–E10. LO = large airways only; LS = large and small airways; NI = neither large nor small airways; SO = small airways only.

value (5). In the present study, remodeling was defined as ASM thickness greater than two SDs of the mean control value; 12 large and 12 small airways were sampled from each case of asthma. The 1,000 iterations of computed samplings of any one large and one small airway would be expected to predict a stable classification of cases when sampling 1–12 airways and defining remodeling on the basis of the mean value. The falling percent of large-only cases in the nonfatal cases (Figure 4E) is consistent with the observation that the majority of large airways do not have ASM remodeling. In the fatal cases (Figure 4F), however, the steady increase in the percent of cases classified as large and small, with a corresponding decrease in the percent of small-only cases, is likely a result of the inclusion of more markedly remodeled large airways. The relative stability of the proportion of large-only cases is consistent with the observation that, on the basis of mean data, the ASM remodeling affects a greater percentage of the large airways than the small airways. Therefore, in fatal asthma, not only do more airways (particularly large airways) have ASM remodeling, but the remodeling is also more severe.

In a study of the variation of ASM remodeling (21), sampling from whole lungs from eight cases of fatal asthma and four cases of nonfatal asthma showed only a small increase in mean ASM area compared with control cases, although there was a shift to the right in the distribution of ASM area/Pbm with a tail of more severe remodeling. These results are consistent with our findings, which demonstrate marked heterogeneity and individual airways with more marked remodeling (greater than three SDs above the control mean). In the same study (21), heterogeneity was expressed as the coefficient of variation (CV) (SD / mean × 100), which was high in both cases without asthma (70.2%) and cases with asthma (98.0%). These values for the coefficient of variation are much higher than those observed within short airway segments of 1 mm (CV = 2–5%) (22), 0.6 mm (CV = 5–10%) (23), or 2 mm (CV = 25–34%) (24) in control cases and fatal asthma.

A further study of the regionality of ASM remodeling (25) showed that spatial correlations of ASM remodeling were present in airways separated by short distances, either in the same transverse plane or in the same branching pathway. In our analysis, we examined the correlations of ASM area in airways that were adjacent in a branching pathway and the correlations between airways that were not adjacent but at similar levels (and sizes) in separate branching pathways (Figure 3). We found that overall correlations were low, although they tended to be higher in adjacent sections in the same branching pathway. These findings support spatial correlation. This may not be surprising, however, because any proposed mechanism of hypertrophy or hyperplasia of smooth muscle, if heterogeneous, is still likely to be regional to some extent. Although we did observe the effect of a pathway in some cases (Figure 2A), this was in a minority of cases, and no clear patterns were evident.

In the present study, the cases of fatal asthma were distinguished by having ASM that involved more airways (particularly large airways), was more severe, and was more confluent. However, there was some overlap between the two asthma groups. Although the amount of smooth muscle around the airways is a determinant of how much the airways can narrow (2), other factors likely contribute to fatal asthma. In addition to more regions of extreme ASM remodeling, these factors include the degree of stimulation of the ASM by inhaled irritants, allergens, or agonists, perception of airway narrowing, and the underlying severity of other pathologies, such as mucus gland hypertrophy and inflammation (26). The degree of stimulation of the ASM by an inhaled allergen is independently related to the dose of the allergen, sensitivity to the allergen, and nonspecific airway responsiveness (27). Therefore, it is possible that a person with mild or unrecognized asthma or no previous asthma symptoms (and presumably very little ASM remodeling) could develop symptoms for the first time or even die of a fatal asthma attack if exposed to a strong enough stimulus, as has been observed in thunderstorm asthma (28).

The strengths of the present study are the large number of asthma cases and control cases, the distribution of asthma severity, and the systematic, extensive sampling of airways. This approach has facilitated the comparison of airways between lobes, within the same pathways (contiguous vs. noncontiguous), and between airway sizes. The availability of whole airway sections reduced the effects of sampling artifacts and allowed the comparison of airways of similar size between individuals. The cases of fatal asthma in this study included those with poorly controlled asthma, undertreated asthma, and those with severe asthma on maximum available therapy. However, in this postmortem study, apart from including or excluding the diagnosis of asthma, clinical details such as current medications, doses and adherence, and objective measures of lung function were not available in all cases.

The causes or mechanisms of the increased ASM in asthma remain unknown. ASM remodeling is related to the severity but not the duration of asthma (3) and is present in early life (29). Asthma is associated with reduced lung function (30) that is present even in infancy (31). These observations suggest that ASM remodeling arises very early in individuals with asthma. The present study did not uncover any specific pattern of ASM remodeling with regard to lobe, bronchial pathway, or airway size that might suggest possible mechanisms of ASM remodeling.

Models of airway narrowing show that the increased ASM mass observed in asthma has the greatest effect on maximal airway narrowing (32). The present study shows that ASM remodeling in asthma is not evenly distributed across the bronchial tree. Incorporating the variability of airway structure and random degrees of maximal airway narrowing into a model of airway narrowing results in marked heterogeneity of reduced ventilation in the lung (33–35). Extending these observations, subsequent modeling has shown that heterogeneity of airway narrowing of central airways may lead to abnormal ventilation in the structurally normal distal lung (9).

Using physiological tests and new imaging techniques, including high-resolution computer-assisted tomography and MRI, numerous studies have demonstrated the marked heterogeneity of ventilation observed in asthma (36–39). The degree of heterogeneity of ventilation is related to asthma severity and airway responsiveness (40–42). Regions of ventilation heterogeneity may vary over time, possibly because of relatively random distal effects of airway narrowing (9, 43). However, many regions are constant over time (8, 44, 45). The persistence of regional heterogeneity suggests a structural basis (39, 46, 47) for these regions that is independent of airway inflammation (39) and is supported by the findings of the present study.

The unpredictable heterogeneous distribution of ASM remodeling that we observed mandates more specific measures to identify airways or lung regions that might be targeted for treatment, such as functional MRI (12, 48). This technique identifies regions of abnormal ventilation but does not identify airway segments with ASM remodeling. Relating sites of ASM remodeling to functional changes more precisely await reliable means of measuring ASM thickness *in vivo* (28, 42). Both the distribution and the absolute increase of ASM mass within the bronchial tree are likely to have additive functions (34).

The widespread and severe ASM remodeling observed in the large airways in severe cases of asthma explains the effectiveness of bronchial thermoplasty, despite its application in a uniform manner (13, 16). The considerable heterogeneity of ASM remodeling of both the large and small airways may explain why bronchial thermoplasty is not effective in all cases and why the more targeted application of bronchial thermoplasty achieves similar results with fewer side effects and fewer treatment sessions (12). Imaging studies using computed tomography and ultrasound can identify localized increased airway wall thickness in midsized and large airways. However, these techniques are unable to distinguish ASM from other airway wall components to accurately localize ASM remodeling. Optical coherence tomography has been shown to define the layer of smooth muscle within the airway wall (14, 15) and this technique offers the possibility of more precisely targeted bronchial thermoplasty (49).

The present study has shown widespread remodeling of the ASM, involving both large and small airways, in cases of severe asthma. In cases of mild–moderate asthma, remodeling of the ASM is more piecemeal yet is likely to contribute to regional functional abnormalities and to airway hyperresponsiveness, which is characteristic of cases of even mild asthma (1). Therefore, approaches to identifying, treating, and assessing the effects of current and new treatments of ASM remodeling may need to be more localized. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

Acknowledgment: The authors would like to acknowledge Karen O. McKay's contribution of airway samples for this study. The authors would also like to acknowledge the contributions of the reviewers of this paper.

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severe asthma. In their study, not surprisingly, high-dose ICS and systemic corticosteroids were significantly associated with severe asthma and low CC16 expression levels in BECs. Critically, adjusting for ICS dose eliminated the association between CC16 concentrations and asthma severity. The authors propose that this may be because of overadjustment. However, it is difficult to untangle the influence of ICS use in this study, which represents a major challenge in translational asthma research in general. Studies of the effect of ICS on BEC CC16 expression in normal individuals and *in vitro* BEC studies would clarify and contextualize the findings of this publication. Finally, the authors present CC16 as a nontraditional Th2 biomarker rather than a negative T2 biomarker. If this is truly the case, then a better understanding of its mechanistic contribution to asthma will be crucial.

Although Li and colleagues from SARP present a promising airway biomarker, there are still important questions that need to be addressed, including identifying the molecular mechanism of CC16 in asthma and the influence of corticosteroids on its utility as a biomarker. However, the approach itself is an important illustration of the path toward refined asthma endotypes. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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A Few Bad Airways Can Wreak Havoc: Recognizing Asthma as a Local Disorder

Stimulating airway smooth muscle (ASM) in an individual with asthma can cause some airways to hyperconstrict in ways healthy airways would not. Although several reasons could contribute to this, there is unequivocal experimental evidence that the ASM surrounding many asthmatic airways is thicker, and more so with severe asthma (1). Starting with Lambert and colleagues (2) and continued with increasing more morphometrically and three-dimensionally consistent airway trees (3–5), computational models consistently show that if one assumes maximum ASM force generation scales proportionately with its thickness, the thickened ASM found in asthmatic airways can lead to hyperresponsiveness. The above, however, is inadequate to fully understand how lung function degrades during an asthma attack in a specific patient or to guide optimal treatment strategies in a patient-specific fashion. The percentage of ASM thickening is not the same everywhere, either in large versus small airways or across airways of similar original diameter throughout the lung or across subjects with a similar clinical classification of asthma severity (6, 7).

Surely all airways do not constrict identically during an asthma attack; ergo, heterogeneous constriction is a fundamental feature of asthma (8, 9). In fact, this feature is the most important contributor to the degradation of lung function that occurs during an attack (3–5). Models predict that ASM thickening enhances the heterogeneity of the resulting constriction (4). Hence, it would seem useful to identify the precise locations of abnormal ASM thickening. Stated another way, understanding the average constriction across all airways is of limited value when conceptualizing more effective future treatment methods. Rather, it is all about the anatomic origins of heterogeneity. This last statement speaks to the heart of insights from the new study by James and colleagues (pp. 452–460) in this issue of the *Journal* (10).

Originally Published in Press as DOI: 10.1164/rccm.202212-2231ED on December 14, 2022

James and colleagues (10) meticulously measured the thickness of the ASM for nine consecutive airway generations along three distinct airway pathways in the lungs of subjects without asthma, subjects with nonfatal asthma (NFA), and subjects with fatal asthma. Each pathway progresses from larger (~9 mm diameter) to smaller (~1 mm diameter) airways. The data allowed them to assess two forms of heterogeneity in ASM thickening: longitudinal (e.g., large vs. small) and spatially distributed heterogeneity (airways of similar generations but distinctly different lung areas). They classified any airway as having abnormal ASM thickness if the thickness was 2 Standard Deviation above the mean from a corresponding normal airway. They found that subjects without asthma had little evidence of thickening of their ASM on average or even in any individual airway along any of the pathways. In contrast, subjects with NFA were far more likely to possess at least one airway, and in many cases a few airways, with highly thickened ASM along one or more pathways, even though the mean ASM across all airways was often within the normal range. Interestingly, they also found that about 20% of subjects with NFA had abnormal thickening only in their small airways. Most of the subjects with fatal asthma showed abnormal thickening in the ASM of both one or more large and one or more small airways and across all three pathways in almost all such subjects. But, even here, averaging across all airways often dampened evidence that there were always at least some individual airways along a pathway with highly abnormal ASM thickness. They conclude that using mean data to compare cases of asthma underestimates the potential severity of disease due to ASM remodeling. This conclusion inherently appreciates that it is the capacity to create heterogeneous constriction that drives asthma severity for subjects with NFA and that only a few airways along a pathway need to possess abnormally thick ASM to accomplish this (i.e., many airways can be within the normal range).

They also report that although there was no correlation of abnormal ASM across airways of the same generation, along different pathways there was some degree of correlation along adjacent airways, suggesting that if an airway in each path had abnormal ASM thickening it was more likely to find another airway close by with the same.

James and colleagues did not report any functional data to support their notion that assessing mean data only might depress one's sense of asthma clinical severity. But there are ample studies that are consistent with this notion. Computational models have shown that when a few airways hyperconstrict heterogeneously the result is a dramatic degradation of lung mechanical function and ventilation distribution in ways that may not at all be evident by simply measuring the overall increase in airway resistance, which reflects mean diameter reduction (3–5). To match measured degradation in mechanics and ventilation distribution simultaneously, Tgavalekos and colleagues (5) only had to hyperconstrict a small percentage of small airways heterogeneously. Constriction of only large airways could not replicate these measurements. Imaging studies (9, 11) have shown that many ventilation defects occur in similar anatomic locations in the same subjects with asthma when imaged longitudinally in time, suggesting elevated ASM thickness might be a local pathology.

Taken together, the study by James and colleagues convincingly supports that asthma is more likely a consequence of local airway abnormalities. James and colleagues showed that more often than not subjects with moderate asthma have abnormal thickening in only a few airways, even while the mean change in ASM thickness is not abnormal. Moreover, the most abnormal airways were in different places from subject to subject.

Regarding the origins of the abnormal ASM thickness, James and colleagues note that many individuals with asthma may already have abnormal ASM thickening at birth. But other factors throughout life can locally elevate ASM around an airway. Inflammatory mediators can upregulate both ASM and extracellular matrix (ECM) remodeling (12), and it seems reasonable that inflammation would not be identical throughout all airways. Similarly, elevated and repeated ASM and airway tension can induce remodeling because of mechanobiological factors upregulating growth factors (12). There is no reason to believe these factors will impact all airways identically. Finally, recent studies show that the ASM and ECM do not operate independently and that stiffening of the ECM can actually amplify the ability of the ASM to create an elevated and sustained force (13). Thickening of the ASM and ECM remodeling could work in tandem then to further create localized abnormalities causing heterogeneity and asthma.

The study by James and colleagues continues the recent trend of raising awareness that the pathological airway conditions in asthma are likely local rather than evenly spread. Consequently, optimal treatments might best target these local abnormalities. In principle, bronchial thermoplasty (BT) could address this, if the clinician knew in advance precisely which airways to target. But BT cannot yet get to all the small airways that are important. Nevertheless, we should focus on creative, perhaps hybrid (imaging plus delivery) methods to treat individuals with asthma on a personalized basis, targeting the specific locations in each individual subject that can create heterogeneity of constriction pattern (14, 15). In sum, preventing or minimizing the heterogeneity should be an explicit goal. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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Interplay between Immune and Airway Smooth Muscle Cells in Obese Asthma

Increasing rates of obesity in children and adults have been associated with the development of severe, difficult-to-control asthma (1). Although some people with asthma and comorbid obesity have type 2–high disease, others experience airway disease associated with insulin resistance and metabolic dysfunction, with little in the way of type 2 inflammation. Asthma in obese people likely consists of many different endotypes of disease, and earlier work by Rastogi and colleagues identified a type of asthma in children with obesity characterized by insulin resistance, augmented T-helper cell type 1 (Th1) function, and upregulation of a pathway related to the Rho-family GTPase cell division cycle 42 (CDC42) in Th1 cells (2). CDC42 is critical in actin cytoskeleton assembly and mediates functions such as vesicle trafficking, orientation of receptors on the cell surface, chemotaxis, and cell adhesion (3). Although the observation of induction in this CDC42 pathway is fascinating, it provides limited insights into how changes in Th1 cell function might be linked to airway disease.

Clearly, airway reactivity is not simply mediated by T cells in isolation; many other cell types are involved. In this context, earlier work by Hakonarson and colleagues is relevant: Cooperative signaling between human airway smooth muscle (ASM) cells and T lymphocytes mediated induction of proasthmatic changes in ASM (4). Furthermore, Orfanos and colleagues identified hypercontractility of ASM cells isolated from individuals with obesity (5), suggesting that ASM changes could be involved in the asthma of obesity. However, the reasons for ASM dysfunction in obesity are not known. In this issue of the *Journal*, Yon and colleagues (pp. 461–474) set out to determine if perhaps there could be a link between Th1 cell dysfunction and ASM dysfunction that might provide insights into the pathogenesis of asthma in children with obesity (6).

Yon and colleagues first studied Th1 cells, then the ASM cells, and finally the interaction between these two cell types. The team used Th1 cells from obese children with asthma that expressed high levels of CDC42 and those from lean children that expressed very low levels of CDC42. The Th1 cells were first stimulated *in vitro* with CD3-CD28. The Th1 cells from obese children tended to have higher levels of the integrin LFA-1 (lymphocyte function–associated antigen 1), and migration in response to the ligand SDF-1 (stromal differentiation factor 1) was independent of CDC42. Although CDC42 is a biomarker identifying these cells, inhibition of this pathway did not affect migration. The investigators also included Th1 cells from three obese and three healthy-weight children without asthma as a control. A higher proportion of T-helper cells from obese children with asthma than from obese children without asthma migrated in response to SDF-1. These data suggest there may be altered expression of cell surface integrins and differences in chemotaxis responses between Th1 cells isolated from lean and obese children that are specific to obese asthma rather than just obesity.

The investigators then studied ASM from obese individuals and lean individuals without asthma obtained from lungs not suitable for transplant. The investigators found that cells from obese individuals expressed higher levels of ICAM-1 (intercellular adhesion molecule 1), the ligand for LFA-1 that mediates adherence of immune cells to smooth muscle. This suggests that ASM from obese individuals is more primed for adherence of immune cells than ASM from lean individuals. Indeed, in coculture experiments, Th1 cells from obese individuals with asthma adhered in greater number to ASM isolated from obese individuals than Th1 cells from

Supported by National Institutes of Health (R01 HL133920).

Originally Published in Press as DOI: 10.1164/rccm.202210-1870ED on October 11, 2022

nature medicine

Resource

https://doi.org/10.1038/s41591-023-02327-2

An integrated cell atlas of the lung in health and disease

Received: 10 March 2022

Accepted: 30 March 2023

Published online: 8 June 2023

Check for updates

A list of authors and their affiliations appears at the end of the paper

Single-cell technologies have transformed our understanding of human tissues. Yet, studies typically capture only a limited number of donors and disagree on cell type definitions. Integrating many single-cell datasets can address these limitations of individual studies and capture the variability present in the population. Here we present the integrated Human Lung Cell Atlas (HLCA), combining 49 datasets of the human respiratory system into a single atlas spanning over 2.4 million cells from 486 individuals. The HLCA presents a consensus cell type re-annotation with matching marker genes, including annotations of rare and previously undescribed cell types. Leveraging the number and diversity of individuals in the HLCA, we identify gene modules that are associated with demographic covariates such as age, sex and body mass index, as well as gene modules changing expression along the proximal-to-distal axis of the bronchial tree. Mapping new data to the HLCA enables rapid data annotation and interpretation. Using the HLCA as a reference for the study of disease, we identify shared cell states across multiple lung diseases, including *SPP1*$^{+}$ profibrotic monocyte-derived macrophages in COVID-19, pulmonary fibrosis and lung carcinoma. Overall, the HLCA serves as an example for the development and use of large-scale, cross-dataset organ atlases within the Human Cell Atlas.

Rapid technological improvements over the past decade have allowed single-cell datasets to grow both in size and number[1]. This has led consortia, such as the Human Cell Atlas, to pursue the generation of large-scale reference atlases of human organs[2,3]. To advance our understanding of health and disease, such atlases must capture variation between individuals that is expected to impact the molecular phenotypes of the cells in a tissue. Whereas the generation of atlases at this scale by single research groups is currently not feasible, integrating datasets generated by the research community at large will enable capture of the diversity of the cellular landscape across individuals.

Several foundational studies have started to map the cellular landscape of the healthy human lung[4–6]. These studies each have a specific bias due to their choice of experimental protocol and technologies, and are therefore not tailored to serve as a universal reference. The studies moreover include only a limited number of samples and individuals, thus lacking the scale and diversity to capture the full cellular heterogeneity present within the lung as well as across individuals.

Integrated single-cell atlases provide novel insights not obtained in individual studies. Recent reference atlases have led to the discovery of unknown cell types[7–9], the identification of marker genes that are reproducible across studies[7,10,11], the comparison of animal and in vitro models with human healthy and diseased tissue[7,12,13] and patient stratification for disease endotypes[14,15]. However, many currently available integrated atlases are limited in the number of human samples[7,8,10–12,16], datasets[16] or cell types[7,9,12,17,18] per organ, as well as donor metadata[12,13,17,19,20] (for example, age, body mass index (BMI) and smoking status), or focus mainly on a specific disease[14,15,17]. These limitations constrain the potential of atlases to serve as a reference, as they fail to represent and catalog the diversity of cellular phenotypes within the healthy organ and across individuals. Moreover, when integrating data from different sources, it is paramount to correctly separate technical biases from biologically relevant information. Yet, the majority of existing atlases have not assessed the quality of their data integration. Nonetheless, successful integration of the available

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https://doi.org/10.1038/s41591-023-02327-2

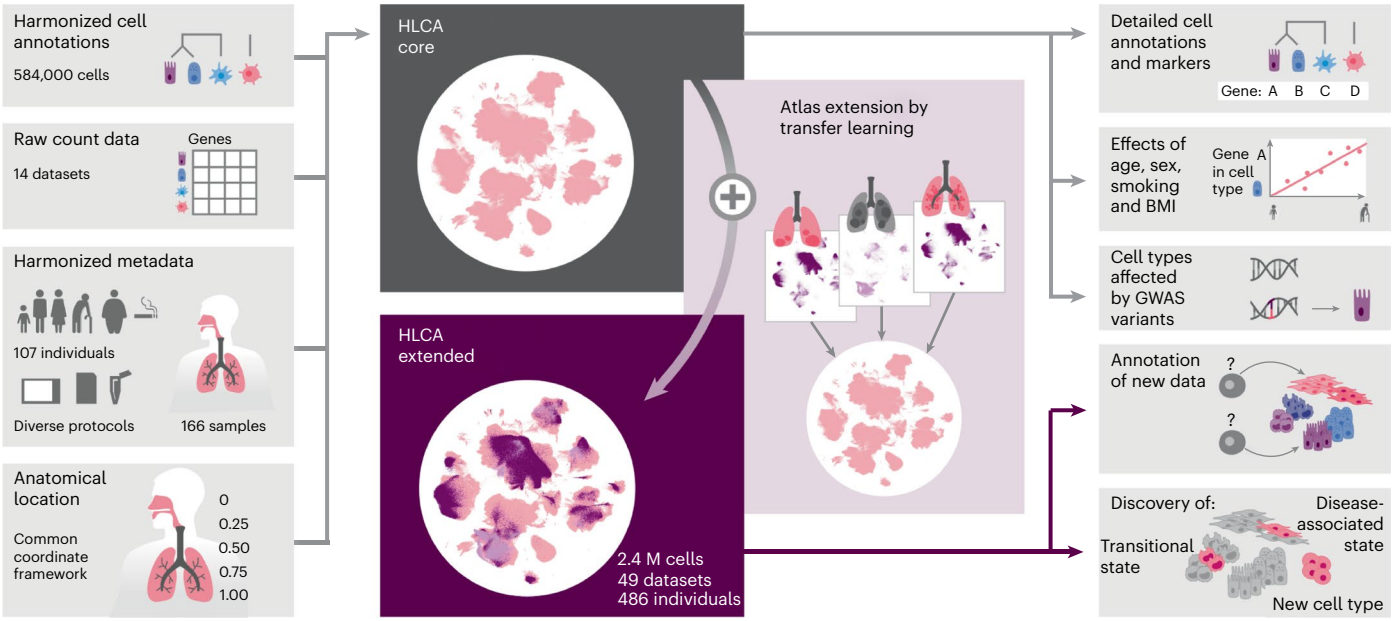


Fig. 1 | HLCA study overview. Harmonized cell annotations, raw count data, harmonized patient and sample metadata and sample anatomical locations encoded into a CCF were collected and generated as input for the HLCA core (left). After integration of the core datasets, the atlas was extended by mapping 35 additional datasets, including disease samples, to the HLCA core, bringing the total number of cells in the extended HLCA to 2.4 million (M). The HLCA core provides detailed consensus cell annotations with matched consensus cell type markers (top right), gene modules associated with technical, demographic and anatomical covariates in various cell types (middle right), GWAS-based association of lung conditions with cell types (middle right) and a reference projection model to annotate new data (middle right) and discover previously undescribed cell types, transitional cell states and disease-associated cell states (right, bottom).

datasets into a single tissue atlas is a critical step in achieving the goals of the Human Cell Atlas[2].

In this resource, we present an integrated single-cell transcriptomic atlas of the human respiratory system, including the upper and lower airways, from published and newly generated datasets (Fig. 1). The Human Lung Cell Atlas (HLCA) comprises data from 486 donors and 49 datasets, including 2.4 million cells, which we re-annotated to generate a consensus cell type reference. The HLCA expands our understanding of the healthy lung and its changes in disease and can be used as a reference for analyzing future lung data. Together, we provide a roadmap for building and using comprehensive, interpretable and up-to-date organ- and population-scale cell atlases.

Results

Data integration establishes the HLCA core

To build the HLCA, we collected single-cell RNA sequencing (scRNA-seq) data and detailed, harmonized technical, biological and demographic metadata from 14 datasets (11 published and three unpublished)[4–6,21–25,26,27]. These datasets include samples from 107 individuals, with diversity in age, sex, ethnicity (harmonized as detailed in Methods), BMI and smoking status (Fig. 2a). Cells were obtained from 166 tissue samples using a variety of tissue donors, sampling methods, experimental protocols and sequencing platforms (Supplementary Tables 1 and 2). Anatomical locations of the samples were projected onto a one-dimensional (1D) common coordinate framework (CCF), representing the proximal (0) to distal (1) axis of the respiratory system, to standardize the anatomical location of origin (Fig. 2a and Supplementary Tables 2 and 3).

Consensus definitions of cell types based on single-cell transcriptomic data across studies—particularly of transitional cell states—are lacking. To enable supervised data integration and downstream integrated analysis, we harmonized cell type nomenclature by building a five-level hierarchical cell identity reference framework (Methods, Supplementary Table 4 and Fig. 2b). We then unified cell type labeling across datasets by mapping the collected cell identity labels for every dataset as provided by the data generator to the hierarchical reference framework, showing varying cell type proportions per sample (Fig. 2c).

To optimally remove dataset-specific batch effects, we evaluated 12 different data integration methods on 12 datasets[4–6,21–25] (Fig. 2d and Supplementary Fig. 1) using our previously established benchmarking pipeline[28]. We used the top-performing integration method, scANVI, to create an integrated embedding of all 584,444 cells of 107 individuals from the collected datasets: the HLCA core (Fig. 3a).

Consensus cell type annotations based on the HLCA core

A large-scale integrated atlas provides the unique opportunity to systematically investigate the consensus in cell type labeling across datasets. To identify areas of consensus and disagreement, we iteratively clustered the HLCA core and investigated donor diversity and cell type label agreement in these clusters using entropy scores (see Methods). Most clusters contained cells from many donors (Extended Data Fig. 1a). Clusters with low donor diversity ($n = 14$) were largely immune cell clusters ($n = 13$), representing donor- or donor group-specific phenotypes. Similarly, a high diversity of (contradictory) cell type labels (high label entropy) can identify both annotation disagreements between studies and clusters of doublets (Methods). Most clusters (61 out of 94) showed low label entropy, suggesting overall agreement of coarse cell type labels across datasets (Fig. 3b). The remaining 33 clusters exhibited high label entropy, highlighting cellular phenotypes that were differently labeled across datasets (Fig. 3b). For example, the immune cluster with the highest label entropy contained many cells that were originally mislabeled as monocytes and macrophages but were actually type 2 dendritic cells (Fig. 3c and Extended Data Fig. 1b). Thus, populations with high label entropy identify mislabeled cell types, indicating the need for consensus re-annotation of the integrated atlas.

As a first step to achieve such a consensus on the diversity of cell types present in the HLCA core, we performed a full re-annotation of the integrated data on the basis of the original annotations and six

expert opinions (consensus annotation; Methods and Fig. 3d). Each of the 61 annotated cell types (Supplementary Table 5) was detected in at least four datasets out of 14, often in specific parts of the respiratory system, and different cell types showed varying fractions of proliferating (*MKI67*$^+$) cells (Extended Data Fig. 2a–c). While our consensus cell type annotations partly correspond to original labels (41% of cells), there were also refinements (28%) and substantial re-annotations (31%; Fig. 3e and Supplementary Fig. 2). To robustly characterize the cell types, we established a universal set of marker genes that generalizes across individuals and studies (Methods, Extended Data Fig. 3 and Supplementary Table 6). The fully re-annotated HLCA core thus combines data from a diverse set of studies to provide a carefully curated reference for cell type annotations and marker genes in healthy lung tissue.

The HLCA recovers rare cell types and identifies novel ones

Rare cell types, such as ionocytes, tuft cells, neuroendocrine cells and specific immune cell subsets, are often difficult to identify in individual datasets. Yet, combining datasets in the HLCA core provides better power for identifying these rare cell types. Ionocytes, tuft and neuroendocrine cells make up only 0.08, 0.01 and 0.02% of the cells in the HLCA core according to the original labels, and were originally identified in only seven, two and four datasets out of 14, respectively. Despite their low abundance, these cells formed three separate clusters of the HLCA core (Fig. 3f). Our re-annotation increases the number of datasets in which these cells are detected up to threefold and identifies both cells falsely annotated as monocytes, tuft cells or neuroendocrine cells, as well as originally undetected rare cells (Fig. 3f and Supplementary Fig. 3a). Importantly, other integration methods tested during our benchmarking, such as Harmony[29] and Seurat's RPCA[30], failed to separate these rare cells into distinct clusters (Supplementary Fig. 3b).

We were further able to detect six cell identities that were not previously found in the human lung or were only recently described in individual studies. These cell types include migratory dendritic cells[31,32] (n = 312 cells, expressing *CCR7*, *LAD1* and *COL19*), hematopoietic stem cells (n = 60, expressing *SPINK2*, *STMN*, *PRSS57* and *CD34*), highly proliferative hillock-like epithelial cells not previously reported in adult human lung (n = 4,600, expressing *KRT6A*, *KRT13* and *KRT14*), the recently described alveolar type 0 cells (n = 1,440, expressing *STFPB*$^+$, *SCGB3A2*$^+$, *SFTPC*^{high} and *SCGB3A1*^{low}) and the closely related preterminal bronchiole secretory cells (n = 4,393, expressing *SFTPB*$^+$, *SCGB3A2*$^+$, *SFTPC*^{low} and *SCGB3A1*^{high}, together with alveolar type 0 cells called transitional club-AT2 cells)[33,34] and a subset of smooth muscle cells (n = 335) that to our knowledge have not previously been described (Fig. 3d,g and Extended Data Fig. 4a–f). These smooth muscle cells, predominantly found in the airways, express canonical smooth muscle markers (*CNN1* and *MYH11*) and also uniquely and consistently express *FAM83D* across datasets (Extended Data Fig. 4e,f). The HLCA core thus enables improved detection and identification of rare cell types, as well as the discovery of unknown cell types.

Donor and experimental factors affect gene expression profiles

Demographic and other metadata covariates affect cellular transcriptional phenotypes[19,25]. Better insight into the impact of these covariates (for example, sex, BMI and smoking) on cell type gene expression can shed light on the contribution of these factors to progression from healthy to diseased states. In addition, technical covariates such as ribosomal and mitochondrial genes exhibit batch-specific variation in expression (Methods and Supplementary Table 7). The diversity in demographics (for example, smoking status, age, harmonized ethnicity and BMI) and experimental protocols represented in the HLCA core enables us to explore the contribution of each technical or biological covariate to cell type-specific gene expression variation (Methods and Supplementary Fig. 4). For many cell types, anatomical location is the biological variable explaining most of the variance between samples (Fig. 4a). Furthermore, sex is most associated with transcriptomic variation in lymphatic endothelial cells, whereas BMI is most associated with variation in B and T cells, harmonized ethnicity in transitional club-AT2 cells and smoking status in innate lymphoid/natural killer cells. Furthermore, for several cell types (for example, mast, AT1 and smooth muscle cells), the tissue dissociation protocol explains most of the variance of all technical as well as biological covariates recorded. These associations provide a systematic overview of the effects of biological and technical factors on the transcriptional state of lung cell types.

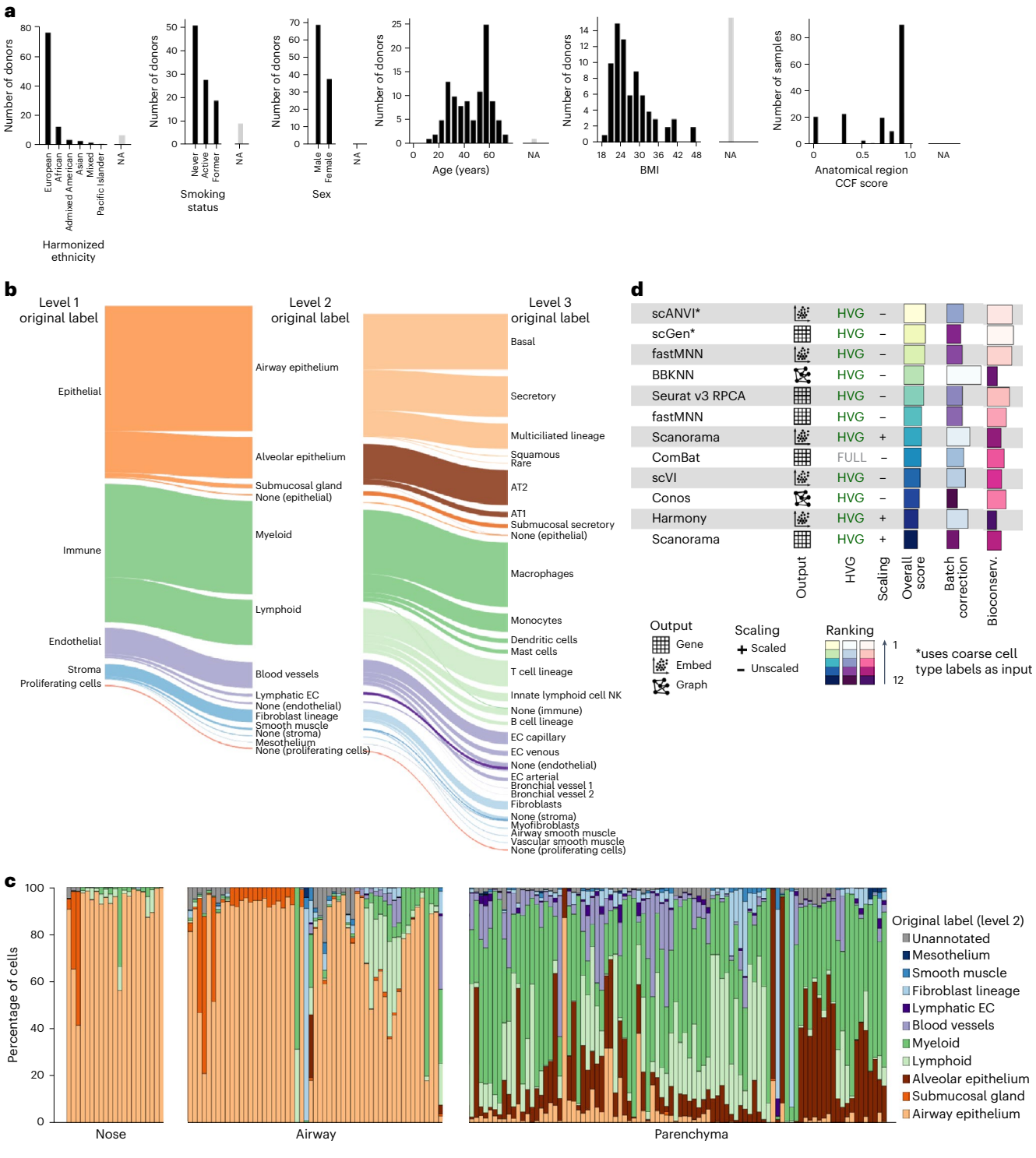
To better characterize how biological variables affect cellular phenotypes, we modeled their cell type-specific effects on the transcriptome at the gene level (Methods). Sex-related differences in lymphatic endothelial cells are dominated by differential expression of genes located on the X and Y chromosomes, but also include a decrease in IFNAR1 in females (Supplementary Table 8), which may be linked to differential interferon responses between the biological sexes[35]. We furthermore found cell type-specific programs that change with proximal (low CCF score) to distal (high CCF score) location along the respiratory tract (Supplementary Tables 8 and 9). For instance, oxidative phosphorylation (including cytochrome c oxidase genes such as *COX7A1*), antigen presentation by major histocompatibility complex class I molecules (including proteasome and protease subunit genes such as *PSMD14* and *PSMB4*), signaling by interleukin-1 and tumor necrosis factor α, as well as planar cell polarity, were downregulated toward more distal locations in secretory, multiciliated and basal cells (Fig. 4b). Some gene programs were specific for a subset of airway epithelial cell types (for example, cornification and keratinization, which were programs that were downregulated in distal multiciliated and secretory cells; including genes such as *KRT8* and *KRT19*). The changes in airway epithelial cell states toward the terminal airways are further illustrated by increased expression of developmental pathway genes such as *NKX2-1*, *NFIB*, *GATA6*, *BMP4* and *SOX9* in multiciliated cells along the proximal-to-distal axis (Fig. 4b), whereas basal cells decrease in number (Fig. 4c)[36]. Similarly, several cell types display

Fig. 2 | Composition and construction of the HLCA core. a, Donor and sample composition in the HLCA core for demographic and anatomical variables. Donors/samples without annotation are shown as not available (NA; gray bars) for each variable. For the anatomical region CCF score, 0 represents the most proximal part of the lung and airways (nose) and 1 represents the most distal (distal parenchyma). Donors show diversity in ethnicity (harmonized metadata proportions: 65% European, 14% African, 2% admixed American, 2% mixed, 2% Asian, 0.4% Pacific Islander and 14% unannotated; see Methods), smoking status (52% never, 16% former, 15% active and 17% NA), sex (60% male and 40% female), age (ranging from 10–76 years) and BMI (20–49; 30% NA). **b**, Overview of the HLCA core cell type composition for the first three levels of cell annotation, based on harmonized original labels. In the cell type hierarchy, the lowest level (1) consists of the coarsest possible annotations (that is, epithelial (48% of cells), immune (38%), endothelial (9%) and stromal (4%)). Higher levels (2–5) recursively break up coarser-level labels into finer ones (Methods). Cells were set to 'none' if no cell type label was available at the level. Cell labels making up less than 0.02% of all cells are not shown. Overall, 94, 66 and 7% of cells were annotated at levels 3, 4 and 5, respectively. **c**, Cell type composition per sample, based on level 2 labels. Samples are ordered by anatomical region CCF score. **d**, Summary of the dataset integration benchmarking results. Batch correction score and biological conservation score each show the mean across metrics of that type, as shown in Supplementary Fig. 1, with metric scores scaled to range from 0 to 1. Both Scanorama and fastMNN were benchmarked on two distinct outputs: the integrated gene expression matrix and integrated embedding (see output). The methods are ordered by overall score. For each method, the results are shown only for their best-performing data preprocessing. Methods marked with an asterisk use coarse cell type labels as input. Preprocessing is specified under HVG (that is, whether or not genes were subsetted to the 2,000 (HVG) or 6,000 (FULL) most highly variable genes before integration) and scaling (whether genes were left unscaled or scaled to have a mean of 0 and a standard deviation of 1 across all cells). EC, endothelial cell; NK, natural killer; Bioconserv., conservation of biological signal.

https://doi.org/10.1038/s41591-023-02327-2

transcriptomic changes in donors with increasing BMI (Fig. 4d and Supplementary Tables 8 and 9). AT2 cells, secretory cells and alveolar macrophages exhibit downregulation of a range of biological processes (Supplementary Fig. 5), including cellular respiration, differentiation and synthesis of peptides and other molecules. In secretory cells, a downregulation of the insulin response pathway is also associated with higher BMI, in line with the insulin resistance observed in donors with obesity[37,38]. In alveolar macrophages, inflammatory responses involving JAK/STAT signaling (previously associated with obesity-induced chronic systemic inflammation[38]) are associated with higher BMI. In contrast, in plasma cells, high BMI is associated with downregulation of gene sets associated with immune response and upregulation of gene sets associated with cellular respiration, the cell cycle and DNA repair. This is consistent with obesity being a known risk factor for multiple myeloma—a plasma cell malignancy[39]. Thus, the HLCA enables a detailed understanding of the effects of anatomical and demographic covariates on the cellular landscape of the lung and their relation to disease.

Biological and technical factors can also affect cell type proportions. Indeed, all cell types show changes in abundance as a function of anatomical location (Fig. 4c and Extended Data Fig. 5). For example, ionocytes are present at comparable proportions in the airway



epithelium, from the larger lower airways (CCF score = 0.36) down to the distal lobular airways (CCF score = 0.81), while being largely absent in the lung parenchyma (CCF score = 0.97). In contrast, neuroendocrine cells are predominantly observed in the larger lower airways but are absent from more distal parts of the bronchial tree (Fig. 4c). In some cases, these proportions are highly dependent on the tissue sampling method and the dissociation protocol used (for example, for smooth muscle FAM83D$^+$ cells; Extended Data Fig. 5). These observations shed light on the effects of biological and technical factors on the abundance of cell types in different parts of the lung and can help guide important choices in study design.

HLCA-based analysis of lung data highlights new cell types

The HLCA core contains an unprecedented diversity of donors, sampling protocols and cell identities, and can serve as a transcriptomic reference for lung research. New datasets can be mapped to this reference to substantially speed up data analysis by transferring consensus cell identity annotations to the new data. We tested this on a recently released multimodal lung dataset[40] (Methods, Fig. 6a and Extended Data Fig. 6). Overall, the transferred labels were correct in the majority of cases, with 68% of the cells correctly labeled, 14% of labels incorrectly labeled and 18% set to unknown due to highly uncertain labeling (Fig. 5b and Methods). Uncertain labels were observed specifically in continuous transitions from one cell type to another and among cellular identities not present in the HLCA core, including rare cell identities (erythrocytes (n = 328), chondrocytes (n = 42), myelinating Schwann cells (n = 7), nonmyelinating Schwann cells (n = 29) and nerve-associated fibroblasts (n = 66); Fig. 5b and Extended Data Fig. 6d). Taken together, these results show that the HLCA core can be used for highly detailed annotation of new datasets, while allowing for the identification of unknown cell types in these datasets based on label transfer uncertainty.

The HLCA provides crucial context for understanding disease

Single-cell studies of disease rely on adequate, matching control samples to allow correct identification of disease-specific changes. To demonstrate the ability of the HLCA core to serve as a comprehensive healthy control and contextualize disease data, we mapped scRNA-seq data from lung cancer samples[41] to the HLCA core (Methods and Extended Data Fig. 7a–c). Using HLCA label transfer, we correctly identified cell states missing from the HLCA core as unknown (cancer cells and erythroblasts). The remaining cells were annotated correctly in 77%, incorrectly in 1% and as unknown in 22% of cases (Extended Data Fig. 7d–g). A finding of the original study was the separation of endothelial cells into tumor-associated and normal cells[41]. Clustering of the projected dataset with the HLCA reference showed that cells expressing the suggested tumor-associated marker *ACKR1* were also abundant in healthy tissue from the HLCA core, specifically in venous endothelial cells (both pulmonary and systemic, Fig. 5c and Supplementary Fig. 6a–c). This suggests that *ACKR1* is a general marker of venous endothelial cells rather than a tumor-specific endothelial cell marker. Similarly, the reported normal endothelial cell marker *EDNRB* characterizes aerocyte capillary endothelial cells, both in tumor and in healthy tissue (Fig. 5c and Supplementary Fig. 6d). As endothelial cell numbers in the original study were low, correctly identifying and distinguishing these cell types without a larger healthy reference is challenging. Thus, by serving as a comprehensive healthy control, the HLCA prevents misinterpretation of limitations in sampling and experimental design as meaningful differences between healthy and diseased tissue.

In addition, the HLCA can provide context to the results of large-scale genetic studies of disease. Genome-wide association studies (GWASs) link disease with specific genomic variants that may confer an increased risk of disease. Previous studies have linked such variants to cell type-specific mechanistic hypotheses, which are often lacking in the initial association study. Yet, these studies fail to include all known lung cell types in their cell type reference[42,43]. To demonstrate the value of the HLCA core in contextualizing genetic data, we mapped association results from four GWASs of lung function or disease[44–47] to the HLCA core cell types, by testing significant enrichment of both weakly and strongly disease-associated variants in regions of genes that characterize each cell type[48] (Fig. 5d, Supplementary Fig. 7 and Methods). We show that genomic variants linked to lung function (forced vital capacity) are associated with smooth muscle (adjusted P value (P_{adj}) = 0.07), alveolar fibroblasts (P_{adj} = 0.07), peribronchial fibroblasts (P_{adj} = 0.07) and myofibroblasts (P_{adj} = 0.07), suggesting that these fibroblast subtypes play a causative role in inherited differences in lung function. We further find a significant association of lung T cells with asthma-associated single-nucleotide polymorphisms (SNPs) (P_{adj} = 0.005). Lung adenocarcinoma-associated variants trend towards AT2 cells (P_{adj} = 0.18) and myofibroblasts are significantly associated with chronic obstructive pulmonary disease (COPD) GWAS SNPs (P_{adj} = 0.04). Thus, by linking genetic predispositions to lung cell types, the HLCA core serves as a valuable resource with which to improve our understanding of lung function and disease.

Finally, the HLCA can be used as a reference for cell type deconvolution of bulk RNA expression samples, which have been shown to reflect cell type proportions more accurately than scRNA-seq datasets[49]. Inferring cell type proportions from bulk RNA samples from nasal brushings and bronchial biopsies using the HLCA core (Supplementary Table 10, Supplementary Fig. 8a and Methods) revealed no significant cell type compositional changes associated with corticosteroid inhalation[50] or asthma[51], respectively (Supplementary Fig. 8b,c and Supplementary Table 11). In contrast, we find that the proportion of capillary endothelial cells in lung resection tissue from the Lung Tissue Database[52] is higher in samples from patients with severe COPD (GOLD stage 3 or 4) than in those from non-COPD controls matched for age and smoking history (P_{adj} = 0.0004). Conversely, alveolar and interstitial macrophages, AT2 cells and dendritic cells decrease in proportion (Fig. 5e, Supplementary Fig. 8d and Supplementary Table 11; P_{adj} = 0.0007, 0.0003, 0.005 and 3.21×10^{-6}, respectively). Finally, smooth muscle shows the largest shift in proportion, increasing significantly in patients with severe COPD ($P = 1.85 \times 10^{-6}$) in line with previous work[53]. As deconvolution of bulk samples using the HLCA can

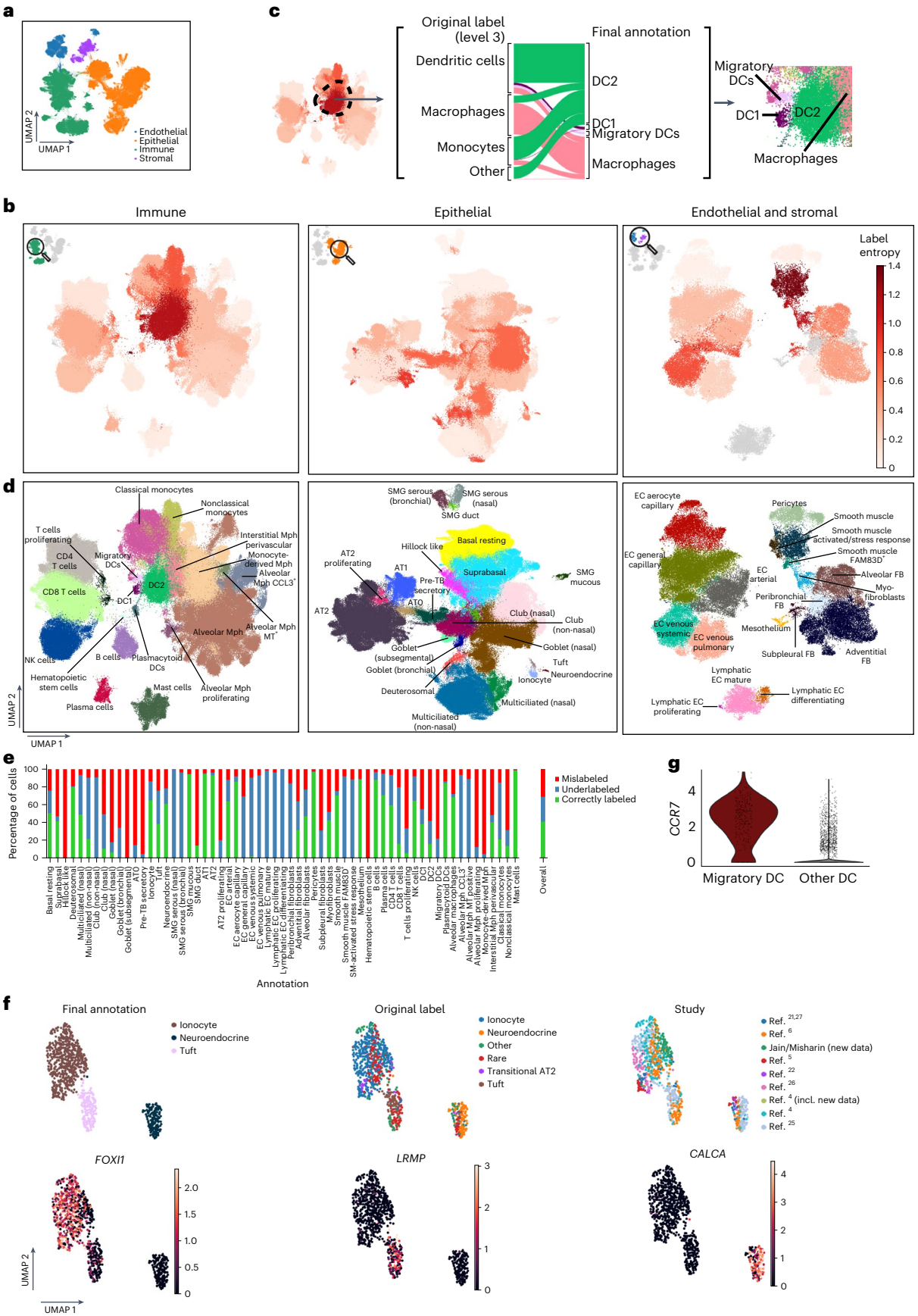
Fig. 3 | The HLCA core conserves detailed biology and enables consensus-driven annotation. a, A UMAP of the integrated HLCA, colored by level 1 annotation. **b**, Cluster label disagreement (label entropy) of Leiden 3 clusters of the HLCA. The HLCA was split into three parts (immune, epithelial and endothelial/stromal) for ease of visualization. Cells from every cluster are colored by label entropy. Clusters with less than 20% of cells annotated at level 3 are colored gray. **c**, Cell type label composition of the immune cluster with the most label disagreement (left), with original labels (middle left) and matching manual re-annotations (middle right). A zoom-in on the UMAP from **b** shows the final re-annotations (right). **d**, UMAPs of the immune, epithelial and endothelial/stromal parts of the HLCA core with cell annotations from the expert manual re-annotation. **e**, Percentage of cells originally labeled correctly, mislabeled or underlabeled (that is, only labeled at a coarser level) compared with final manual re-annotations. The percentages were calculated per manual annotation, as well as across all cells (right bar). **f**, UMAP of HLCA clusters annotated as rare epithelial cell types (that is, ionocytes, neuroendocrine cells and tuft cells). Final annotations, original labels and the study of origin are shown (top), as well as the expression of ionocyte marker *FOXI1*, tuft cell marker *LRMP* and neuroendocrine marker *CALCA* (bottom). **g**, Log-normalized expression of the migratory dendritic cell marker *CCR7* in cells identified during re-annotation as migratory dendritic cells, versus other dendritic cells. AT, alveolar type; DC, dendritic cell; FB, fibroblast; Mph, macrophage; MT, metallothionein; SM, smooth muscle; SMG, submucosal gland; TB, terminal bronchiole.

https://doi.org/10.1038/s41591-023-02327-2

reveal disease-specific changes in cell type composition, we provide publicly available preprocessed cell type signature matrices based on the HLCA core (https://github.com/LungCellAtlas/HLCA).

Extending the HLCA by projecting new data

As knowledge of cell types in the lung expands, and the sizes of newly generated datasets increase, annotations in the HLCA core will need



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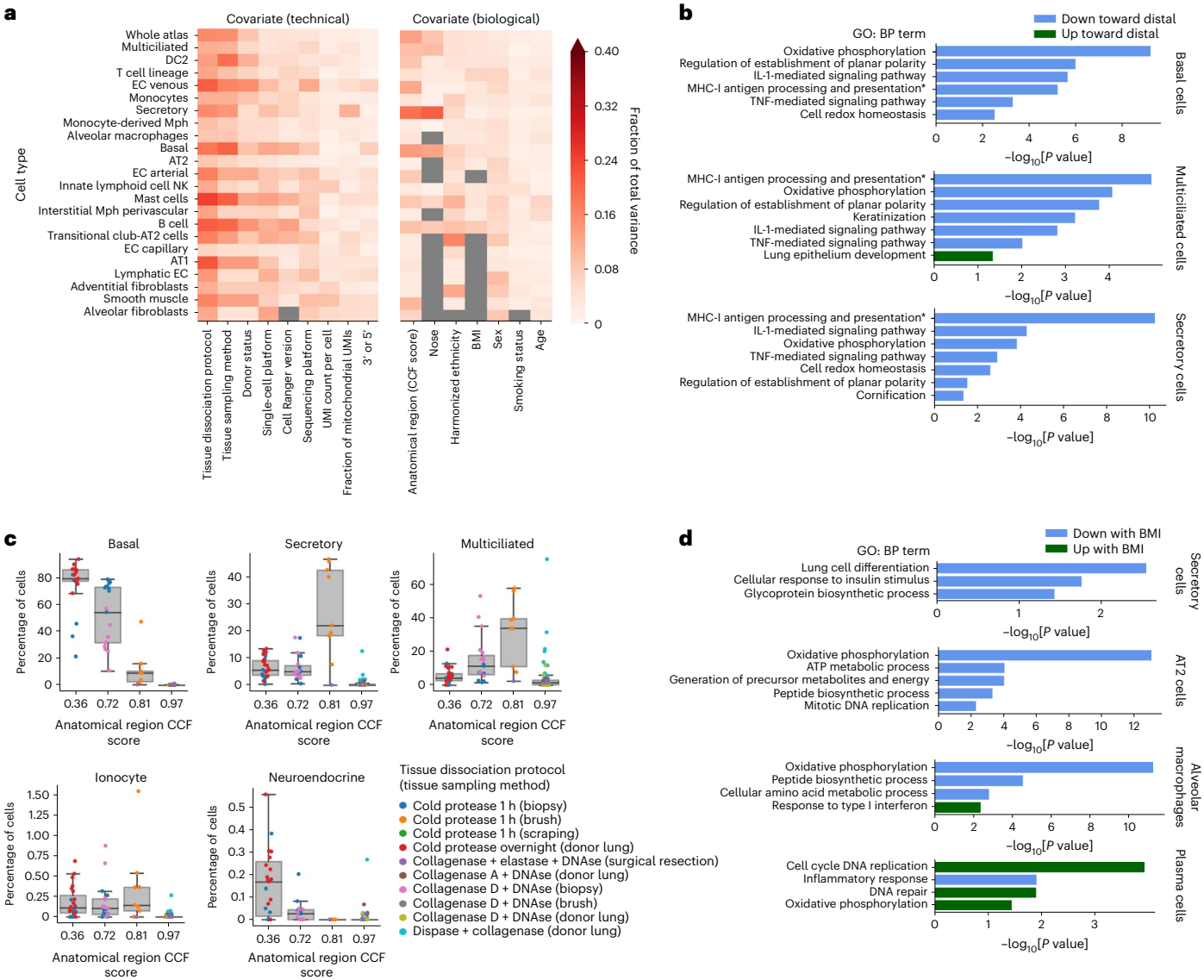


Fig. 4 | Demographic and technical variables driving interindividual variation. a, Fraction of total inter-sample variance in the HLCA core integrated embedding that correlates with specific covariates. Covariates are split into technical (left) and biological covariates (right). Cell types are ordered by the number of samples in which they were detected. Only cell types present in at least 40 samples are shown. Tissue sampling method represents the way a sample was obtained (for example, surgical resection or nasal brush). Donor status represents the state of the donor at the moment of sample collection (for example, organ donor, diseased alive or healthy alive). The heatmap is masked gray where fewer than 40 samples were annotated for a specific covariate or where only one value was observed for all samples for that cell type. **b**, Selection of gene sets that are significantly associated with anatomical location CCF score, in different airway epithelial cell types. All gene set names are Gene Ontology biological process (GO: BP) terms. Sets upregulated toward distal lungs are shown in green, whereas sets downregulated are shown in blue. The full name of the term marked by an asterisk is 'Antigen processing and presentation of exogenous peptide antigen via MHC-I'. **c**, Cell type proportions per sample, along the proximal-to-distal axis of the respiratory system. The lowest and highest CCF scores shown (0.36 and 0.97) represent the most proximal and most distal sampled parts of the respiratory system, respectively (trachea and parenchyma), excluding the upper airways. The dots are colored by the tissue dissociation protocol and tissue sampling method used for each sample. The boxes show the median and interquartile range of the proportions. Samples with proportions more than 1.5 times the interquartile range away from the high and low quartile are considered outliers. Whiskers extend to the furthest nonoutlier point. $n = 23$, 19, 9 and 90 for CCF scores 0.36, 0.72, 0.81 and 0.97, respectively. **d**, Selection of gene sets significantly up- (green) or downregulated (blue) with increasing BMI, in four different cell types. For **b** and **d**, *P* values were calculated using correlation-adjusted mean-rank gene set tests (Methods) and false discovery rate corrected using the Benjamini–Hochberg procedure. IL-1, interleukin-1; MHC-I, major histocompatibility complex class I; TNF, tumor necrosis factor.

to be further refined. The HLCA and its annotations can be updated by learning from new data projected onto the reference. We simulated such an HLCA update using the previously projected healthy lung dataset, specifically focusing on the cell identities that were distinguished based on their tissue location in matched spatial transcriptomic data (spatially annotated cell types)[40]. These cell identities were present at very low frequencies (median: 0.005% of all cells; Supplementary Fig. 9a). Both spatially annotated mesenchymal cell types with more than 40 cells (immune-recruiting fibroblasts and chondrocytes) and two rare cell types (myelinating Schwann cells and perineurial nerve-associated fibroblasts) were recovered in distinct clusters (spatially annotated clusters), and three of these (all except chondrocytes) also contained cells from the HLCA core, thereby enabling a refinement of existing HLCA core annotations using the spatial context from the projected

dataset (Fig. 5f and Supplementary Fig. 9b,c). In this manner the HLCA core and its annotations can be refined by mapping new datasets to the atlas and incorporating annotations from these new datasets into the reference.

Mapping data to the HLCA highlights disease-related states

To extend the atlas and include samples from lung disease, we mapped 1,797,714 cells from 380 healthy and diseased individuals from 37 datasets (four unpublished and 33 published[21,24,26,27,33,40,41,54–70]) to the HLCA core using scArches[71], bringing the HLCA to a total of 2.4 million cells from 486 individuals (Fig. 6a and Supplementary Table 1). Label transfer from the HLCA core to the newly mapped datasets enabled detailed cell type annotation across datasets even for rare cells, including 2,048 migratory dendritic cells identified across 28 datasets with label transfer, whereas this cell type was originally labeled in only two of 12 labeled datasets (Extended Data Fig. 8).

Out of 37 new datasets, 27 were observed to map well to the HLCA, as evaluated by the mean label transfer uncertainty score (Fig. 6b, Supplementary Fig. 10a and Methods). The remaining ten datasets were often from coronavirus disease 2019 (COVID-19) studies or, unlike the HLCA core, contained pediatric samples (Fig. 6b,c and Supplementary Fig. 10b). In these datasets, higher uncertainty values may be attributable to true biological differences between the mapped data and the HLCA core adult, healthy lung samples. Overall, the successfully mapped datasets include disease samples, as well as single-nucleus and single-cell data from multiple chemistries (Fig. 6b), demonstrating the potential of the HLCA core as a universal reference.

Pulmonary diseases are characterized by the emergence of unique disease-associated transcriptional phenotypes[4,21,22,24,72]. We observed higher levels of label transfer uncertainty in datasets from diseased lungs (Fig. 6b, condition), possibly flagging cell types changed in response to disease. Specifically, labels of alveolar fibroblasts and alveolar macrophages, which interact to form a dysregulated cellular circuit in idiopathic pulmonary fibrosis (IPFs)[21,22,24], are transferred with higher uncertainty in IPF samples than in samples from healthy controls from the same dataset[64] (Fig. 6d and Extended Data Fig. 9a,b). Furthermore, uncertainty scores separate cells—derived from donors with IPF—within these cell types into more and less affected subsets: the genes more highly expressed in the high-uncertainty subset are also lowly expressed in healthy samples (Fig. 6e). Genes downregulated in high-uncertainty IPF macrophages are associated with homeostatic functions of tissue-resident alveolar macrophages and lipid metabolism (*PPARG*, *FABP4* and others)[22,24,58], while upregulated genes are associated with extracellular matrix remodeling and scar formation in the context of lung fibrosis (*SPP1*, *PLA2G7* and *CCL2*; Supplementary Tables 12 and 13 and Extended Data Fig. 9b,c)[22,24,58]. Thus, the HLCA core can be used to annotate new data, identify previously unreported populations, and—using label transfer uncertainty scores—help to detect disease-affected cell states and corresponding gene expression programs. This vastly speeds up analysis and interpretation of new data, automatically prioritizing the most relevant populations. Automated mapping of new data to the HLCA core can be done by any user via an interactive web portal (https://github.com/LungCellAtlas/HLCA) or using code tutorials as provided online.

The HLCA reveals common aberrant cell states across diseases

Similar to healthy cellular states, the HLCA can provide insight into disease-specific states that are consistent across demographics and experimental protocols. To demonstrate this, we determined which cell types are consistently affected by IPF across datasets, extending the previous IPF analysis to five independent datasets. We found that cells labeled as alveolar fibroblasts consistently show high uncertainty levels in IPF samples compared with controls across all mapped IPF datasets that include controls[58,62,64] (Extended Data Fig. 10a). Clustering of alveolar fibroblasts from the HLCA core and all IPF datasets[21,24,58,62,64] shows that cells from patients with IPF predominantly cluster together in a single cluster (Fig. 6f,g and Extended Data Fig. 10b) characterized by high expression of genes previously associated with IPF[64,73,74] (*CCL2*, *COL1A1*, *CTHRC1* and *MMP19*), as well as further fibrosis-associated markers (*SERPINE1*, an inhibitor of extracellular matrix breakdown[75], and *HIF1A*, a chronic hypoxia response gene[76]; Fig. 6h and Supplementary Table 14). These marker genes are consistently expressed across datasets (Extended Data Fig. 10c), confirming that the identification of this IPF-specific alveolar fibroblast state is reproducible.

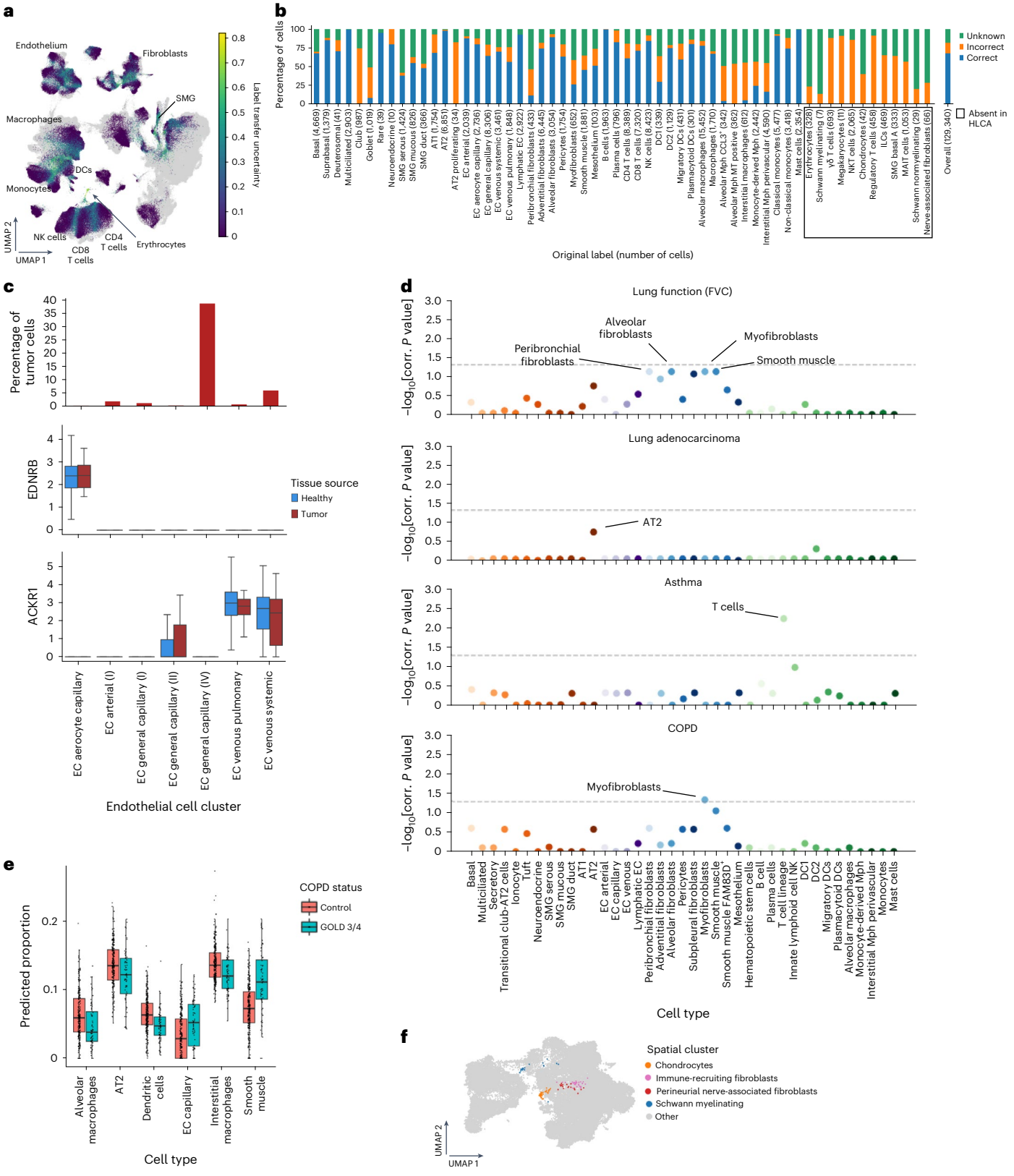
The HLCA contains data across more than ten lung diseases, providing the unique opportunity to discover cellular states shared across diseases. Discovering such common diseased cellular states could improve our understanding of lung diseases and accelerate the identification of effective treatments. For example, profibrotic *SPP1*$^{+}$ monocyte-derived macrophages (MDMs) have previously been reported in COVID-19, IPF and cancer[26,77,78]. To test whether similar cross-disease MDM states could be discovered in the HLCA, we performed clustering of all MDMs from the HLCA (Fig. 6i). We identified four main MDM subtypes (Methods and Supplementary Table 15), each showing distinct gene expression and disease enrichment patterns, and representing different stages of monocyte-to-MDM differentiation and adaptation to the disease microenvironment. First, an early and inflammatory MDM state was observed that was high in the expression of *CCL2*, a gene involved in the recruitment of immune cells. This cluster predominantly contained cells from bronchoalveolar lavage fluid samples collected early during the course of COVID-19 pneumonia (cluster 2; *IL1RN*^{high} and *S100A12*^{high}; Fig. 6i–k and Extended Data Fig. 10d–h). We further observed an MDM subset expressing inflammation and phagocytosis-associated genes (cluster 4; *CCL18*,

Fig. 5 | The HLCA core serves as a reference for label transfer and data contextualization. a, UMAP of the jointly embedded HLCA core (gray) and the projected healthy lung dataset (colored by label transfer uncertainty). HLCA cell types surrounding regions of high uncertainty are labeled. **b**, Percentage of cells from the newly mapped healthy lung dataset that are annotated either correctly or incorrectly by label transfer annotation or annotated as unknown, split by original cell type label (number of cells in parentheses). Cell type labels not present in the HLCA are boxed. **c**, Top, percentage of cells derived from tumor tissue, per endothelial cell cluster from the joint HLCA core and lung cancer data embedding. Only clusters with at least ten tumor cells are shown. Clusters are named based on the dominant HLCA core cell type annotation in the cluster. Middle, box plot showing the expression of *EDNRB* in endothelial cell clusters, split by tissue source. Bottom, as in the middle plot but for the expression of *ACKR1*. Numbers of cells per group were as follows: 6,574 (endothelial cell aerocyte capillary), 7,379 (endothelial cell arterial (I)), 10,906 (endothelial cell general capillary (I)), 3,440 (endothelial cell general capillary (II)), 2,859 (endothelial cell general capillary (III)), 6,318 (endothelial cell venous pulmonary) and 7,161 (endothelial cell venous systemic). **d**, Association of HLCA cell types with four different lung phenotypes based on previously performed GWASs. The horizontal dashed lines indicate a significance threshold of $\alpha = 0.05$. *P* values were calculated using linkage disequilibrium score regression (Methods) and multiple testing corrected with the Benjamini–Hochberg procedure. **e**, Cell type proportions in lung bulk expression samples as estimated from HLCA-based cell type deconvolution, comparing controls ($n = 281$) versus donors with severe COPD (GOLD stage 3/4; $n = 83$). **f**, UMAP of fibroblast-dominated clusters from the jointly embedded HLCA core and mapped healthy lung dataset, colored by spatial cluster, with cells outside of the indicated clusters colored in gray. For all boxplots, the boxes show the median and interquartile range. Data points more than 1.5 times the interquartile range outside the low and high quartile are considered outliers. In **c**, these are not shown (see Supplementary Fig. 6 for full results), whereas in **e**, they are shown. Whiskers extend to the furthest nonoutlier point. corr., corrected; FVC, forced vital capacity; MAIT cells, mucosal-associated invariant T cells; NKT cells, natural killer T cells.

https://doi.org/10.1038/s41591-023-02327-2

IL18, *C1QA* and *TREM2*) and enriched for samples from patients with COVID-19 pneumonia, as well as samples from patients with lung carcinoma (Fig. 6i–k and Extended Data Fig. 10d–h). A third MDM subset represented a more differentiated MDM phenotype, as indicated by the expression of *MARCO* and *MCEMP1*, dominated by cells from non-diseased samples (cluster 3; Fig. 6i–k and Extended Data Fig. 10d,f).

The final MDM subset was dominated by IPF samples. Interestingly, this cluster was also enriched for cells from patients who died late in the course of COVID-19 and developed post-COVID-19 lung fibrosis, as well as cells from patients with lung carcinoma (cluster 0; Fig. 6i–k and Extended Data Fig. 10g–i). This multidisease cluster is marked by high expression of *SPP1*, *LPL* and *CHIT1*—markers that have been shown



https://doi.org/10.1038/s41591-023-02327-2

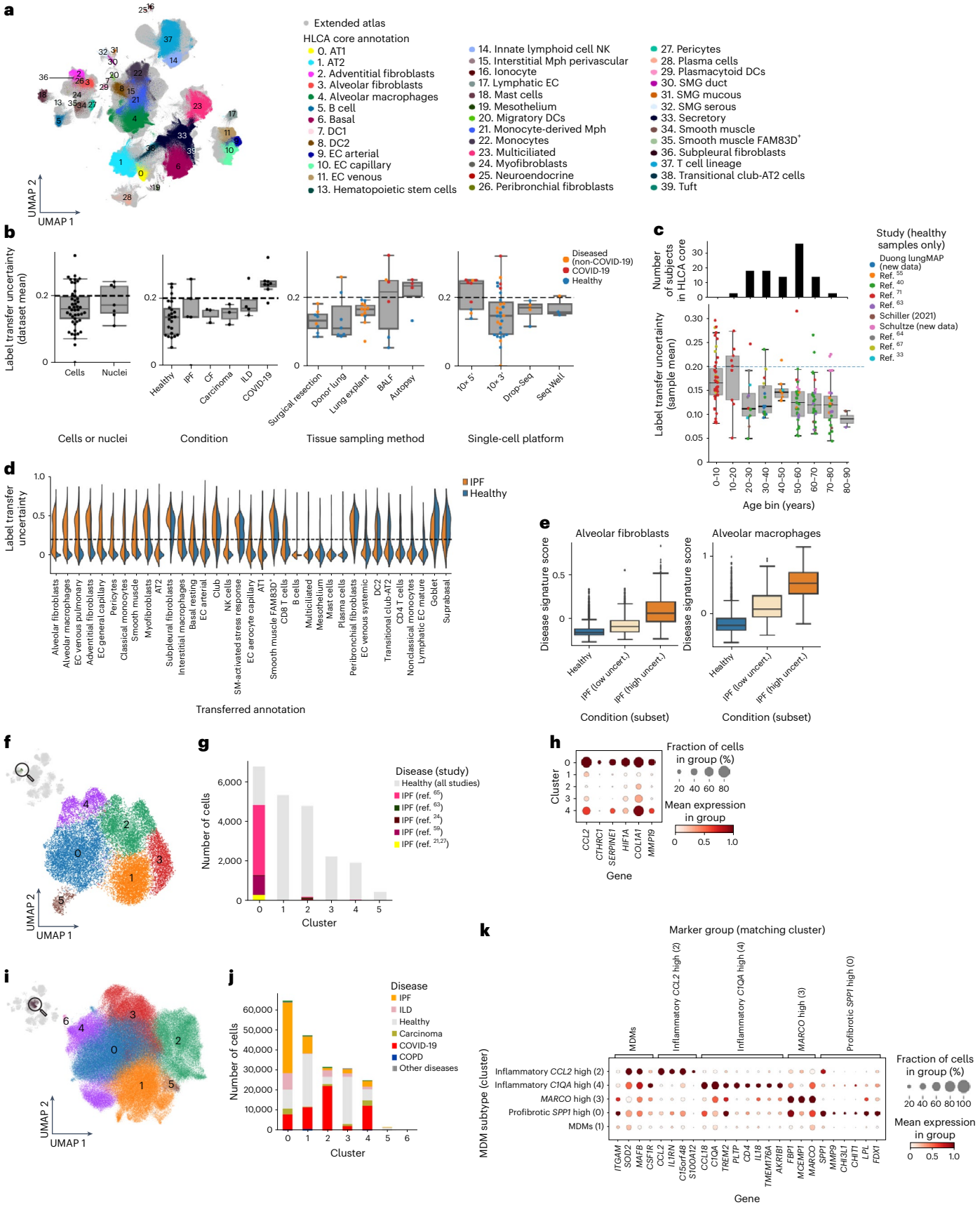


Fig. 6 | The extended HLCA enables the identification of disease-associated cell states. a, UMAP of the extended HLCA colored by coarse annotation (HLCA core) or in gray (cells mapped to the core). **b**, Uncertainty of label transfer from the HLCA core to newly mapped datasets, categorized by several experimental or biological features. Categories with fewer than two instances are not shown. The numbers of datasets per category were as follows: 30 cells, 7 nuclei, 23 healthy, 5 IPF, 3 CF, 3 carcinoma, 4 ILD, 8 surgical resection, 7 donor lung, 12 lung explant, 6 bronchoalveolar lavage fluid, 4 autopsy, 9 10x 5′, 31 10x 3′, 4 Drop-Seq and 3 Seq-Well. **c**, Bottom, mean label transfer uncertainty per mapped healthy lung sample in the HLCA extension, grouped into age bins and colored by study. The numbers of mapped samples per age bin were as follows: 43 for 0–10 years, 33 for 10–20 years, 31 for 20–30 years, 23 for 30–40 years, 19 for 40–50 years, 12 for 50–60 years, 9 for 60–70 years, 8 for 70–80 years and 2 for 80–90 years. Top, bar plot showing the number of donors per age group in the HLCA core. **d**, Violin plot of label transfer uncertainty per transferred cell type label for a single mapped IPF dataset[64], split into cells from healthy donors (blue) and donors with IPF (orange). **e**, Uncertainty-based disease signature scores among alveolar fibroblasts and alveolar macrophages, split into cells from control donors (n = 10,453 and 1,812, respectively), and low-uncertainty cells (n = 1,419 and 200, respectively) and high-uncertainty cells (n = 1,172 and 162, respectively) from donors with IPF. **f**, UMAP embedding of alveolar fibroblasts (labeled with manual annotation (core) or label transfer (five IPF datasets)) colored by Leiden cluster. **g**, Composition of the clusters shown in **f** by study, with cells from control samples colored in gray. **h**, Expression of marker genes for IPF-enriched cluster 0 per alveolar fibroblast cluster. Cluster 5 was excluded as 96% of its cells were from a single donor. **i**, UMAP of all MDMs in the HLCA, colored by Leiden cluster. **j**, Composition of the MDM clusters from **i** by disease. **k**, Expression of cluster marker genes among all MDM clusters excluding donor-specific clusters 5 and 6. For **h** and **k**, mean counts were normalized such that the highest group mean was set to 1 for each gene. For **b**, **c** and **e**, the boxes show the median and interquartile range. Data points more than 1.5 times the interquartile range outside the low and high quartile are considered outliers. Whiskers extend to the furthest nonoutlier point. BALF, bronchoalveolar lavage fluid; CF, cystic fibrosis; Drop-Seq, droplet sequencing; ILD, interstitial lung disease; Mph, macrophages; SM, smooth muscle; uncert., uncertainty.

to play a causal role in the development of lung fibrosis[22,79–81] (Fig. 6k), one of which (*CHIT1*) is currently being investigated as a therapeutic target for IPF[82]. The expression of these markers is consistent across diseases and studies (Extended Data Fig. 10f), suggesting that also in cancer and late-stage COVID-19 samples a subset of MDMs adopt a fibrosis-associated phenotype. Together, this analysis shows that the HLCA enables a better understanding of cellular states shared between diseases and thereby has the potential to accelerate the discovery of effective disease treatments.

Discussion

In this study, we built the HLCA: an integrated reference atlas of the human respiratory system. While previous studies have described the cellular heterogeneity within the human lung[4–6,24,58], study-specific biases due to experimental design and a limited number of sampled individuals constrain their capacity to capture population variation and serve as a universal reference. The HLCA integrates data from 49 datasets to produce such a reference of 2.4 million cells, covering all major lung scRNA-seq studies published to date. The core of this atlas consists of a fully integrated healthy reference of 14 datasets with 61 cell identities, including rare and novel cell types, representing a data-derived consensus annotation of the cellular landscape of the human lung. We leveraged the unprecedented complexity of the HLCA to recover cell type-specific gene modules associated with covariates such as lung anatomical location, age, sex, BMI and smoking status. By projecting data onto the HLCA, we showed that the HLCA enables a fast and detailed annotation of new datasets, as well as the identification of unique, disease-associated cell states and cell states common to multiple diseases. The HLCA is publicly available as a resource for the community, together with an online platform for automated mapping of new data. Taken together, the HLCA is a universal reference for single-cell lung research that promises to accelerate future studies into pulmonary health and disease.

The ultimate goal of a human lung cell atlas reference is to provide a comprehensive overview of all cells in the healthy human lung, as well as their variation from individual to individual. Despite its overall diversity, the HLCA is limited by the biological, demographic and experimental diversity in the foundational single-cell studies. For example, 65% of the HLCA core data are from individuals of European harmonized ethnicity, highlighting the urgent need for diversification of the population sampled in lung studies. Moreover, ethnicity metadata were based on self-reports and harmonized across datasets, which is an imperfect approach to representing the diversity of the atlas. SNP-based inference of genetic ancestry constitutes a more objective and therefore preferable approach to the grouping of individuals based on genetic background and would aid in better assessing the genetic diversity captured in the atlas. Overall, more diverse samples will enrich the atlas, diversify captured cell identities and improve the quality of the HLCA as a reference for new datasets. Such a reference will also enable comparison with model systems such as mice, cell lines or organoids, although further method development may be required to map across diverse in vitro and clinical datasets.

The constituent datasets of the HLCA vary widely in experimental design, such as the sample handling protocol or single-cell platform used, causing dataset-specific batch effects. The quality of the HLCA hinges on the choice of data integration method, with methods such as Seurat's RPCA[30] and Harmony[29] failing to correctly group rare cell identities into separate clusters. Nevertheless, also in the HLCA, certain subsets of T cells (regulatory T cells and γδ T cells) could not be identified as separate clusters, showing the limitations of the current HLCA in capturing cellular heterogeneity for a subset of immune cell types. Mapping additional datasets with high-resolution annotations (for example, derived from multimodal data) could provide the power to detect these cell identities in the atlas. Indeed, the HLCA must be viewed as a live resource that requires continuous updates. While we showed that mapping new, spatially annotated data to the HLCA core can refine HLCA annotations, this new knowledge must be consolidated by regular updates of the HLCA with new datasets (including epigenomic, spatial and imaging data) and refinements of HLCA annotations based on additional expert opinions. Thereby, the HLCA can serve as a community- and data-driven platform for open discussion on lung cell identities as the respiratory community progresses in charting the cellular landscape of the lung. In this process, we envision that the HLCA will be completed in two phases: first on the level of cellular variation (when no new consensus cell types can be found) and then in the description of individual variation (when population diversity is fully represented).

Taken together, the HLCA provides a central single-cell reference of unprecedented size. It offers a model framework for building integrated, consensus-based, population-scale atlases for other organs within the Human Cell Atlas. The HLCA is publicly available, and combined with an open-access platform to map new datasets to the atlas, this resource paves the way toward a better and more complete understanding of both health and disease in the human lung.

Online content

Any methods, additional references, Nature Portfolio reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41591-023-02327-2.

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Methods

Ethics approval and consent

Ethics approval information per study was as follows. For the pooled data from refs. 21,27, approval was given by the Vanderbilt Institutional Review Board (IRB) (numbers 060165 and 171657) and Western IRB (number 20181836). All samples were collected from declined organ donors who were also consented for research. For ref. 6, the study was approved by the Comité de Protection des Personnes Sud Est IV (approval number 17/081). Informed written consent was obtained from all participants involved. For Jain_Misharin_2021 (A.V.M., M.J. and N.S.M., newly generated dataset), the protocol was approved by the Northwestern University IRB (STU00214826). Written informed consent was obtained from all study participants. For ref. 5, patient tissues were obtained under a protocol approved by Stanford University's Human Subjects Research Compliance Office (IRB 15166). Informed consent was obtained from each patient before surgery. For ref. 22, healthy control lungs were obtained under a protocol approved by the University of Pittsburgh Committee for Oversight of Research and Clinical Training Involving Decedents (CORID protocol 718) and following rejection as candidate donors for transplant (IRB STUDY 19100326). For ref. 23, tissue samples were obtained from the Cambridge Biorepository for Translational Medicine (CBTM) with approval from the National Research Ethics Services (NRES) Committee of East of England−Cambridge South (15/EE/0152). Tissue samples were obtained with informed consent from the donor families. For ref. 26, the protocol was approved by the Northwestern University IRB (STU00212120). Written informed consent was obtained from all individuals in the study. For the pooled data from ref. 4 and associated unpublished data, the protocol was approved by the IRB (Algemeen Beoordelings- en Registratieformulier number NL69765.042.19). Patients gave informed consent. For ref. 25, the National Jewish Health IRB approved the research under IRB protocols HS-3209 and HS-2240. Informed consent was obtained from authorized family members of all donors. For ref. 4, approval was given by the NRES Committee of East of England−Cambridge South (Research Ethics Committee (REC) reference: 15/EE/0152). Informed consent for use of the tissue was obtained from the donors' families. For Barbry_unpubl (P.B., L.-E.Z., M.J.A., A.C., C.B. et al., newly generated dataset), the protocol was approved by the Centre Hospitalier Universitaire de Nice. Nasal and tracheobronchial samples were collected from patients with IPF after obtaining their informed consent. For ref. 26, approved was given by the IRB of Northwestern University (STU00212120, STU00213177, STU00212511 and STU00212579). For inclusion in this study, patients or their designated medical power of attorney provided informed consent. For Duong_lungMAP_unpubl (T.E.D., K.Z., X.S., J.S.H. and G.P., newly generated dataset), all postmortem human donor lung samples were obtained from the Biorepository for Investigation of Neonatal Diseases of the Lung (BRINDL), supported by the National Heart, Lung, and Blood Institute (NHLBI) LungMAP Human Tissue Core housed at the University of Rochester. Consent can be found on the repository's website (brindl.urmc.rochester.edu/). For ref. 54, the study was conducted in accordance with the Declaration of Helsinki and Department of Health and Human Services Belmont Report. The use of biomaterial and data for this study was approved by the local ethics committee of the Medical Faculty Heidelberg (S-270/2001 and S-538/2012). All individuals gave informed consent for inclusion before they participated in the study. For ref. 55, human lung tissues were procured under each institution's approved IRB protocol (numbers 00035396 (Cedars-Sinai Medical Center), 03-1396 (University of North Carolina at Chapel Hill), 1172286 (Cystic Fibrosis Foundation and WIRB-Copernicus Group Western IRB) and 16-000742 (University of California, Los Angeles)). Informed consent was obtained from lung donors or their authorized representatives. For ref. 57, the study was approved and monitored by the National Jewish Health IRB (FWA00000778). Written informed consent was obtained from all participants. For ref. 58, the study protocol was approved by the Partners Healthcare IRB (protocol 2011P002419). For ref. 60, lung tissue was obtained under a protocol approved by the University of Pittsburgh IRB (IRB STUDY 19100326) during transplantation surgery. For ref. 59, the study was conducted according to the principles expressed in the Declaration of Helsinki. Ethical approval was obtained from Ethics Committee Research UZ/KU Leuven (S63881). All participants provided written informed consent for sample collection and subsequent analyses. For ref. 40, approval was given by the NRES Committee of East of England−Cambridge South (15/EE/0152). The CBTM operates in accordance with UK Human Tissue Authority guidelines. Samples were obtained from deceased transplant organ donors by the CBTM with informed consent from the donor families. For ref. 70, ethical approval was given through the Living Airway Biobank, administered through the University College London Great Ormond Street Institute of Child Health (REC reference: 19/NW/0171; Integrated Research Application System (IRAS) project ID: 261511; North West Liverpool East REC), REC reference 18/SC/0514 (IRAS project ID: 245471; South Central Hampshire B REC; administered through the University College London Hospitals NHS Foundation Trust), REC reference 18/EE/0150 (IRAS project ID: 236570; East of England−Cambridge Central REC; administered through Great Ormond Street Hospital NHS Foundation Trust) and REC reference 08/H0308/267 (administered through the Cambridge University Hospitals NHS Foundation Trust), as well as by the local R&D departments at all hospitals. All of the study participants or their surrogates provided informed consent. For ref. 61, all protocols were reviewed and approved by the IRB at the Memorial Sloan Kettering Cancer Center (IRB protocol 14-091). Noninvolved lung, tumor tissues and metastatic lesions were obtained from patients with lung adenocarcinoma undergoing resection surgery at the Memorial Sloan Kettering Cancer Center after obtaining informed consent. For ref. 69, samples underwent IRB review and approval at the institutions where they were originally collected. Specifically, the Dana-Farber Cancer Institute approved protocol 13-416, the partners Massachusetts General Hospital and Brigham and Women's Hospital approved protocols 2020P000804, 2020P000849 and 2015P002215, the Beth Israel Deaconess Medical Center approved protocols 2020P000406 and 2020P000418 and New York Presbyterian Hospital/Columbia University Irving Medical Center approved protocols IRB-AAAT0785, IRB-AAAB2667 and IRB-AAAS7370. Secondary analysis of samples at the Broad Institute was covered under Massachusetts Institute of Technology IRB protocols 1603505962 and 1612793224, or the Not Human Subjects Research protocol ORSP-3635. Donor identities were encoded at the hospitals before shipping to or sharing with the Broad Institute for sample processing or data analysis, respectively. For ref. 62, the study was approved by the local ethics committee of the Ludwig Maximilian University of Munich (EK 333-10 and 382-10). Written informed consent was obtained from all patients. For Schiller_2021 (H.B.S., J.G.-S., C.H.M., B.H.K., M.A. et al., newly generated dataset), the study was approved by the local ethics committee of the Ludwig Maximilian University of Munich (EK 333-10 and 382-10). Written informed consent was obtained from all patients. For Schultze_unpubl (J.L.S., C.S.F., T.S.K. and E.C., newly generated dataset), human lung tissue was available for research purposes following ethical approval from Hannover Medical School (ethical vote of the German Centre for Lung Research (DZL) number 7414, 2017). All patients in this study provided written informed consent for sample collection and data analysis. For ref. 63, samples were obtained under the Cells and Mediators IRB protocol (2003P002088). All individuals provided written informed consent. For ref. 64, the studies described were conducted according to the principles of the Declaration of Helsinki. The study was approved by the University of California, San Francisco IRB. Written informed consent was obtained from all individuals. For ref. 65, peripheral blood was obtained from healthy consenting adult volunteers by venipuncture through a protocol approved by the Columbia University IRB. All relevant ethical regulations for work with

human participants were complied with. For ref. 66, donor lung samples were provided through the federal United Network for Organ Sharing via the National Disease Research Interchange and International Institute for the Advancement of Medicine and entered into the NHLBI LungMAP BRINDL at the University of Rochester Medical Center, overseen by the IRB as RSRB00047606. For for the pooled data from ref. 33 and associated unpublished data, human lung tissue collection was approved by the Duke University IRB (Pro00082379) and University of North Carolina Biomedical IRB (03-1396) under exempt protocols. Consent was obtained to use human tissues for research purposes. For ref. 41, the study was approved by the local ethics committee at University Hospitals Leuven (B322201422081) and all of the relevant ethical regulations were complied with. Only patients with untreated, primary, nonmetastatic lung tumors who underwent lung lobe resection with curative intent and who provided informed consent were included in this study. For ref. 67, all of the research involving human participants was approved by the Northwestern University IRB. Samples from patients with COVID-19, viral pneumonia and other pneumonia, as well as controls without pneumonia, were collected from participants enrolled in the Successful Clinical Response in Pneumonia Therapy study STU00204868. All study participants or their surrogates provided informed consent. For ref. 56, the IRB of the University of Cincinnati College of Medicine approved all human-relevant studies. For ref. 68, the study was conducted according to the principles expressed in the Declaration of Helsinki. Ethical approval was obtained from the REC of Shenzhen Third People's Hospital (2020-112). All participants provided written informed consent for sample collection and subsequent analyses. Further study details can be found in Supplementary Table 1.

Single-cell sequencing and preprocessing of data

Several previously unpublished datasets were used for the HLCA and generated as follows.

Barbry_unpubl. Participants recruited by the Pneumology Unit of Nice University Hospital were sampled between 1 and 15 December 2020. The full procedure, including patient inclusion criteria, is detailed at https://www.clinicaltrials.gov/ct2/show/NCT04529993. Nasal and tracheobronchial samples were collected from patients with IPF after obtaining their informed consent, following a protocol approved by the Centre Hospitalier Universitaire de Nice. The data were derived from the clinical trial registered at ClinicalTrials.gov under reference NCT04529993. This study was described as an interventional study instead of an observational study because the participants were volunteers and all assigned to a specific bronchoscopy not related to routine medical care. Participants were prospectively assigned to a procedure (bronchoscopy) according to a specific protocol to assess our ability to sample the airway. No other procedures were included in this study. Metadata of the donors' sex was based on self-report. The libraries were prepared as described in Deprez et al.[6] and yielded an average of 61,000 ± 11,000 cells per sample, with a viability above 95%. The single-cell suspension was used to generate single-cell libraries following the v3.1 protocol for 3′ chemistry from 10x Genomics (CG000204). Sequencing was performed on a NextSeq 500/550 sequencer (Illumina). Raw sequencing data were processed using the Cell Ranger 6.0.0 pipeline, with the reference genome GRCh38 and annotation using Ensembl98. For each sample, cells with fewer than 200 transcripts or more than 40,000 transcripts were filtered out, as well as genes expressed in fewer than three cells. Normalization and log transformation were done using the standard Scanpy[83] pipeline. Principal component analysis (PCA) was performed on 1,000 highly variable genes (HVGs) to compute 50 principal components, and the Louvain algorithm was used for clustering. These clusters were then annotated by hand for each sample. Raw counts and the thus obtained cell annotations were used as input for the HLCA.

Schiller_2021. Tumor-free, uninvolved lung samples (peritumor tissues) were obtained during tumor resections at the lung specialist clinic Asklepios Fachkliniken München-Gauting and accessed through the bioArchive of the Comprehensive Pneumology Center in Munich. The study was approved by the local ethics committee of the Ludwig Maximilian University of Munich (EK 333-10 and 382-10), and written informed consent was obtained from all patients. All fresh tissues from patients in a given time frame without any specific selection criteria were included, and only patients with obvious chronic lung disease as comorbidity based on their lung function parameters before tumor resection were excluded. Metadata of the donors' sex were based on self-report.

Single-cell suspensions for scRNA-seq were generated as previously described[62]. In brief, lung tissue samples were cut into smaller pieces, washed with phosphate-buffered saline (PBS) and enzymatically digested using an enzyme mix composed of dispase, collagenase, elastase and DNAse for 45 min at 37 °C while shaking. After inactivating the enzymatic activity with 10% fetal calf serum (FCS)/PBS, dissociated cells were passed through a 70 µm cell strainer, pelleted by centrifugation (300*g*; 5 min) and subjected to red blood cell lysis. After stopping the lysis with 10% FCS/PBS, the cell suspension was passed through a 30 µm strainer and pelleted. Cells were resuspended in 10% FCS/PBS, assessed for viability and counted using a Neubauer hematocytometer. The cell concentration was adjusted to 1,000 cells per µl and ~16,000 cells were loaded on a 10x Genomics Chip G with Chromium Single Cell 3′ v3.1 gel beads and reagents (3′ GEX v3.1; 10x Genomics). Libraries were prepared according to the manufacturer's protocol (CG000204_RevD; 10× Genomics). After a quality check, scRNA-seq libraries were pooled and sequenced on a NovaSeq 6000 instrument.

The generation of count matrices was performed using the Cell Ranger computational pipeline (v3.1.0; STAR v2.5.3a). The reads were aligned to the GRCh38 human reference genome (GRCh38; Ensembl99). Downstream analysis was performed using the Scanpy[83] package (version 1.8.0). We assessed the quality of our libraries and excluded barcodes with fewer than 300 genes detected, while retaining those with a number of transcripts between 500 and 30,000. Furthermore, cells with a high proportion (>15%) of transcript counts derived from mitochondrial-encoded genes were removed. Genes were considered if they were expressed in at least five cells. Raw counts of cells that passed filtering were used as input for the HLCA.

Duong_lungMAP_unpubl. All postmortem human donor lung samples were obtained from BRINDL, supported by the NHLBI LungMAP Human Tissue Core housed at the University of Rochester. Consent, tissue acquisition and storage protocols can be found on the repository's website (brindl.urmc.rochester.edu/). Data were collected as part of the Human Biomolecular Atlas Program (HuBMAP). Metadata of the donor's sex were based on self-report. For isolation of single nuclei, ten cryosections (40 µm thickness) from O.C.T.-embedded tissue blocks stored at −80 °C were shipped on dry ice and processed according to a published protocol[84]. Single-nucleus RNA-seq was completed using 10x Chromium Single Cell 3′ Reagent Kits v3, according to a published protocol[84,85]. Raw sequencing data were processed using the 10x Cell Ranger v3 pipeline and the GRCh38 reference genome. For downstream analysis, mitochondrial transcripts and doublets identified by DoubletDetection[86] version 2.4.0 were removed. Samples were then combined and cell barcodes were filtered based on the genes detected (>200 and <7,500) and the gene unique molecular identifier (UMI) ratio (gene.vs.molecule.cell.filter function) using Pagoda2 (github.com/hms-dbmi/pagoda2). Also using Pagoda2 for clustering, counts were normalized to total counts per nucleus. For batch correction, gene expression was scaled to dataset average expression. After variance normalization, all significantly variant genes (n = 4,519) were used for PCA. Clustering was done at different k values (50, 100 or 200) using the top 50 principal components and the infomap community detection

algorithm. Then, principal component and cluster annotations were imported into Seurat[30] version 4.0.0. Differentially expressed genes for all clusters were generated for each *k* resolution using Seurat FindAllMarkers (only.pos = TRUE, max.cells.per.ident = 1000, logfc.threshold = 0.25, min.pct = 0.25). Clusters were manually annotated based on distinct differentially expressed marker genes. Raw counts and the thus obtained cell annotations were used as input for the HLCA.

Pooled data from ref. 4 and unpublished data. These data were a combination of published[4] and unpublished data. In both cases, healthy volunteers were recruited for bronchoscopy at the University Medical Center in Groningen after giving informed consent and according to the protocol approved by the IRB (ABR number NL69765.042.19). Inclusion criteria and tissue processing were performed as previously described[4]. In short, all donors were 20–65 years old and had a history of smoking <10 pack-years. Metadata of the donors' sex were based on self-report. To exclude respiratory disease, the following criteria were used: absent history of asthma or COPD; no use of asthma- or COPD-related medication; a negative provocation test (concentration of methacholine that provokes a 20% decrease in the forced expiratory volume in 1 s (FEV_1) > 8 mg ml^{-1}); no airflow obstruction (FEV_1/forced vital capacity ≥ 70%); and an absence of lung function impairment (that is, FEV_1 ≥ 80% predicted). All donors underwent a bronchoscopy under sedation using a standardized protocol[87]. Nasal brushes were obtained from the lateral inferior turbinate in a subset of the volunteers immediately before bronchoscopy using a Cyto-Pak CytoSoft nasal brush (Medical Packaging Corporation). Six macroscopically adequate endobronchial biopsies were collected for this study, located between the third and sixth generation of the right lower and middle lobe. Bronchial brushes were obtained from a different airway at similar anatomical locations using a Cellebrity bronchial brush (Boston Scientific). Extracted biopsies and bronchial and nasal brushes were processed directly, with a maximum of 1 h delay. Bronchial biopsies were chopped biopsies using a single-edge razor blade. A single-cell solution was obtained by tissue digestion using 1 mg ml^{-1} collagenase D and 0.1 mg ml^{-1} DNase I (Roche) in Hanks' Balanced Salt Solution (Lonza) at 37 °C for 1 h with gentle agitation for both nasal brushes and bronchial biopsies. Single-cell suspensions were filtered and forced using a 70 µm nylon cell strainer (Falcon), followed by centrifugation at 550*g* and 4 °C for 5 min and one wash with PBS containing 1% bovine serum albumin (BSA; Sigma–Aldrich). The single-cell suspensions used for 10x Genomics scRNA-seq analysis were cleared of red blood cells using a red blood cell lysis buffer (eBioscience) followed by live cell counting and loading of 10,000 cells per lane. We used 10x Genomics Chromium Single Cell 3′ Reagent Kits v2 and v3 according to the manufacturers' instructions. Raw sequencing data were processed using the Cell Ranger 3.1.0-based HLCA pipeline, with the reference genome GRCh38 and annotation using Ensembl98. Ambient RNA correction was performed with FastCAR[88], using an empty library cutoff of 100 UMI and a maximum allowed contamination chance of 0.05, ignoring the mitochondrial RNA. Data were merged and processed using Seurat[30], filtering to libraries with >500 UMIs and >200 genes and to the libraries containing the lowest 95% of mitochondrial RNA per sample and <25% mitochondrial RNA, normalized using sctransform[89] while regressing out variation correlating with the percentage of mitochondrial RNA per cell. In general, 15 principal components were used for the clustering, at a resolution of 0.5 to facilitate manual annotation of the dataset. Clusters in the final object that were driven by single donors were removed. Raw counts and cell annotations were used as input for the HLCA.

Jain_Misharin_2021. Nasal epithelial samples were collected from healthy volunteers who provided informed consent at Northwestern Medicine in Chicago. The protocol was approved by the Northwestern University IRB (STU00214826). Healthy volunteers were recruited to match a cohort of patients with cystic fibrosis for the ongoing study at Northwestern University (with M.J. as the principal investigator). In both studies, A.V.M. did not influence participant recruitment and did not introduce biases in sample selection. Metadata of the donors' sex were based on self-report. Briefly, donors were seated and asked to extend their neck. A nasal curette (Rhino-Pro; VWR) was inserted into either nare and gently slid in the direction of posterior to anterior ~1 cm along the lateral inferior turbinate. Five curettes were obtained per participant. The curette tip was then cut and placed in 2 ml hypothermosol and stored at 4 C until processing. A single-cell suspension was generated using the cold-active dispase protocol reported by Deprez et al.[6] and Zaragosi and Barbry[90] with slight modification. Specifically, ethylenediaminetetraacetic acid (EDTA) was omitted and cells were dispersed by pipetting 20 times every 5 min using a 1 ml tip instead of tritration using a 21/23 G needle. The final concentration of protease from *Bacillus licheniformis* was 10 mg ml^{-1}. The total digestion time was 30 min. Following the wash in 4 ml 0.5% BSA in PBS and centrifugation at 400*g* for 10 min, cells were resuspended in 0.5% BSA in PBS and counted using a Nexcelom K2 Cellometer with acridine orange/propidium iodide reagent. This protocol typically yields ~300–500,000 cells with a viability of >95%. The resulting single-cell suspension was then used to generate single-cell libraries following the protocol for 5′ V1 (CG000086 Rev M; 10x Genomics) or V2 chemistry (CG000331 Rev A; 10x Genomics). Excess cells from two of the samples were pooled together to generate one additional single-cell library. After a quality check, the libraries were pooled and sequenced on a NovaSeq 6000 instrument. Raw sequencing data were processed using the Cell Ranger 3.1.0 pipeline, with the reference genome GRCh38 and annotation using Ensembl98. To assign sample information to cells in the single-cell library prepared from two samples, we ran souporcell[91] version 2.0 for that library and two libraries that were prepared from these samples separately. We used common genetic variants prepared by the souporcell authors to separate cells into two groups by genotype for each library, and Pearson correlation between the identified genotypes across libraries to establish correspondence between genotype and sample. Cell annotations were assigned to cell clusters based on expert interpretation of marker genes for each cluster. Cell clusters were derived with the Seurat[30] version 3.2 workflow in which samples were normalized with sctransform[89], 3,000 HVGs were selected and integrated and clusters were derived from 30 principal components using the Louvain algorithm with default parameters. Clusters with a low number of UMIs and high expression of ribosomal or mitochondrial genes were excluded as low quality. Raw counts and the thus obtained cell annotations were used as input for the HLCA.

Schultze_unpubl. Human lung tissue wabus available for research purposes following ethical approval from Hannover Medical School (Nr. 7414, 2017). All patients in this study provided written informed consent for sample collection and data analyses. At Hannover Medical School, patients with lung cancer were recruited in the course of their operation (that is, surgical tumor resection was performed according to the ethical vote of the German Centre for Lung Research, ethical vote 7414 and data safety guidelines). There was no bias in patient recruitment since the samples were collected as fresh native tissue following surgical tumor resection and according to the availability of surplus adjacent nonmalignant lung tissue, which was resected in parallel to the tumor tissue. Metadata of the donors' sex were based on self-report or reported by medical professionals during consenting. Fresh adjacent normal tumor-free lung tissues from patients with non-small cell lung cancer tumors were obtained by the Lung Research group (D. Jonigk, Pathology, Hannover Medical School) and processed for single-cell isolation immediately. Lung tissue was chopped with a scalpel and scissors and digested using BD Tumor Dissociation Reagent (BD Biosciences) for 30 min in a 37 °C water bath. The digestion was stopped with 1% FCS and 2 mM EDTA in PBS without Ca^{2+}/Mg^{2+} and cells were filtered over

a 70 µm cell strainer (BD Falcon). Erythrocytes were removed using a human MACSxpress Erythrocyte Depletion Kit (Miltenyi Biotec) and cells were filtered using a 40 µm cell strainer (BD Falcon). The viability of the cells was assessed microscopically and by flow cytometry using a LIVE/DEAD Fixable Yellow Dead Cell Stain Kit (Invitrogen) and was ~84%. The single-cell suspension was processed for scRNA-seq and library preparation with the Seq-Well protocol[92]. Library pools with fewer than 100 cells were discarded and merged into one object. The Seurat v3.2 pipeline was used to further analyze the data. Genes in fewer than five cells in the dataset, as well as the mitochondrial genes *MT-RNR1* and *MT-RNR2*, were removed. Cells with fewer than 200 genes were discarded, whereas cells with <5% mitochondrial genes or <30% intronic reads were kept for further analysis. The data were log normalized and 2,000 variable genes were calculated for each sample for integration with Seurat's Canonical Correlation Analysis algorithm[93]. The data were scaled, 50 principle components were selected and the data were clustered with 0.6 resolution. Cluster annotation revealed a low-quality cluster that was subsequently removed and the process (the calculation of variable genes, calculation of 30 principal components, clustering with 0.4 resolution) was repeated. Raw counts of the cells that passed all filtering were provided as input for the HLCA.

HLCA core data collection

To accommodate data protection legislation, scRNA-seq datasets of healthy lung tissue were shared by dataset generators as raw count matrices, thereby obviating the need to share genetic information. Count matrices were generated using varying software (Supplementary Table 1). Previously published scRNA-seq data were partly realigned by the dataset generators: the raw sequencing data of four previously published studies were realigned to GRCh38 using Ensembl84 for the HLCA[5,6,25,40]. For two of these studies[5,6], the Cell Ranger 3.1.0-based HLCA pipeline was used for realignment. For the remaining two[25,40], data were processed as previously described[25,40], but with the reference genome and genome annotation adapted to the HLCA (GRCh38; Ensembl84). All other datasets in the HLCA core were originally already aligned to GRCh38 (Ensembl84) except data from ref. 22 (GRCh38; Ensembl93) (Supplementary Table 1). For ref. 6, the count matrices provided had ambient RNA removed, as described previously.

Metadata collection (HLCA core)

For all of the datasets from the HLCA core, a preformatted sample metadata form was filled out by the dataset providers for every sample, containing metadata such as the ID of the donor from whom the sample came, the donor's self-reported ethnicity, the type of sample, the sequencing platform and so on (Supplementary Table 2). Ethnicity metadata were based on self-reported ethnicity for live donors or retrieved from medical records or assigned by the organ procurement team in the case of organ donors, as collected in the individual studies. For donor ethnicity, the following categories of self-reported ethnicity were used during metadata collection: Black, white, Latino, Asian, Pacific Islander and mixed. To conform to pre-existing 1,000 Genomes ancestry superpopulations[94], these self-reported ethnicity categories were then harmonized with the superpopulation categories as follows: Black was categorized as African, white as European and Latino as admixed American, while keeping the category Asian (merging the superpopulations East Asians and South Asians as this granularity was missing from the collected self-reported ethnicity data) and keeping Pacific Islander, as this category did not correspond to any of the superpopulations but does constitute a separate population in HANCESTRO[95]. We refer to the resulting categories as harmonized ethnicity. Both self-reported ethnicity (as collected) and harmonized ethnicity per donor are detailed in Supplementary Table 2. Cell type annotations from dataset providers were included in all datasets. For tissue dissociation protocols, protocols were grouped based on: (1) enzyme(s) used for tissue dissociation; and (2) the digestion time in cases where large differences were observed between protocols (that is, cold protease protocols were split into two groups: 30–60 min versus overnight).

General data preprocessing for the HLCA core

Patients with lung conditions affecting larger parts of the lung, such as asthma or pulmonary fibrosis, were excluded from the HLCA core and later added to the extended atlas. For the HLCA core, all matrices were gene filtered based on Cell Ranger Ensembl84 gene-type filtering[96] (resulting in 33,694 gene IDs). Cells with fewer than 200 genes detected were removed (removing 2,335 cells and 21 extra erythrocytes with close to 200 genes expressed as these hampered SCRAN normalization; see below), along with genes expressed in fewer than ten cells (removing 5,167 out of 33,694 genes).

Total count normalization with SCRAN

To normalize for differences in total UMI counts per cell, we performed SCRAN normalization[97]. Since SCRAN assumes that at least half of the genes in the data being normalized are not differentially expressed between subgroups of cells, we performed SCRAN normalization within clusters. To this end, we first performed total count normalization, by dividing each count by its cell's total count and multiplying by 10,000. We then performed a log transformation using natural log and pseudocount 1. A PCA was subsequently performed. Using the first 50 principal components, a neighborhood graph was calculated with the number of neighbors set to $k = 15$. Data were subsequently clustered with Louvain clustering at a resolution of $r = 0.5$. SCRAN normalization was then performed on the raw counts, using the Louvain clusters as input clusters and with the minimum mean (library size adjusted) average count of genes to be used for normalization set to 0.1. The resulting size factors were used for normalization. For the final HLCA (and not the benchmarking subset), cells with abnormally low size factors (<0.01) or abnormally high total counts after normalization (>10×10^5) were removed from the data (267 cells in total).

Cell type reference creation and metadata harmonization

To harmonize cell type labels from different datasets in the HLCA core, a common reference was created to which original cell type labels were mapped (Supplementary Table 4). To accommodate labels at different levels of detail, the cell type reference was made hierarchical, with level 1 containing the coarsest possible labels (immune, epithelial and so on) and level 5 containing the finest possible labels (for example, naive CD4 T cells). Levels were created in a data-driven fashion, recursively breaking up coarser-level labels into finer ones where finer labels were available.

To map anatomical location to a 1D CCF score that could be used for modeling, a distinction was made between upper and lower airways. First, an anatomical coordinate score was applied to the upper airways, starting at 0 and increasing linearly (with a value of 0.5) between each of the following anatomical locations: inferior turbinate, nasopharynx, oropharnyx, vesibula and larynx. The trachea received the next anatomical coordinate score using the same linear increment as in the upper airways (a score of 2.5). In the lower airways, the coordinate score within the bronchial tree was based on the generation airway, with the trachea being the first generation and the total number of generations assumed to be 23 (ref. 98). The alveolar sac was assigned the coordinate score of the 23rd generation airway. The coordinate score of each generation airway was calculated by taking the $\log_2$ value of the generation and adding it to the score of the trachea. Using this methodology, the alveolus received an anatomical coordinate score of 7.02. To calculate the final CCF coordinate, the coordinate scores (ranging from 0 to 7.02) were scaled to a value between 0 (inferior turbinate) and 1 (alveolus). Samples were then mapped to this coordinate system using the best approximation of the sampling location for each of the samples of the core HLCA (Supplementary Table 3).

Data integration benchmarking

For computational efficiency, benchmarking was performed on a subset of the total atlas, including data from ten studies split into 13 datasets (ref. 22 was split into 10xv1 and 10xv2 data; ref. 25 was split into 10xv2 and 10xv3 data; and the pooled data from ref. 21 and associated unpublished data were split into two based on the processing site). The data came from 72 donors, 124 samples and 372,111 cells. Preprocessing of the benchmarking data included the filtering of cells (minimum number of total UMI counts: 500) and genes (minimum number of cells expressing the gene: 5).

For integration benchmarking, the scIB benchmarking framework was used[99] with default integration parameter settings unless otherwise specified. All benchmarked methods except scGen (that is, BBKNN, ComBat, Conos, fas[99] tMNN, Harmony, Scanorama, scANVI, scVI and Seurat RPCA) were run at least twice: on the 2,000 most HVGs; and on the 6,000 most HVGs. Of these methods, all that did not require raw counts as input were run twice on each gene set: once with gene counts scaled to have a mean of 0 and standard deviation of 1; and once with unscaled gene counts. scVI and scANVI, which require raw counts as input, were not run on scaled gene counts. scGen was only tested on 2,000 unscaled HVGs. For HVG selection, first, HVGs were calculated per dataset using Cell Ranger-based HVG selection[100] (default parameter settings: min_disp=0.5, min_mean=0.0125, max_mean=3, span=0.3, n_bins=20). Then, genes that were highly variable in all datasets were considered overall highly variable, followed by genes highly variable in all datasets but one, in all datasets but two and so on until a predetermined number of genes were selected (2,000 or 6,000 genes).

For scANVI and scVI, genes were subset to the HVG set and the resulting raw count matrix was used as input. For all other methods, SCRAN-normalized (as described above) data were used. Genes were then subset to the precalculated HVG sets. For integration of gene-scaled data, all genes were scaled to have mean of 0 and standard deviation of 1.

Two integration methods allowed for input of cell type labels to guide the integration: scGen and scANVI. As labels, level 3 harmonized cell type labels were used (Supplementary Table 4), except for blood vessel endothelial, fibroblast lineage, mesothelial and smooth muscle cells, for which we used level 2 labels. Since scGen does not accept unlabeled cells, cells that did not have annotations available at these levels (that is, cells annotated as cycling, epithelial, stromal or lymphoid cells with no further annotations; 4,499 cells in total) were left out of the benchmarking data.

The dataset rather than the donor of the sample was used as the batch parameter. The maximum memory usage was set to 376 Gb and all methods requiring more memory were excluded from the analysis. The quality of each of the integrations was scored using 12 metrics, with four metrics measuring the batch correction quality and eight metrics quantifying the conservation of biological signal after integration (Supplementary Fig. 1; metrics previously described[28]). Overall scores were computed by taking a 0.4:0.6 weighted mean of batch effect removal to biological variation conservation (bioconservation), respectively. Methods were ranked based on overall score (Supplementary Fig. 1).

Splitting of studies into datasets

For integration of the data into the HLCA core, we first determined for which cases studies had to be split into separate datasets (which were treated as batches during integration). Reasons for possible splitting were: (1) different 10x versions used within a study (for example, 10xv2 versus 10xv3); or (2) the processing of samples at different institutes within a study. To determine whether these covariates caused batch effects within a study, we performed principal component regression[101]. To this end, we preprocessed single studies separately (total count normalization to median total counts across cells and subsequent PCA with 50 principal components). For each study, we then calculated the fraction of the variance in the first 50 principal components that could be explained (PC_{expl}) by the covariate of interest (that is, 10x version or processing institute):

$$PC_{expl} = \frac{\sum_{i=1}^{50} \text{var}(\text{cov})}{\sum_{i=1}^{50} \text{var}(PC_i)}$$

where $\text{var}(PC_i|\text{cov})$ is the variance in scores for the ith principal component across cells that can be explained by the covariate under consideration, based on a linear regression.

Then, 10x version or processing institute assignments were randomly shuffled between samples and PC_{expl} was calculated for the randomized covariate. This was repeated over ten random shufflings and the mean and standard deviation of PC_{expl} were then calculated for the covariate. If the nonrandomized PC_{expl} was more than 1.5 standard deviations above the randomized PC_{expl}, we considered the covariate a source of batch effect and split the study into separate datasets. Thus, both Jain_Misharin_2021 and ref. 22 were split into 10xv1 and 10xv2; ref. 25 was split into 10xv2 and 10xv3; and ref. 21 and its pooled unpublished data were not split based on 10x version nor on processing location.

Integration of HLCA core datasets with scANVI

For integration of the datasets into the HLCA core, coarse cell type labels were used as described for integration benchmarking (AT1, AT2, arterial endothelial cell, B cell lineage, basal, bronchial vessel 1, bronchial vessel 2, capillary, multiciliated, dendritic, fibroblast lineage, $KRT5^{-}KRT17^{+}$ epithelial, lymphatic endothelial cell, macrophages, mast cells, megakaryocytes, mesothelium, monocytes, neutrophils, natural killer/natural killer T cells, proliferating cells, rare, secretory, smooth muscle, squamous, submucosal secretory, T cell lineage, venous and unlabeled), except cells with lacking annotations were set to unlabeled instead of being removed. scANVI was run on the raw counts of the 2,000 most HVGs (calculated as described above), using datasets as the batch variable to enable the conservation of interindividual variation. The following parameter settings were used: number of layers: 2; number of latent dimensions: 30; encode covariates: True; deeply inject covariates: False; use layer norm: both; use batch norm: none; gene likelihood: nb; n epochs unsupervised: 500; n epochs semi-supervised: 200; and frequency: 1. For the unsupervised training, the following early-stopping parameters were used: early stopping metric: elbo; save best state metric: elbo; patience: 10; threshold: 0; reduce lr on plateau: True; lr patience: 8; and lr_factor: 0.1. For the semisupervised training, the following early-stopping parameter settings were used: early stopping metric: accuracy; save best state metric: accuracy; on: full dataset; patience: 10; threshold: 0.001; reduce lr on plateau: True; lr_patience: 8; and lr_factor: 0.1. The integrated latent embedding generated by scANVI was used for downstream analysis (clustering and visualization). For gene-level analyses (differential expression and covariate effect modeling), uncorrected counts were used.

UMAP embedding and clustering

To cluster the cells in the HLCA core, a nearest neighbor graph was calculated based on the 30 latent dimensions that were obtained from the scANVI output, with the number of neighbors set to $k = 30$. This choice of k, while improving clustering robustness, could impair the detection of very rare cell types. Coarse Leiden clustering was done on the graph with a resolution of $r = 0.01$. For each of the resulting level 1 clusters, a new neighbor graph was calculated using scANVIs 30 latent dimensions, with the number of neighbors again set to $k = 30$. Based on the new neighbor graph, each cluster was clustered into smaller level 2 clusters with Leiden clustering at a resolution of $r = 0.2$. The same was done for levels 3 and 4 and (where needed) 5, with k set to 15, 10 and 10, respectively, and the resolution set to 0.2. Clusters were named based on their parent clusters and sister clusters (for example, cluster 1.2 is the third biggest subcluster (starting at 0) of cluster 1).

For visualization, a 2D UMAP[102] of the atlas was generated based on the 30 nearest neighbors graph.

Calculating cluster entropy of cell type labels and donors

To quantify cluster cell type label disagreement for a specific level of annotation, the label Shannon entropy was calculated on the cell type label distribution per cluster as

$$-\sum_{i=1}^{k} p(x_i)\log[p(x_i)],$$

where $x_1 \ldots x_k$ are the set of labels at that annotation level and $p(x_i)$ is the fraction of cells in the cluster that was labeled as x_i. Cells without a label at the level under consideration were not included in the entropy calculation. If <20% of cells were labeled at the level under consideration, the entropy was set to not available for the figures. The entropy of donors per cluster (that is, diversity of donors in a cluster) was calculated in the same way.

Thresholds for high label/donor entropy and doublet clusters

To set a threshold for high label entropy, we calculated the label entropy of a hypothetical cluster with 75% of cells given one label and 25% of cells given another label, as a cluster with <75% of cells with the same label suggests substantial disagreement in terms of cell type labeling. Clusters with a label entropy above that level (0.56) were considered to have high label entropy. Six small clusters with high label entropy even at the coarsest level of annotation highlighted doublet populations (identified via simultaneous expression of lineage-specific marker genes; for example, expression of both epithelial (AT2) and stromal (smooth muscle) marker genes) not labeled as such in the original datasets. These clusters were removed from the HLCA core, bringing the total number of clusters to 94. To set a threshold for low donor entropy, we calculated the label entropy for a hypothetical cluster with 95% of cells from one donor and the remaining 5% of cells distributed over all other donors, as clusters with >95% of the cells from the same cluster could be considered single-donor clusters, possibly caused by remaining batch effects or by donor-specific biology that is difficult to interpret. Clusters with a donor entropy below that level (0.43) were considered clusters with low donor entropy.

Rare cell type analysis

To determine how well rare cell types (ionocytes, neuroendocrine cells and tuft cells) were clustered together and separate from other cell types after integration, we calculated recall (the percentage of all cells annotated as a specific rare cell type that were grouped into the cluster) and precision (the percentage of cells from the cluster that were annotated as a specific rare cell type) for all level 3 clusters. Nested clustering on Harmony[29,102] and Seurat's RPCA[30] output was done based on PCA of the corrected gene counts, recalculating the principal components for every parent cluster when performing clustering into smaller children clusters, with clustering performed as described above under 'UMAP embedding and clustering'. The level 3 clusters with the highest sensitivity for each cell type are included in Supplementary Fig. 3b.

Manual cell type annotation

Re-annotation of cells in the HLCA core was done by six investigators with expertise in lung biology (E.M., M.C.N., A.V.M., L.-E.Z., N.E.B. and J.A.K.) based on original annotations and differentially expressed genes of the HLCA core clusters. Annotation was done per cluster, using finer clusters where these represented specific known cell types or states rather than donor-specific variation. Annotations of cell identities were hierarchical (as was the harmonized cell type reference) and each cluster was annotated at the finest known level, whereafter coarser levels could automatically be inferred (for example, a cell annotated as a $CD8^+$ T cell was then automatically annotated as of T cell lineage at level 3, lymphoid cell lineage at level 2 and immune cell lineage at level 1). The number of cells per cell type is shown for all levels in Supplementary Table 5.

Mislabeling of original cells was identified by comparing final annotations with original harmonized labels and checking whether these were contradictory (and not only done at different levels of detail). Out of 61 final cell types, 18 included mostly mislabeled cells, 12 of which were previously known cell types. Despite consisting of mostly mislabeled cells from the original datasets, individual experts agreed on the annotation of these cell types: for all previously known cell types with a high proportion of mislabeled cells, the majority of experts agreed on the final annotation for the atlas, or only differed in the granularity of annotation.

Marker gene selection

Marker genes were calculated based on per-sample, per-cell-type pseudo-bulks, calculating the mean (normalized and log-transformed) expression per pseudo-bulk for every gene. Pseudo-bulks were only calculated for a sample if it had at least ten cells of the cell type under consideration. An exception was made for cell types with fewer than 100 cells in total, for which the minimum number of cells per sample was set to 3. Pseudo-bulks rather than cell-level counts were used to ensure equal weighing of every sample in the differential expression test, thus statistically testing cell type-specific changes in expression that were significant across samples rather than cells. As pseudo-bulks represent the mean of a repeated draw from a single distribution, based on the central limit theorem, we expect pseudo-bulk gene counts to be normally distributed, and a *t*-test was therefore used to test differential gene expression, comparing a single cell type with all other cell types in the atlas (marker iteration 1). To further filter out differentially expressed genes that were not consistently expressed across samples, we applied a filtering step to remove genes expressed in <80% of the pseudo-bulks, or genes expressed in <50% of cells per pseudo-bulk (with the filtering based on the mean across pseudo-bulks). Similarly, to ensure specificity of gene expression, additional filtering was done to remove genes expressed in >20% of other pseudo-bulks. For many cell types, marker genes unique to a single cell type across the entire atlas could not be found. To nonetheless collect a robust and unique set of marker genes for every cell type, a hierarchical approach was taken, subsetting the atlas to four compartments (epithelial, endothelial, immune and stromal, for each of which a marker set was calculated) before calculating cell type-specific marker genes and filtering on uniqueness only within the compartment (marker iteration 2). When necessary, a second subsetting step was done, now subsetting to the next coarsest cell type set within the compartment (for example, lymphatic endothelial cells) and repeating the same procedure (marker iteration 3). Finally, filtering criteria were loosened for the remaining cell types for which no unique markers could be found in any of the iterations (marker iterations 4 and 5). Exact filtering parameters per iteration can be found in Supplementary Table 16. For lymphatic endothelial cell subtypes, one subtype contained sufficient cells for only a single sample, hampering a pseudo-bulk-based approach. Therefore, lymphatic endothelial cell subset markers (mature, differentiating and proliferating) were chosen based on known literature, after checking consistency with expression patterns observed in the HLCA lymphatic endothelial cells.

Variance between individuals explained by covariates

To quantify the extent to which different technical and biological covariates correlated with interindividual variation in the atlas, we calculated the variance explained by each covariate for each cell type. We first split the data in the HLCA core by cell type annotation, merging substates of a single cell type into one (Supplementary Table 5; includes the number of cells per cell type). For every cell type, we excluded samples that had fewer than ten cells of the sample. We then

summarized covariate values per sample for every cell type depending on the variable, taking the mean across cells from a sample for scANVI latent components (integration results), UMI counts per cell and fractions of mitochondrial UMIs, while for all other covariates (for example, BMI and tissue sampling method) each sample had only one value; therefore, these values were used.

Next, we performed principal component regression on every covariate, as described previously (see the section 'Splitting of studies into datasets'), but now using scANVI latent component scores instead of principal component scores for the regression, yielding a fraction of latent component variance explained per covariate. Samples that did not have a value for a given covariate (for example, where the BMI was not recorded for the donor) were excluded from the regression. Categorical covariates were converted to dummy variables. Cell type–covariate pairs for which only one value was observed for the covariate were excluded from the analysis.

Quantification of the correlation or dependence between variables within a cell type and identification of the minimum number of samples needed to control for spurious correlation are described below.

Covariate dependence for interindividual variance

To check the extent to which covariates correlated with each other, thereby possibly acting as confounders in the principal component regression scores, we determined dependence between all covariate pairs for every cell type. If at least one covariate was continuous, we calculated the fraction of variance in the continuous covariate that could be explained by the other covariate (dummying categorical covariates) and took the square root (equal to Pearson's r for two continuous covariates). For two categorical covariates, if both covariates had more than two unique values, we calculated normalized mutual information between the covariates using scikit-learn[103], since a linear regression between these two covariates is not possible.

Finding the minimum number of samples for variance modeling

To control for spurious correlations between interindividual cell type variation and covariates due to low sample numbers, we assessed the relationship between sample number and mean variance explained across all covariates for every cell type. We found that for cell types sampled in fewer than 40 samples the mean variance explained across all covariates showed a high negative correlation with the number of samples (Supplementary Fig. 4a). We reasoned that for these cell types correlations between interindividual variation and our covariates were inflated due to undersampling. Moreover, we note that at lower sample numbers technical and biological covariates often strongly correlate with each other across donors (Supplementary Fig. 4c). This might lead to the attribution of true biological variation to technical covariates, and vice versa, complicating the interpretation of observed interindividual cell type variation. Consequently, we consider 40 a recommended minimum number of samples to avoid spurious correlations between observed interindividual variation and tested covariates, and excluded results from cell types with fewer samples.

Cell type filtering covariate encoding for gene-level modeling

To select cell types for which covariate effects could be confidently modeled at the gene level, we followed the same procedure for every cell type: we filtered out all genes that were expressed in fewer than 50 cells and all samples that had fewer than ten cells of the cell type. We furthermore filtered out datasets with fewer than two donors and refrained from modeling categories in covariates that had fewer than three donors in their category for that cell type.

We encoded smoking status as a continuous covariate, setting never to 0, former to 0.5 and current to 1. Anatomical region was encoded into anatomical region CCF scores as described earlier. As we noted that changes from the nose to the rest of the airways and lungs were often independent from continuous changes observed in the lungs only, we encoded nasal as a separate covariate, setting samples from the nose to 1 and all others to 0. BMI and age were rescaled, such that the 10th percentile of observed values across the atlas was set to 0 and the 90th percentile was set to 1 (25 and 64 years for age, respectively, and 21.32 and 36,86 for BMI).

To determine whether covariance between covariates was low enough for modeling, we calculated the variance inflation factor (VIF) between covariates at the donor level. The VIF quantifies multicollinearity among covariates of an ordinary least squares regression and a high VIF indicates strong linear dependence between variables. If the VIF was higher than 5 for any covariate for a specific cell type, we concluded that covariance was too high and excluded that cell type from the modeling. As many cell types lacked sufficient representation of harmonized ethnicities other than European, including harmonized ethnicity in the analysis simultaneously decreased the samples that could be included in the analysis to only those with ethnicity annotations; hence, we excluded harmonized ethnicity from the modeling.

Modeling gene-level interindividual variation and GSEA

To model the effects of demographic and anatomical covariates (sex, age, BMI, harmonized ethnicity, smoking status and anatomical location of the sample) on gene expression, we employed a generalized linear mixed model. We used sample-level pseudo-bulks (split by cell type), since the covariates modeled also varied at the sample or donor level and not at the cell level. Modeling these covariates at the cell level (that is, treating single cells as independent samples even when they come from the same sample) has been shown to inflate P values[104,105]. First, we split the lung cell atlas by cell type annotation, pooling detailed annotations into one subtype (for example, grouping all lymphatic endothelial cell subtypes into one) (Supplementary Table 5; includes the number of cells per cell type). Subsequent filtering, covariate encoding and exclusion of cell types due to covariate dependence are described above.

Gene counts were summed across cells for every sample, within cell type. Sample-wise sums (that is, pseudo-bulks) were normalized using edgeR's calcNormFactors function, using default parameter settings. We then used voom[106], a method designed for bulk RNA-seq that estimates observation-specific gene variances and incorporates these into the modeling. Specifically, we used a voom extension (differential expression testing with linear mixed models) that allows for mixed-effects modeling and modeled gene expression as:

$$\log[\text{normcount}] \sim 1 + \text{age} + \text{sex} + \text{BMI} + \text{smoking} + \text{nose} + \text{CCF score} + (1|\text{dataset})$$

where dataset is treated as a random effect to correct for dataset-specific changes in expression and all other effects are modeled as fixed effects. Resulting P values were corrected for multiple testing within every covariate using the Benjamini–Hochberg procedure.

To identify more systematic patterns across genes and changes happening at the gene set level, a gene set enrichment analysis was performed using correlation-adjusted mean-rank gene set tests[107]. The analysis was performed in R using the cameraPR function in the limma package[108], with the differential expression test statistic. Gene Ontology biological process terms[109,110] were tested separately for each comparison. These sets were obtained from MSigDB (version 7.1)[111], as provided by the Walter and Eliza Hall Institute (https://bioinf.wehi.edu.au/MSigDB/index.html).

Mapping of GWAS results to the HLCA cell types

To stratify GWAS results from several lung diseases by lung cell type, we applied stratified linkage disequilibrium score regression in single cells (sc-LDSC)[48]. sc-LDSC can link GWAS results to cell types by calculating,

for each cell type, whether disease-associated variants are enriched in genomic regions of cell-type specific genes (i.e. the region of each gene and its surrounding base pairs), while taking into account the genetic signal of proximal linkage disequilibrium-associated regions. Here cell-type specific genes are defined as genes differentially expressed in the cell type of interest[48]. In contrast with simple enrichment testing of only significantly disease-associated genes from a GWAS among genes differentially expressed in a cell type, this method takes into account all SNPs included in the GWAS. Thus, consistent enrichment of weakly disease-associated genes (that would not individually pass significance tests) in a cell type could still lead to a significant association between the disease and the cell type. In this way, sc-LDSC provides more statistical power to detect associations between cell types and heritable phenotypes such as lung diseases.

To perform sc-LDSC on the HLCA, first a differential gene expression test was performed for every grouped cell type (Supplementary Table 5) in the HLCA using a Wilcoxon rank-sum test, testing against the rest of the atlas. The top 1,000 most significant genes with positive fold changes were stored as genes characterizing that cell type (cell type genes) and used as input for LDSC[48]. Gene coordinates of cell type genes were obtained based on the GRCh37.13 genome annotation. For SNP data (names, locations and linkage-related information), the 1000 Genomes European reference (GRCh37) was used, as previously described[48]. Only SNPs from the HapMap 3 project were included in the analysis. For identification of SNPs in the vicinity of cell type genes, we used a window size of 100,000 base pairs. Genes from X and Y chromosomes, as well as human leukocyte antigen genes, were excluded because of their unusual genetic architecture and linkage patterns. For linkage disequilibrium score calculation, a 1 cM window was used. Significance of the link between a phenotype and a cell type was calculated using LDSC[48]. *P* values yielded by LDSC were corrected for multiple testing for every disease tested using the Benjamini–Hochberg correction procedure. As a negative control, the analysis was performed with a GWAS of depression and no cell types were found to be significant (Supplementary Fig. 7). The numbers of cases and controls per GWAS study were as follows: $n = 2{,}668$ cases and 8,591 controls for IPF; $n = 35{,}735$ cases and 222,076 controls for COPD; $n = 11{,}273$ cases and 55,483 controls for lung adenocarcinoma; $n = 321{,}047$ individuals for lung function; $n = 88{,}486$ cases and 447,859 controls for asthma; and $n = 113{,}769$ cases and 208,811 controls for depression (used as negative control).

Generating cell type signature matrices for deconvolution

To enable deconvolution of bulk expression data on the basis of the HLCA, HLCA cell type signature matrices were generated. One generic-purpose signature matrix was created per sublocation of the respiratory system (that is, one parenchyma, one airway and one nose tissue matrix; Supplementary Table 10). Additionally, a script to generate custom reference sets from the HLCA data is provided together with the HLCA code on GitHub (https://github.com/LungCellAtlas/HLCA) to tailor the deconvolution signature matrix to any specific research question.

Cell types were included in the bulk deconvolution signature matrix on the basis of cell proportions (constituting >2% of cells within samples of the corresponding tissue in the HLCA core). In addition, cell types were merged when they were deemed too transcriptionally similar. For each included cell type, 200 cells were randomly sampled from the HLCA core, while all cells were included for cell types with fewer than 200 cells present in the HLCA core. Cells were sampled from the matching anatomical location (for example, nose T cells rather than parenchymal T cells were used for the nose signature matrix). Signature matrices were constructed using CIBERSORTx[112] (version 1.0) according to default settings, and no cross-platform batch correction was applied. The reference data were optimized by deconvolution of pseudo-bulk samples constructed from the HLCA core data, assessing deconvolution performance per included cell type based on the correlation of predicted proportions with ground truth composition (Supplementary Fig. 8a).

The following cell types were included in the deconvolution: endothelial cell arterial, endothelial cell capillary, lymphatic endothelial cell, basal and secretory (merged), multiciliated lineage, AT2, B cell lineage, innate lymphoid cell (ILC) natural killer and T cell lineage (merged), dendritic cells, alveolar macrophages, interstitial macrophages, mast cells, fibroblast lineage, smooth muscle, endothelial cell venous and monocytes (for the parenchyma); basal resting and suprabasal (merged), multiciliated lineage, club, goblet, dendritic cells, hillock like and T cell lineage (for the nose); and endothelial cell venous, CD4 T cells, fibroblasts, smooth muscle, basal and secretory (merged), multiciliated lineage, endothelial cell capillary, interstitial macrophages, B cell lineage, natural killer cells, CD8 T cells, dendritic cells, alveolar macrophages, mast cells and monocytes (for the airway). Capillary endothelial cells and interstitial macrophages (airway) were excluded from statistical testing due to poor performance in the benchmark. Venous endothelial cells and monocytes (parenchyma), hillock-like cells and T cell lineage cells (nose) and B cell lineage cells, natural killer cells, CD8 T cells, dendritic cells, alveolar macrophages, mast cells and monocytes (airways) were excluded from statistical testing due to >60% zero proportions.

Deconvolution of bulk expression data using the HLCA core

The parenchymal signature matrix was used to deconvolve RNA expression data of samples from the Lung Tissue Database[52] (GEO accession number GSE23546) using only lung tissue samples from patients with COPD GOLD stages 3 and 4 ($n = 27$ and 56, respectively) and matched controls ($n = 281$). The Lung Tissue Database dataset was run on the Rosetta/Merck Human RSTA Custom Affymetrix 2.0 microarray platform (HuRSTA-2a520709; GPL10379). As this platform has multiple probe sets for each gene, we focused on the probe sets that were derived from curated RefSeq records (with NM_ accession prefixes) when present to maximize the accuracy of the deconvolution. Where genes did not have probe sets based on curated RefSeq records or had multiple probe sets mapping to curated RefSeq records, the probe set with the highest average microarray intensity across samples was selected. Quantile normalization of the data and subsequent deconvolution were performed using CIBERSORTx. A Wilcoxon rank-sum test between control and GOLD stage 3/4 samples was performed to identify statistically significant compositional changes in advanced-stage COPD compared with control tissue. GOLD 3/4 and control samples were matched for age and smoking history. Cell types with >60% of samples predicted to have a proportion of zero were excluded from the Wilcoxon test, as the high number of tied ranks (zeros in both groups) would result in inflated *P* values. *P* values were multiple testing corrected using the Benjamini–Hochberg procedure.

The same procedure was followed for a dataset of nasal brush bulk RNA-seq samples from asthmatic donors pre- and postinhalation of corticosteroids ($n = 54$ and 26, respectively)[50] and a dataset of airway biopsy bulk RNA-seq samples from asthmatic donors and controls ($n = 95$ and 38, respectively)[51]. As these consisted of RNA-seq data, no quantile normalization was applied.

Extension of the HLCA core by mapping of new data

To map unseen scRNA-seq and single-nucleus RNA-seq data to the HLCA, we used scArches, our transfer learning-based method that enables mapping of new data to an existing reference atlas[71]. scArches trains an adaptor added to a reference embedding model, thereby enabling it to generate a common embedding of the new data and the reference, allowing reanalysis and de novo clustering of the joint data. The data to map were subsetted to the same 2,000 HVGs that were used for HLCA integration and embedding, and HVGs that were absent in the new data were set to 0 counts for all cells. Raw counts were used as

input for scArches, except for the ref. 40 dataset, for which ambient RNA removal was run previously on the raw counts. Healthy lung data[40] were split into two datasets: 3′ and 5′ based. Lung cancer data[41] were also split into two datasets: 10xv1 and 10xv2.

The model that was learned previously for HLCA integration using scANVI was used as the basis for the scArches mapping. scArches was then run to train adaptor weights that allowed for mapping of new data into the existing HLCA embedding, using the following parameter settings: freeze-dropout: true; surgery_epochs: 500; train base model: false; metrics to monitor: accuracy and elbo; weight-decay: 0; and frequency: 1. The following early-stopping criteria were used: early stopping metric: elbo; save best state metric: elbo; on: full dataset; patience: 10; threshold: 0.001; reduce lr on plateau: True; lr patience: 8l and lr_factor: 0.1.

Gene name harmonization

To enable cross-dataset gene-level analysis, harmonization of gene names from different datasets (using different reference genome builds and genome annotations; Supplementary Table 1) was necessary. Both annotation sources (for example, Ensembl or RefSeq) and annotation versions (for example, Ensembl release 84 or Ensembl release 91) contribute to the variation between different gene naming schemes. Therefore, both annotation sources and versions, including outdated ones, need to be taken into account to enable the mapping of all gene names to a single naming scheme.

For the harmonization of gene names, we aimed to map all original gene names to the target scheme HUGO Gene Nomenclature Committee gene name, corresponding to the Ensembl release 107 publication. To find the most likely match between each original gene name and a target gene name, we first downloaded Ensembl releases 79 to 107, which included for each release: (1) all Ensembl gene IDs from the downloaded release (for example, ENSG00000081237.21); (2) corresponding Ensembl transcript and protein IDs (for example, ENST00000442510.8 and ENSP00000411355.3); (3) matching Ensembl IDs from the previous release; (4) matching gene IDs from other genome annotation sources (for example, RefSeq); and (5) matching gene, transcript and protein identifiers from various external resources, such as UniProt, the HUGO Gene Nomenclature Committee and the Consensus Coding Sequence Project. We then constructed a graph, with each Ensembl ID, other genome annotation ID and external resource identifier represented by a single node per release. Nodes were then connected based on the matching (points 2–5) provided by Ensembl, weighing edges based on Ensembl similarity scores where available. For each original gene name from the HLCA datasets, the path with the lowest mean edge weight from that gene name to a gene name from the target names (Ensembl release 107) was selected to find the most likely matching gene name from the target (Supplementary Table 17). Genes for which no target could be found were excluded from downstream analysis. When multiple genes were matched with the same target gene name, counts from the original genes were summed.

Identification of genes associated with common batch effects

To identify the genes most commonly exhibiting batch-specific expression, the HLCA was split by cell type and a differential expression analysis was performed (based on a Wilcoxon rank-sum test) in each cell type, comparing cells from one dataset (batch) with those from all other datasets and repeating this for all datasets. Datasets that had fewer than ten cells of the cell type or fewer than three samples with cells of the cell type were excluded from the test. For each test, genes were filtered such that only genes that were significantly upregulated were retained. Next, the fraction of included datasets in which a gene was significantly upregulated in the cell type (affected dataset fraction) was calculated for all genes. To find genes that were often batch effect associated across many cell types, the mean of the affected dataset fractions was calculated across cell types for each gene.

Cell type label transfer from the HLCA core to new datasets

To perform label transfer from the HLCA core to the mapped datasets from the extended HLCA, we used the scArches k nearest neighbor-based label transfer algorithm[71]. Briefly, a k nearest neighbor graph was generated from the joint embedding of the HLCA core and the new, mapped dataset, setting the number of neighbors to $k = 50$. Based on the abundance and proximity in a cell's neighborhood of reference cells of different types, the most likely cell type label for that cell was selected. Furthermore, a matching uncertainty score was calculated based on the consistency of reference annotations among the k nearest neighbors of the cell of interest

$$u_{c,y,\mathrm{N}_c} = 1 - p\left(Y = y | X = c, \mathrm{N}_c\right)$$

where $u_{c,y,\mathrm{Nc}}$ is the uncertainty score for a query cell c with transferred label y; N_c is its set of k nearest neighbors; and $p(\mathrm{Y} = y|\mathrm{X} = c, \mathrm{N}_c)$ is the weighted (by edge weights) proportion of N_c with label y, as previously described[113]. Thus, high consistency in HLCA core annotations leads to low uncertainty scores and low consistency (that is, mixing of distinct reference annotations) leads to high uncertainty scores. For label transfer to lung cancer and healthy, spatially annotated projected data (Fig. 5b and Extended Data Fig. 7g), cells with an uncertainty score above 0.3 were set to unknown.

Disagreement between original labels and transferred annotations (that is, transferred annotations with high certainty but not matching the original label) in the data from ref. 40 highlighted three different cases: annotations not included in the mapped data (for example, preterminal bronchiole secretory cells, which were labeled as club and goblet in the mapped data; these may not be incorrect label transfers but cannot be verified by label comparison alone); cell types that are part of a continuum, with cutoffs between cell types chosen differently in the reference than in the projected data (for example, macrophage subtypes); and cell types missing in the HLCA core that have high transcriptional similarity to other cell types that are present in the HLCA, which was observed for several finely annotated immune cell identities. For example, γδ T cells, ILCs, megakaryocytes, natural killer T cells and regulatory T cells were not annotated in the HLCA core, as these cell types could not be distinguished with confidence in the integrated object and were often lacking in the constituent datasets. Indeed, cell types from the T cell/ILC/natural killer lineages are known to be particularly difficult to annotate using transcriptomic data only[16]. Therefore, cells annotated with these labels in the projected dataset were largely incorrectly annotated as $CD4^+$ T cells, $CD8^+$ T cells and natural killer cells through label transfer (Fig. 5b and Extended Data Fig. 6e)

Calibration of uncertainty cutoff for classifying as unknown

For the extended atlas, we calibrated the uncertainty score cutoff by determining which uncertainty levels indicate possible failure of label transfer. To determine the uncertainty score at which technical variability from residual batch effects impairs correct label transfer, we evaluated how label transfer performed at the level of datasets, as these predominantly differ in experimental design. To determine an uncertainty threshold indicative of possible failure of label transfer, we harmonized original labels for 12 projected datasets[54,58,59,64,66] (one unpublished: Duong_lungMAP_unpubl) and assessed the correspondence between original labels with the transferred annotations. Only cells with level 3 or 4 original annotations were considered, as these levels represent informative annotations while not representing the finest detail. Level 5 annotations will often display high uncertainty levels due to high annotation granularity rather than remaining batch effects. To assess the optimal uncertainty cutoff for labeling a new cell as unknown, we used these results to generate a receiver operating characteristic curve. We chose a cutoff around the elbow point, keeping the false positive rate below 0.5 (uncertainty cutoff = 0.2; true positive rate = 0.879; false positive rate = 0.495) to best distinguish correct

from incorrect label transfers (Supplementary Fig. 10a). False positives were either due to incorrect label transfer or incorrect annotations in the original datasets. Cells with an uncertainty higher than 0.2 were set to unknown.

Identifying clusters with spatially annotated cell types

The ref. 40 study of healthy lung included cell type annotations based on matched spatial transcriptomic data. Many of these annotations were not present in the HLCA core. To determine whether these spatial cell types could still be recovered after mapping to the HLCA core, we looked for clusters specifically grouping these cells. We focused on six spatial cell types: perineurial nerve-associated fibroblasts; endoneurial nerve-associated fibroblasts; immune-recruiting fibroblasts; chondrocytes; myelinating Schwann cells; and nonmyelinating Schwann cells. As these cell types were often present at very small frequencies, we performed clustering at different resolutions to determine whether these cells were clustered separately at any of these resolutions. We clustered at resolutions of 0.1, 0.2, 0.5, 1, 2, 3, 5, 10, 15, 20, 25, 30, 50, 80 and 100, with the number of neighbors set to $k = 30$ for resolutions under 25 and $k = 15$ for resolutions of 25 or higher, to enable the detection of smaller clusters. Minimum recall (the percentage of cells with the spatial cell type annotation captured in the cluster) and minimum precision (the percentage of cells from ref. 40 in the cluster that had the spatial cell type annotation) were both set to 25%. The cluster with the highest recall was selected for every spatial cell type (unless this cluster decreased precision by >33% compared with the cluster with the second highest recall). If the precision of the next best cluster was doubled compared with the cluster with the highest recall and recall did not decrease by >20%, this cluster was selected.

Disease signature score calculation

To learn disease-specific signatures based on label transfer uncertainty scores, cells from the mapped data with the same transferred label (either alveolar fibroblasts or alveolar macrophages) were split into low-uncertainty cells (<0.2) and high-uncertainty cells (>0.4), excluding cells between these extremes (for alveolar fibroblasts, $n = 11{,}119$ (<0.2) and $n = 2{,}863$ (>0.4); for alveolar macrophages, $n = 1{,}770$ (<0.2) and $n = 577$ (>0.4)). We then performed a differential expression analysis on SCRAN-normalized counts using a Wilcoxon rank-sum test with default parameters, comparing high- and low-uncertainty cells. The 20 most upregulated genes based on log-fold changes were selected after filtering out genes with a false discovery rate-corrected P value (using the Benjamini–Hochberg procedure) above 0.05 and genes with a mean expression below 0.1 in the high-uncertainty group. To calculate the score of a cell for the given set of genes, the average expression of the set of genes was calculated, after which the average expression of a reference set of genes was subtracted from the original average, as described previously[114]. The reference set consists of a randomly sampled set of genes for each binned expression value. The resulting score was considered the cell's disease signature score.

Cross-dataset analysis of IPF-associated cell states

To uncover the cell identities affected in IPF, label transfer uncertainty was analyzed for three mapped datasets from the extended HLCA[58,62,64] that included both IPF and healthy samples. Cell types of interest were determined based on the largest increase in mean label transfer uncertainty in IPF compared with healthy samples, while checking for consistency in increments across the three datasets. This highlighted alveolar fibroblasts as the main cell type of interest. To find IPF-specific alveolar fibroblast states, all alveolar fibroblasts from the abovementioned datasets and two more IPF datasets[21,24] (for which no healthy data were mapped, as these were already in the core) were clustered together with the alveolar fibroblasts from the HLCA core. For clustering, a k nearest neighbor graph was calculated on the joint scArches-derived 30-dimensional embedding space setting $k = 30$, after which the cells were clustered using the Leiden algorithm with a resolution of 0.3. The resolution was chosen such that datasets were not isolated in single clusters, thus avoiding clustering driven solely by dataset-specific batch effects. One cluster (cluster 5) was small ($n = 460$ cells) and displayed low donor entropy (0.17), indicating that it almost exclusively came from a single donor (96% of cells from HLCA core donor 390C). It was therefore excluded from further analysis. To perform differential gene expression analysis, gene counts were normalized to a total of 7,666 counts (the median number of counts across the HLCA) and then log transformed with a pseudocount of 1. Finally, a Wilcoxon rank-sum test was used on the normalized data to detect differentially expressed genes for cluster 0 ($n = 6{,}765$ cells versus a total of $n = 14{,}731$). The results were filtered such that genes expressed in <30% of cells of the cluster of interest were excluded, as well as genes that were expressed in >20% of cells outside of the cluster and genes with a multiple testing-corrected P value (using the Benjamini–Hochberg procedure) above 0.05 (Supplementary Table 14).

Multidisease analysis

To investigate whether the HLCA can be used to identify disease-associated cell states shared across multiple diseases, MDMs from the HLCA core, together with all cells from the mapped datasets labeled as MDMs based on label transfer, were jointly analyzed. Datasets and diseases with fewer than 50 MDMs were excluded from the analysis. The cells were subsequently clustered as described above for the cross-dataset IPF analysis. Finally, a Wilcoxon rank-sum test was used on the normalized data to detect differentially expressed genes per cluster (number of cells per cluster: $n = 64{,}915$ (cluster 0), 47,539 (cluster 1), 32,027 (cluster 2), 31,097 (cluster 3), 25,267 (cluster 4), 1,998 (cluster 5) and 307 (cluster 6)). The results were filtered as described above (Supplementary Table 15).

Version information

The following tools and versions were used: R (version 4.1.1 for covariate modeling and version 4.0.3 for GSEA); edgeR (version 3.28.1); lme4 (version 1.1-27.1); LDSC (version 1.0.1); Limma (version 3.46.0); Scanpy (version 1.9.1); scArches (version 0.3.5); scIB (version 0.1.1); scikit-learn (version 0.24.1); and scvi-tools (scANVI; version 0.8.1).

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

The HLCA (raw and normalized counts, integrated embedding, cell type annotations and clinical and technical metadata) is publicly available and can be downloaded via cellxgene (https://cellxgene.cziscience.com/collections/6f6d381a-7701-4781-935c-db10d30de293). The HLCA core reference model and embedding for the mapping of new data to the HLCA can moreover be found on Zenodo (https://doi.org/10.5281/zenodo.7599104). The original, published datasets that were included in the HLCA can also be accessed under GEO accession numbers GSE135893, GSE143868, GSE128033, GSE121611, GSE134174, GSE150674, GSE151928, GSE136831, GSE128169, GSE171668, GSE132771, GSE126030, GSE161382, GSE155249, GSE135851, GSE145926 and GSE178360, GSE227136, GSE158127, European Genome-phenome Archive study IDs EGAS00001004082, EGAS00001004344, EGAD00001005064 and EGAD00001005065 and URLs https://www.synapse.org/#!Synapse:syn21041850, https://data.humancelllatlas.org/explore/projects/c4077b3c-5c98-4d26-a614-246d12c2e5d7, https://www.ncbi.nlm.nih.gov/projects/gap/cgi-bin/study.cgi?study_id=phs001750.v1.p1, https://www.nupulmonary.org/covid-19-ms2/?ds=full&meta=SampleName, https://figshare.com/articles/dataset/Single-cell_RNA-Seq_of_human_primary_lung_and_bronchial_epithelium_cells/11981034/1, https://covid19.lambrechtslab.org/downloads/

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Code availability

The HLCA pipeline for processing the sequencing data for counting matrices, used for a subset of HLCA datasets (Methods), is available at https://github.com/LungCellAtlas/scRNAseq_pipelines. All further code used for the HLCA project can be found in the HLCA reproducibility GitHub repository (https://github.com/LungCellAtlas/HLCA_reproducibility). The landing page of the HLCA, including up-to-date links, can be found at https://github.com/LungCellAtlas/HLCA. Automated mapping to the HLCA and label transfer can be done with scArches[71] at FASTGenomics (https://beta.fastgenomics.org/p/hlca) or using the code and tutorial in the HLCA mapping GitHub repository (https://github.com/LungCellAtlas/mapping_data_to_the_HLCA). Links to additional and updated platforms will be published on the HLCA landing page (see above). Automated mapping to the HLCA and label transfer with Azimuth[16,71] (not shown in this manuscript) can be done at azimuth.hubmapconsortium.org. Label transfer with CellTypist[117] (not shown in this manuscript) can be done at https://www.celltypist.org/models.

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Acknowledgements

This publication is part of the Human Cell Atlas (www.humancellatlas.org/publications/). This work was supported by the Chan Zuckerberg Initiative (CZI; LLC Seed Network grant CZF2019-002438 (Lung Cell Atlas 1.0) to P.B., M.D.L., A.V.M., M.C.N., D.P.S., J.R., P.R.T., K.B.M., F.J.T. and H.B.S.); National Institutes of Health (NIH; R01HL145372) and Department of Defense (W81XWH-19-1-0416) (to J.A.K. and N.E.B.); Fondation pour la Recherche Médicale (DEQ20180339158), Conseil Départemental des Alpes Maritimes (2016-294DGADSH-CV), Inserm Cross-cutting Scientific Program HuDeCA 2018, Agence Nationale de la Recherche SAHARRA (ANR-19-CE14-0027), ANR-19-P3IA-0002-3IA, National Infrastructure France Génomique (ANR-10-INBS-09-03) and PPIA 4D-OMICS (21-ESRE-0052) (to P.B.); H2020-SC1-BHC-2018-2020 Discovair (grant agreement 874656) (to P.B., K.B.M., S.A.T., M.C.N., F.J.T., M.P., H.B.S. and J.L.); NIH 1U54HL145608-01 (to M.D.L., K.Z., X.S., J.S.H. and G.P.); Wellcome (WT211276/Z/18/Z) and Sanger core grant WT206194 (to K.B.M. and S.A.T.); ESPOD fellowship of the European Molecular Biology Laboratory European Bioinformatics Institute and Sanger Institute (to E.M.); R01 HL153312, U19 AI135964, P01 AG049665, R01 HL158139, R01 ES034350 and U54 AG079754 (to A.V.M.); Lung Foundation Netherlands project numbers 5.1.14.020 and 4.1.18.226 (to M.C.N.); NIH grants R01HL146557 and R01HL153375 (to P.R.T.); German Center for Lung Research and Helmholtz Association (to H.B.S.); Helmholtz Association's Initiative and Networking Fund through Helmholtz AI (ZT-I-PF-5-01) and Bavarian Ministry of Science and the Arts in the framework of the Bavarian Research Association ForInter (Interaction of Human Brain Cells) (to F.J.T.); Doris Duke Charitable Foundation (to J.A.K.); Joachim Herz Foundation (to L.D.); Ministry of Economic Affairs and Climate Policy by means of the Public–Private Partnership (to T.M.K.); 3IA Cote d'Azur PhD program (to A.C.); R01 HL135156, R01 MD010443, R01 HL128439, P01 HL132821, P01 HL107202, R01 HL117004 and Department of Defense grant W81WH-16-2-0018 (to M.A.S.); HL142568 and HL14507 from the NHLBI (to D.S.); P50 AR060780-06A1 (to R.L. and T.T.); Medical Research Council Clinician Scientist Fellowship (MR/W00111X/1) (to M.Z.N.); Jikei University School of Medicine (to M.Y.); University College London Birkbeck Medical Research Council Doctoral Training Programme (to K.B.W.); CZI (to J.W., Y.X. and N.K.); 5U01HL148856 (to J.W. and Y.X.); R01 HL153045 (to Y.X.); R01HL127349, R01HL141852 and U01HL145567 (to N.K.); 2R01HL068702 (to D.P.S. and J.R.); 5R01HL14254903 and 4UH3CA25513503 (to T.J.D.); R21HL156124, R56HL157632 and R21HL161760 (to A.M.T.); NIH U54 AG075931 and 5R01 HL146519 (to O.E.); Swedish Research Council and Cancerfonden (to C.S.); CZI Deep Visual Proteomics (to P.H.); U01HL148861-03 (to G.P.); CZI 2021-237918 (to J.S.H., P.R.T., H.B.S. and F.J.T.); CZIF2022-007488 from the CZI Foundation (F.J.T., S.A.T., M.D.L. and K.B.M.); European Respiratory Society and European Union's Horizon 2020 Research and Innovation Programme under Marie Sklodowska-Curie grant agreement number 847462 (to J.G.-S. and A.J.O.); and Fondation de l'Institut Universitaire de Cardiologie et de Pneumologie de Québec (to Y.B.). We thank E. Spiegel from the Core Facility Statistical Consulting at the Helmholtz Center Munich Institute of Computational Biology for statistical consulting.

Author contributions

L.S., D.C.S. and C.R.-S. performed disease analysis on the extended HLCA. M.D.L., L.S. and D.C.S. performed integration benchmarking. T.E.G. performed bulk deconvolution. A.F. helped with bulk deconvolution. L.S. and L.Z. performed gene set enrichment analyses. L.S. performed all of the other analyses on the integrated data. M.D.L., A.V.M., M.C.N., L.S. and F.J.T. wrote the manuscript. M.D.L., A.V.M., M.C.N., L.S. and F.J.T. supervised analysis of the integrated data. M. Berg, L.A., T.M.K., L.-E.Z., C.J.T., M.-J.A., J.G.-S., C.H.M., L.P., M.C., T.S.K., E.C., B.H.K., L.B.W., C.M., Y.C. and A.A. generated unpublished data. N.E.B., P.B., T.E.D., K.B.M., A.V.M., M.C.N., H.B.S., P.R.T., J.L.S., C.S.F., J.A.K., S.A.T., M.J., M.V.B., S.L., K.Z., X.S., J.S.H. and G.P. supervised the unpublished data generation and analysis. N.E.B., M.D.L., A.V.M., M.C.N., L.S., J.A.K., E.M. and L.-E.Z. annotated the integrated HLCA core. M.A., M. Berg, A.C.A.G., N.S.M., L.-E.Z., C.H.M., A.C., C.B., T.S.K., E.C., P.K.L.M., A.J.G., L.T.B. and M. Banchero preprocessed an unpublished dataset. E.M., C.B., N.D.J., K.J.T., T.T., A.W.-C., M.Y., K.B.W., P.K.L.M., M.G., L.T.B., A.J.O., M.V.B. and L.B.W. gathered sample and patient metadata. N.D.J., K.J.T., N.H. and A.J.G. realigned the published data. M.D.L., L.S., D.C.S., L.Z., C.R.-S., L.H., L.D., C.D.D., I.L.I. and C.T.-L. curated the data. L.S., T.W., N.S.M. and C.B. set up a shareable Cell Ranger pipeline. M.L. provided scArches support. L.S. and M.v.P. set up an automated scArches mapping pipeline. C.X. set up CellTypist automated annotation for public use. K.I. performed gene name harmonization. W.T., Y.B. and D.D.S. contributed published bulk RNA-seq data. Y.J. generated and assessed the quality of batch-corrected expression matrices. All authors reviewed the manuscript.

Funding

Open access funding provided by Helmholtz Zentrum München - Deutsches Forschungszentrum für Gesundheit und Umwelt (GmbH).

Competing interests

P.R.T. serves as a consultant for Surrozen, Cellarity and Celldom and is currently acting Chief Executive Officer of Iolux. F.J.T. consults for Immunai, Singularity Bio, CytoReason and Omniscope and has ownership interest in Dermagnostix and Cellarity. In the past 3 years, M.D.L. was a contractor for the CZI and received remuneration for talks at Pfizer and Janssen Pharmaceuticals. J.A.K. reports grants/contracts from Boehringer Ingelheim and Bristol Myers Squibb, consulting fees from Janssen and Boehringer Ingelheim and study support from Genentech and is a member of the scientific advisory board of APIE Therapeutics. In the past 3 years, S.A.T. has received remuneration for consulting and Scientific Advisory Board membership from Genentech, Roche, Biogen, GlaxoSmithKline, Foresite Labs and Qiagen. S.A.T. is a co-founder and board member of and holds equity in Transition Bio. D.S. is a founder of Pliant Therapeutics and a member of the Genentech Scientific Advisory Board and has a sponsored research agreement with AbbVie. N.K. served as a consultant to Boehringer Ingelheim, Third Rock, Pliant, Samumed, NuMedii, Theravance, LifeMax, Three Lakes Partners, Optikira, AstraZeneca, RohBar, Veracyte, Augmanity, CSL Behring, Galapagos and Thyron over the past 3 years and reports equity in Pliant and Thyron, grants from Veracyte, Boehringer Ingelheim and Bristol Myers Squibb and nonfinancial support from miRagen and AstraZeneca. N.K. owns intellectual property on novel biomarkers and therapeutics in IPF licensed to biotechnology. O.R.-R. is a co-inventor on patent applications (PCT/US2016/059233, PCT/US2018/064553, PCT/US2018/060860, PCT/US2017/016146, PCT/US2019/055894, PCT/US2018/064563, PCT/US2020/032933) filed by the Broad Institute for inventions related to single-cell genomics. O.R.-R. has been an employee of Genentech since 19 October 2020 and has equity in Roche. O.E. serves in an advisory capacity to Pieris Pharmaceuticals, Blade Therapeutics, Delta 4 and YAP Therapeutics. Y.B. holds a Canada

Research Chair in the Genomics of Heart and Lung Diseases. The remaining authors declare no competing interests.

Additional information

Extended data is available for this paper at https://doi.org/10.1038/s41591-023-02327-2.

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41591-023-02327-2.

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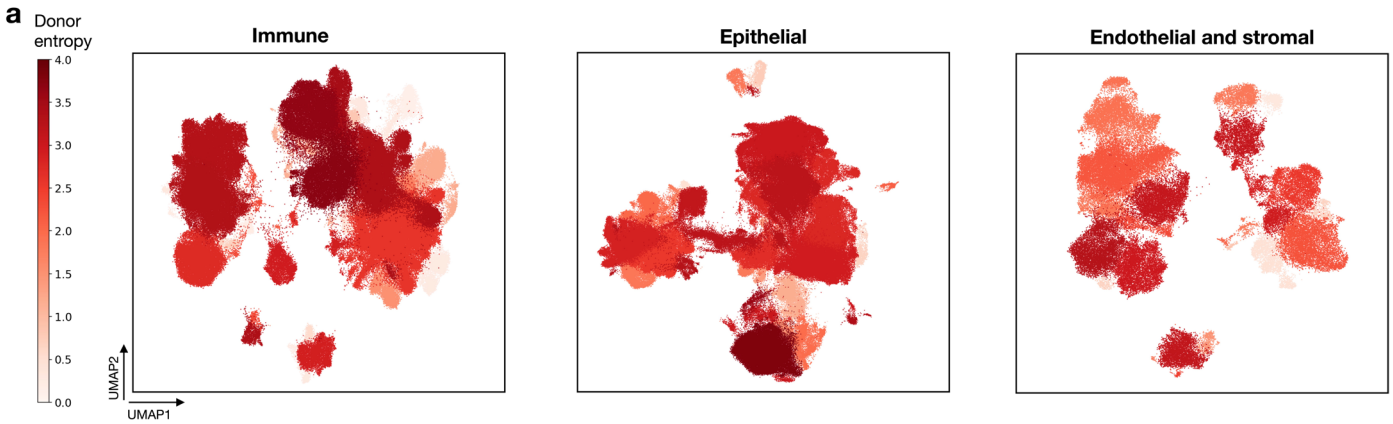
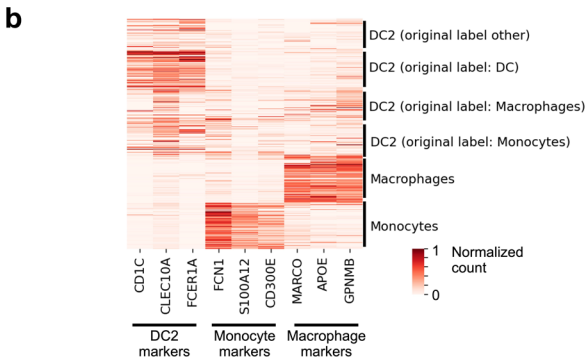
Peer review information *Nature Medicine* thanks Shalev Itzkovitz, Deyou Zheng and the other, anonymous, reviewer(s) for their contribution to the peer review of this work. Primary Handling Editor: Anna Maria Ranzoni, in collaboration with the *Nature Medicine* team.

https://doi.org/10.1038/s41591-023-02327-2





Extended Data Fig. 1 | HLCA cluster donor diversity and marker expression for a cluster with high cell type label disagreement. a, Donor diversity is calculated for every cluster as entropy of donor proportions in the cluster, with high entropy indicating the cluster contains cells from many different donors. Most clusters (80 out of 94) contain cells from many donors (median 47 donors per cluster, range 2–102), as illustrated by high donor entropy (>0.43), whereas 14 clusters show low donor diversity. These are largely immune cell clusters (n=13, of which 7 macrophage clusters, 4 T cell clusters and 2 mast cell clusters), representing donor- or group-specific phenotypes. Matching cell type annotations are shown in Fig. 3d. **b**, Marker expression among cells from the immune cluster with highest disagreement in original cell type labels (high 'label entropy'). DC2, monocyte and macrophage marker expression is shown for cells from Fig. 3c. Cells are labeled by their final annotation, as well as their original label. Log-normalized counts are scaled such that for each gene the 99th expression percentile, as calculated among all cells included in the heatmap, is set to 1. DC: dendritic cell.

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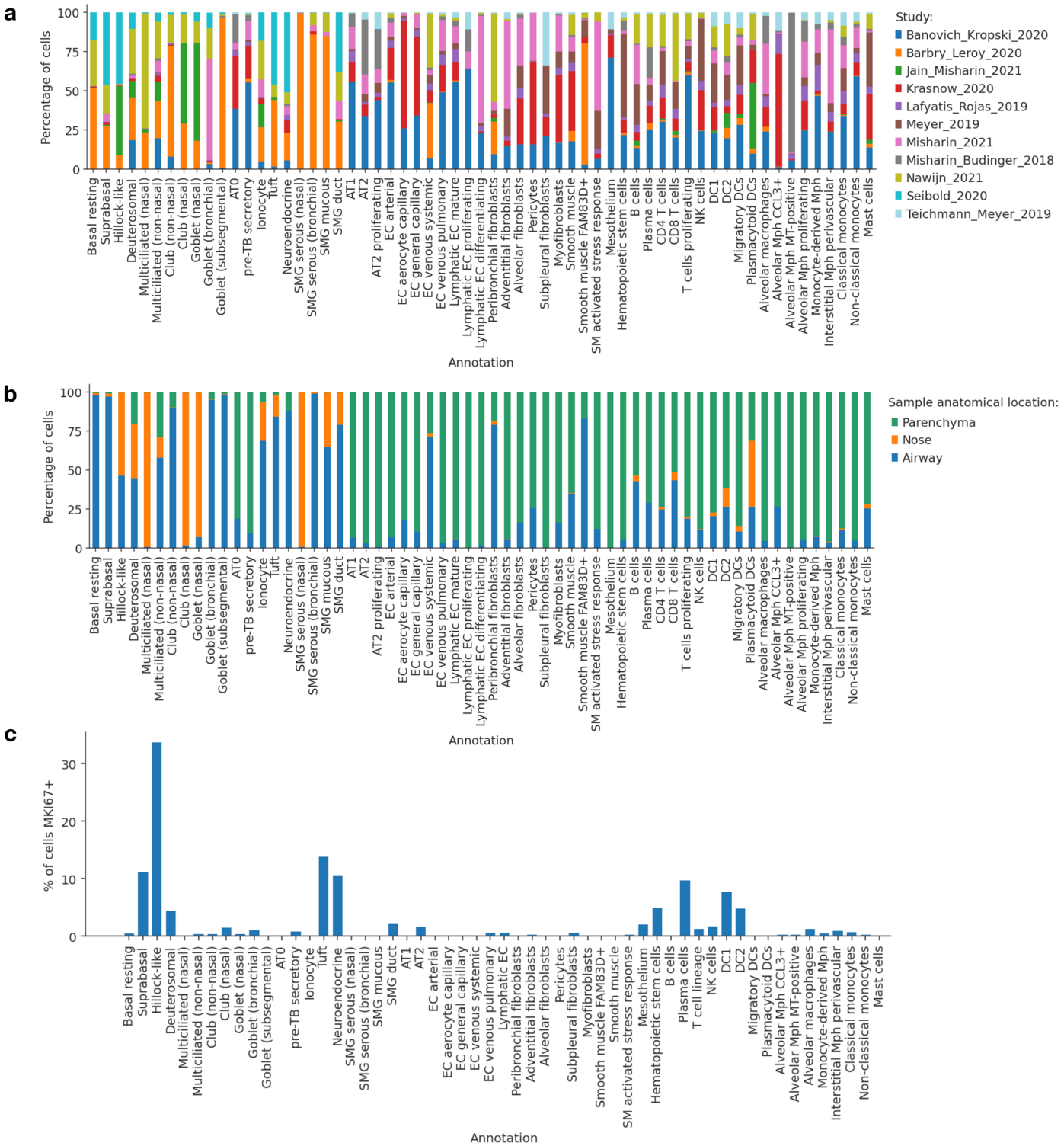

Extended Data Fig. 2 | HLCA core cell type composition details. a, Percentage of cells from each of the 11 studies included in the HLCA core, shown per cell type (3 studies include 2 separate datasets). Each cell type was detected in at least 4 out of 14 datasets, with a median of 11 datasets in which a cell type was detected, and a maximum of 14. **b**, Percentage of cells from each of the three anatomical locations, shown per cell type. **c**, Percentage of cells with at least one UMI count for *MKI67*, a marker gene of proliferating cells, shown per cell type. AT: alveolar type. TB: terminal bronchiole. SMG: submucosal gland. DC: dendritic cell. Mph: macrophage. NK: natural killer. MT: metallothionein. SM: smooth muscle. EC: endothelial cell.

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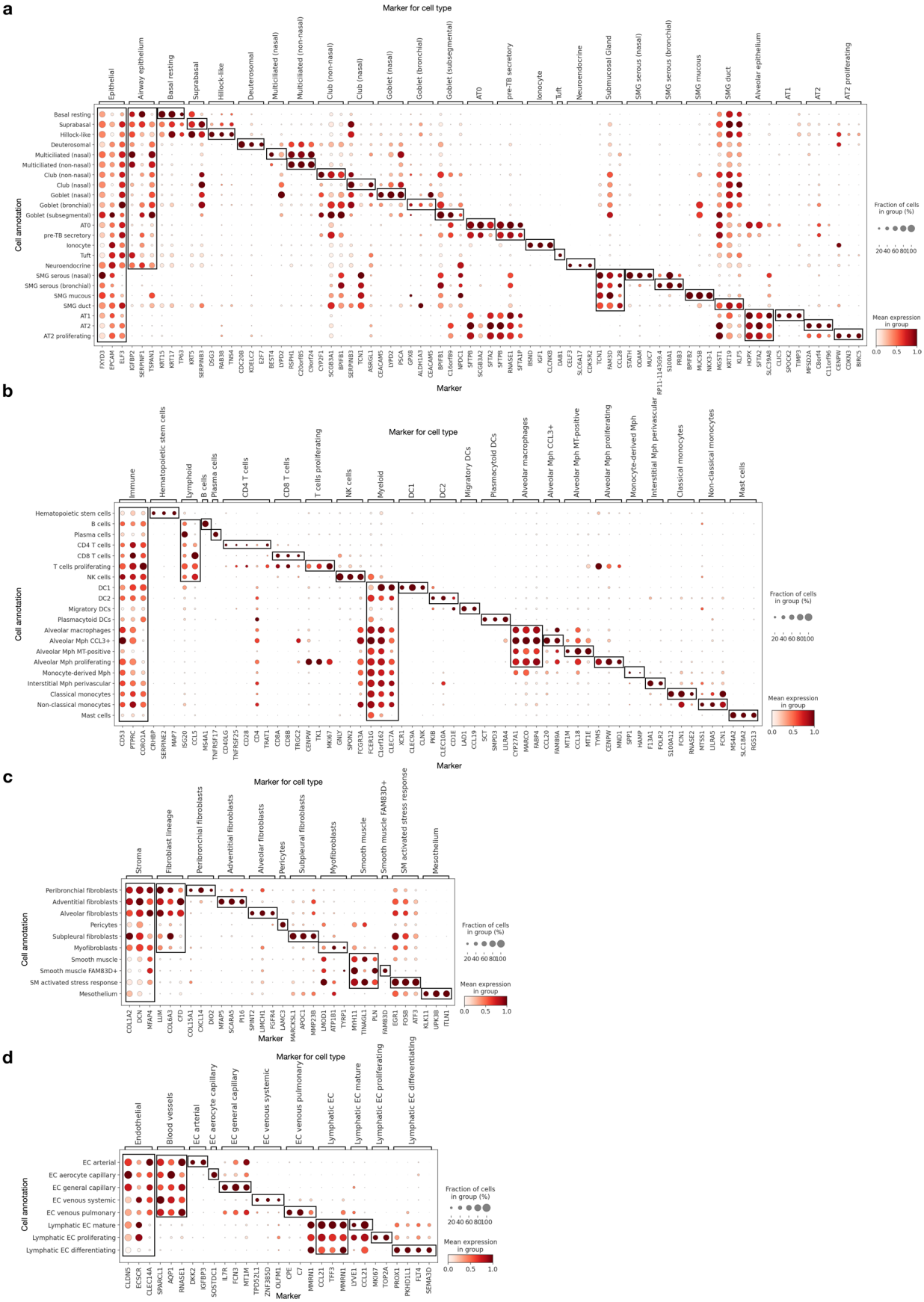

Extended Data Fig. 3 | See next page for caption.

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Extended Data Fig. 3 | Marker gene expression for all 61 cell types in the HLCA core. Expression is shown within each cell type compartment. **a**, Epithelial cell type markers, **b**, Immune cell type markers, **c**, Stromal cell type markers, **d**, Endothelial cell type markers. Expression was normalized such that the maximum group expression of cells within the compartment for each marker was set to 1. Marker gene sets include both sets that mark groups of cell types (for example 'epithelial') and single cell types (for example 'basal resting'). For each marker gene set, cell types identified by the set are boxed. AT: alveolar type. TB: terminal bronchiole. SMG: submucosal gland. DC: dendritic cell. Mph: macrophage. NK: natural killer. MT: metallothionein. SM: smooth muscle. EC: endothelial cell.

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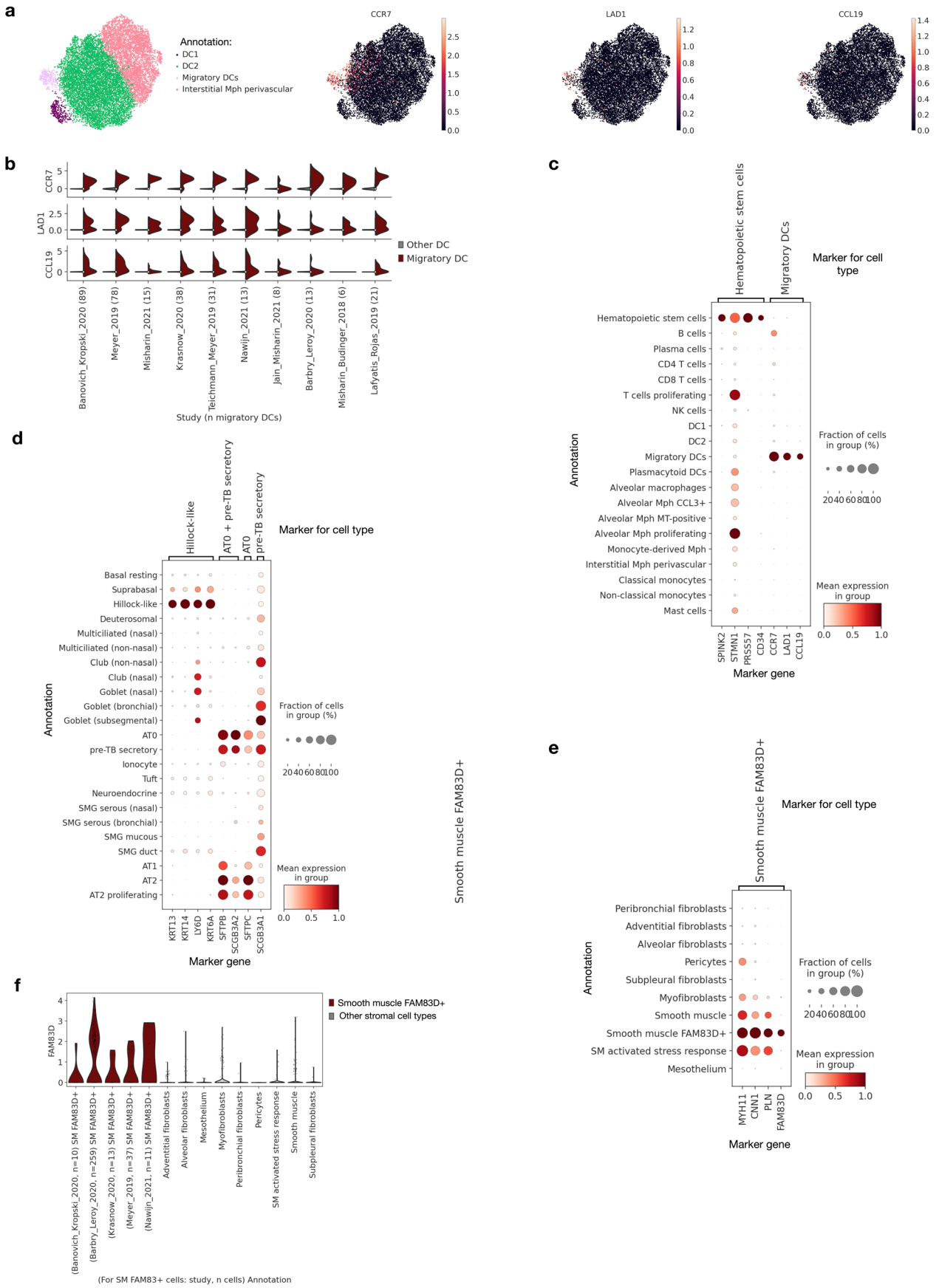

Extended Data Fig. 4 | See next page for caption.

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Extended Data Fig. 4 | Marker expression of several rare and novel cell types detected in the HLCA. a, A UMAP embedding of all cells annotated as dendritic cells, colored by final detailed annotation (left), and by expression of three migratory DC marker genes (right, *CCR7*, *LAD1*, and *CCL19*). **b**, Expression of migratory DC marker genes from **a** among migratory DCs (red, right half of violins) versus other DCs (gray, left half of violins), split by study. Number of migratory DCs per study is specified in the x-axis labels. **c**, Expression of markers for two novel immune cell types (hematopoietic stem cells and migratory DCs, found in 9 and 10 out of 11 studies, respectively), shown per stromal cell type. **d**, Expression of markers for three novel epithelial cell types (hillock-like, AT0, and pre-TB secretory cells, found in 9, 9, and 11 out of 11 studies, respectively), shown per epithelial cell type. Two markers shared between AT0 and pre-TB secretory cells are also included. **e**, Expression of markers for a novel stromal cell type ('smooth muscle FAM83D+', found in 8 out of 11 studies), including three general smooth muscle marker genes and one marker gene uniquely expressed in FAM83D+ smooth muscle cells (*FAM83D*), shown per stromal cell type. For **c**-**e**, gene counts were normalized such that the maximum expression of a group of cells in the plot was set to 1. **f**, *FAM83D* expression across stromal cell types. Cells annotated as FAM83D+ smooth muscle are split by study. Studies with fewer than 3 smooth muscle FAM83D+ cells are not shown. DC: dendritic cell. Mph: macrophage. MT: metallothionein. AT: alveolar type. SMG: submucosal gland. TB: terminal bronchiole.

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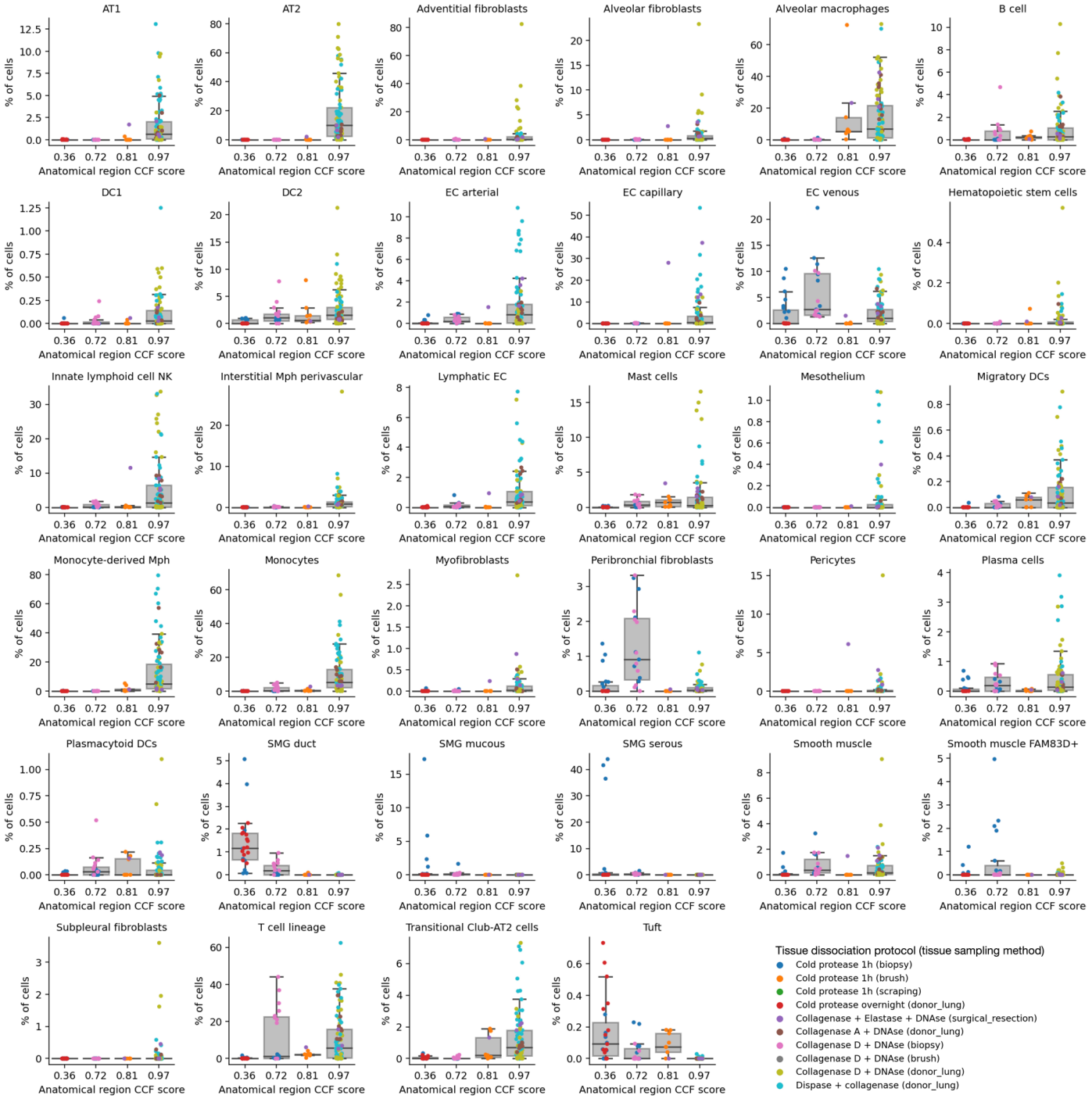

Extended Data Fig. 5 | Cell type proportions per sample along the proximal-to-distal axis of the lung. All cell types not included in Fig. 4b are shown. The lowest and highest CCF score shown (0.36, 0.97) represent the most proximal and most distal sampled parts of the respiratory system, respectively (trachea and parenchyma), excluding the upper airways. Dots are colored by the tissue dissociation protocol and tissue sampling method used for the sample. Boxes show median and interquartile range of the proportions. Samples with proportions more than 1.5 times the interquartile range away from the high and low quartile are considered outliers. Whiskers extend to the furthest non-outlier point. n=23, 19, 9 and 90 for CCF score 0.36, 0.72, 0.81 and 0.97, respectively. AT: alveolar type. DC: dendritic cell. EC: endothelial cell. NK: natural killer. Mph: macrophages. SMG: submucosal gland.

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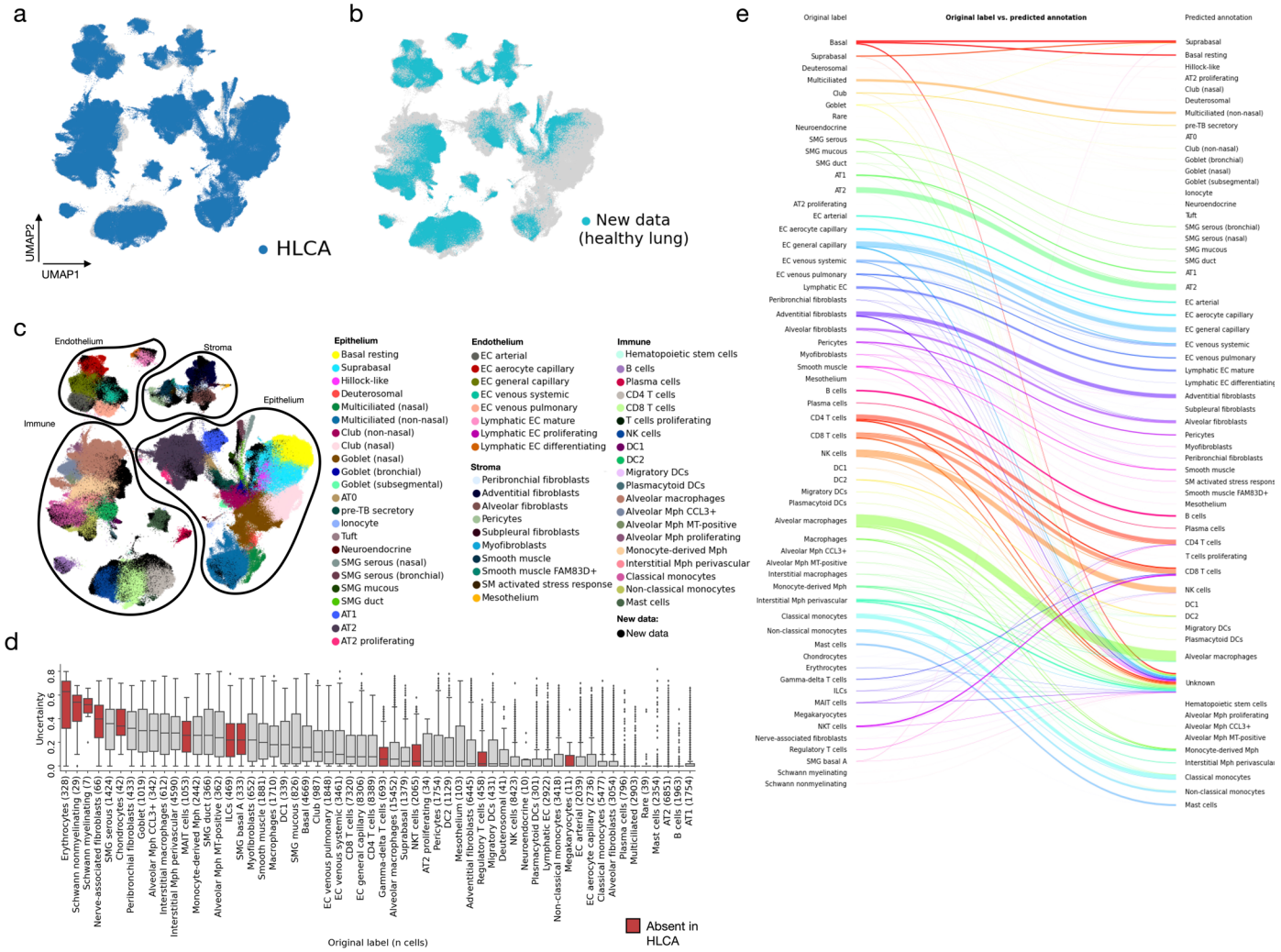

Extended Data Fig. 6 | Mapping of unseen healthy lung scRNA-seq data to the HLCA core. a, UMAP of the jointly embedded HLCA core (dark blue, plotted on top) and the newly mapped healthy lung data (gray). **b**, Same as a, but now plotting cells from the HLCA in gray, and cells from the new data on top in light blue. **c**, Same as a, but now coloring cells from the HLCA core by their final annotation, and coloring cells from the new data in black. Cells from each of the compartments are outlined to ease visual identification of cell types by colors. **d**, Uncertainty of label transfer (ranging from 0 to 1) for cells from the mapped data, subdivided by original cell type label. Number of cells per label is shown between brackets. Cell labels are ordered by mean uncertainty. Boxes of cell labels not present in the HLCA core are colored red. Boxes show median and interquartile range of uncertainty. Cells with uncertainties more than 1.5 times the interquartile range away from the high and low quartile are considered outliers and plotted as points. Whiskers extend to the furthest non-outlier point. **e**, Sankey plot of original labels of cells from the mapped dataset versus predicted annotations based on label transfer. Cells with uncertainty >0.3 are labeled 'unknown'. AT: alveolar type. DC: dendritic cells. EC: endothelial cells. ILCs: innate lymphoid cells. MAIT cells: mucosal-associated invariant T cells. MT: metallothionein. Mph: macrophages. NK: natural killer. NKT cells: natural killer T cells. SM: smooth muscle. SMG: submucosal gland. TB: terminal bronchiole.

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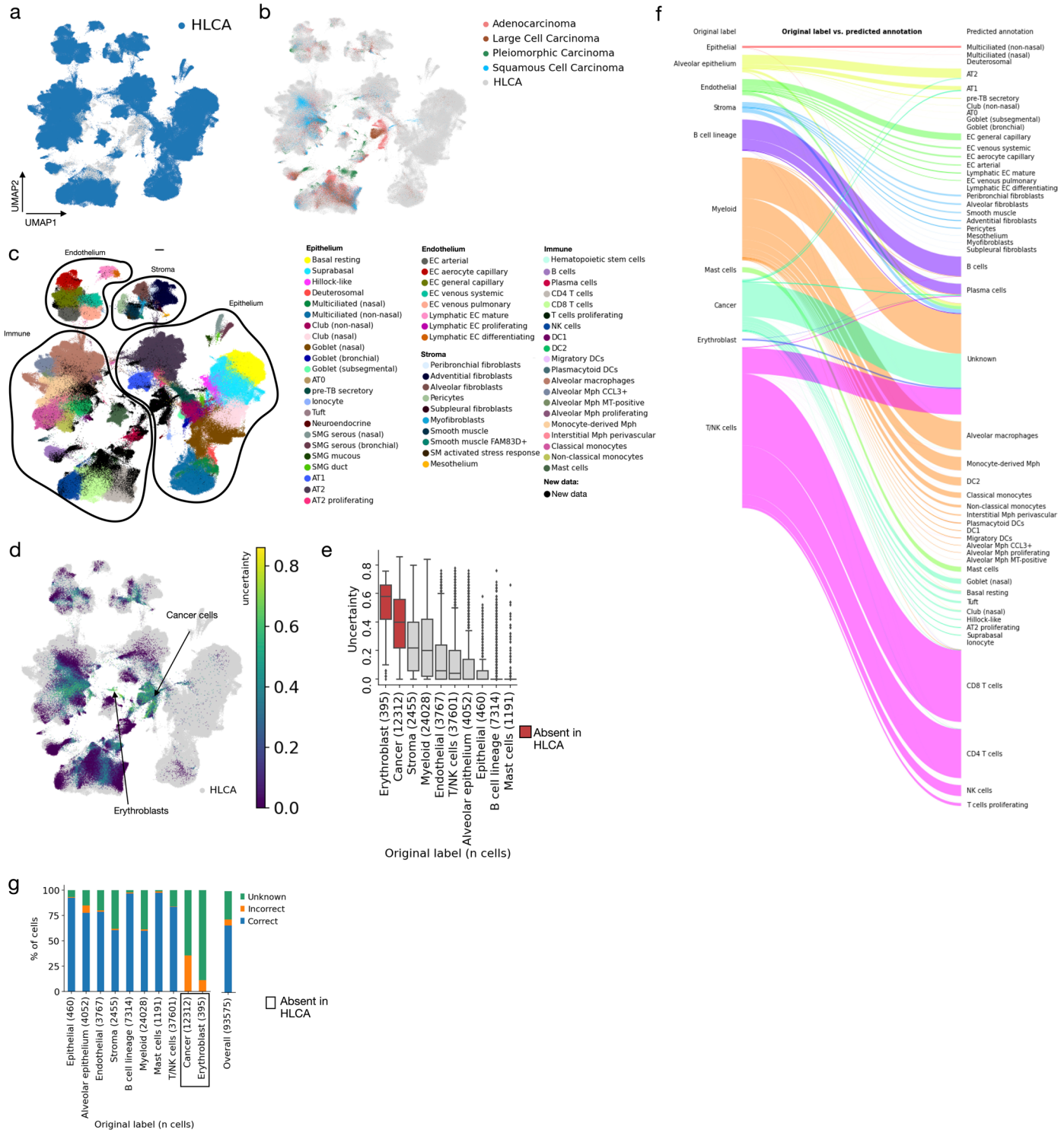

Extended Data Fig. 7 | See next page for caption.

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Extended Data Fig. 7 | Mapping of unseen lung cancer data to the HLCA. a, UMAP of the jointly embedded HLCA (dark blue, plotted on top) and lung cancer data (gray). **b**, Same as a, but now plotting cells from the HLCA core in gray. Cells from the mapped data are plotted on top, and colored by the cancer type of the patient. **c**, Same as **a**, but now coloring cells from the HLCA core by their final final annotation, and coloring cells from the mapped cancer data in black. Cells from each of the compartments are outlined to ease visual identification of cell types by colors. **d**, Uncertainty of label transfer, shown for all cells from the mapped data. Regions dominated by high-uncertainty cells are labeled by the original cell type label. Cells from the HLCA core are colored in gray. **e**, Uncertainty of label transfer (ranging from 0 to 1) for the mapped cells, subdivided by original cell type label. Number of cells per label is shown between brackets. Boxes of cell type labels not present in the HLCA core are colored red. Cell types are ordered by mean uncertainty. Boxes show median and interquartile range of uncertainty. Cells with uncertainties more than 1.5 times the interquartile range away from the high and low quartile are considered outliers and plotted as points. Whiskers extend to the furthest non-outlier point. **f**, Sankey plot of original labels of the mapped data versus predicted annotations based on label transfer. Cells with uncertainty >0.3 are labeled 'unknown'. **g**, Percentage of cells from newly mapped healthy lung dataset that are either annotated correctly or incorrectly by label transfer annotation (matched at the level of the original labels), or annotated as unknown, subdivided by original cell type label. The number of cells in the mapped dataset labeled with each label are shown between brackets after cell type names. Cell type labels not present in the HLCA are boxed. AT: alveolar type. DC: dendritic cells. EC: endothelial cells. MT: metallothionein. Mph: macrophages. NK: natural killer. SM: smooth muscle. SMG: submucosal gland. TB: terminal bronchiole.

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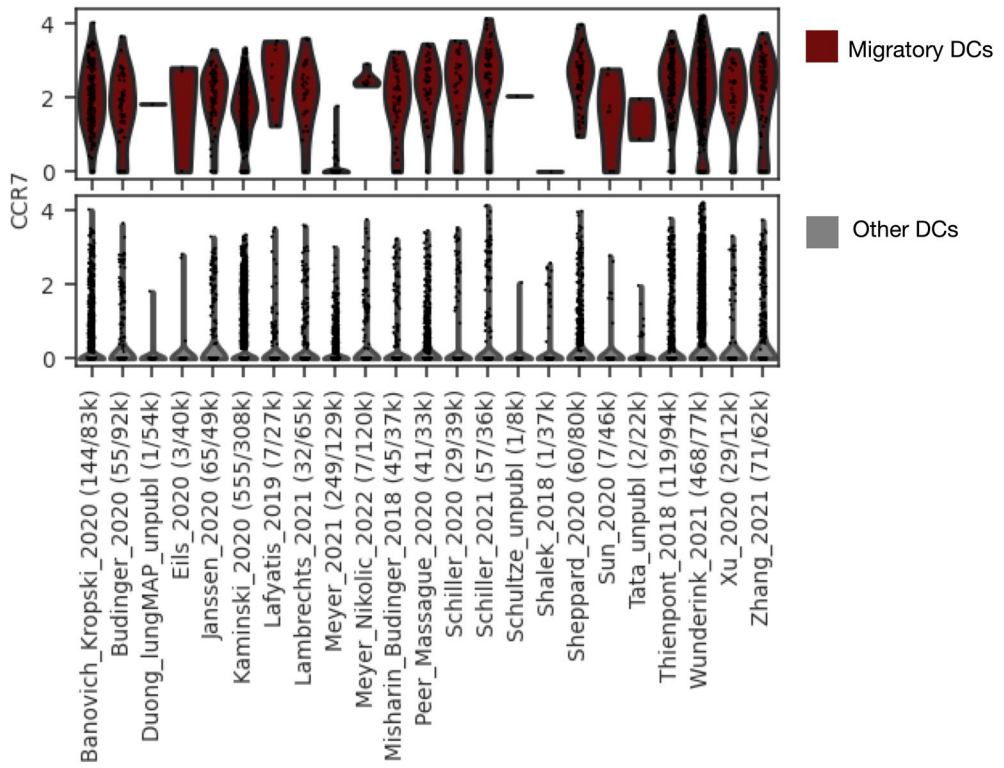

Extended Data Fig. 8 | Expression of *CCR7* among cells annotated as migratory DCs by label transfer. Expression of *CCR7* is shown for all cells that were annotated as migratory DCs with low uncertainty (<0.2) (top) and all other cells annotated as DC (bottom) by label transfer from the HLCA core to the extended HLCA. Cells are grouped based on study of origin (some studies contain multiple datasets). X-tick labels show study, number of cells annotated as migratory DCs, and number of total cells (in thousands) per study. *CCR7* counts shown are counts that were normalized based on the total count among 2000 genes used for mapping to the HLCA core, and then log-transformed. DCs: dendritic cells.

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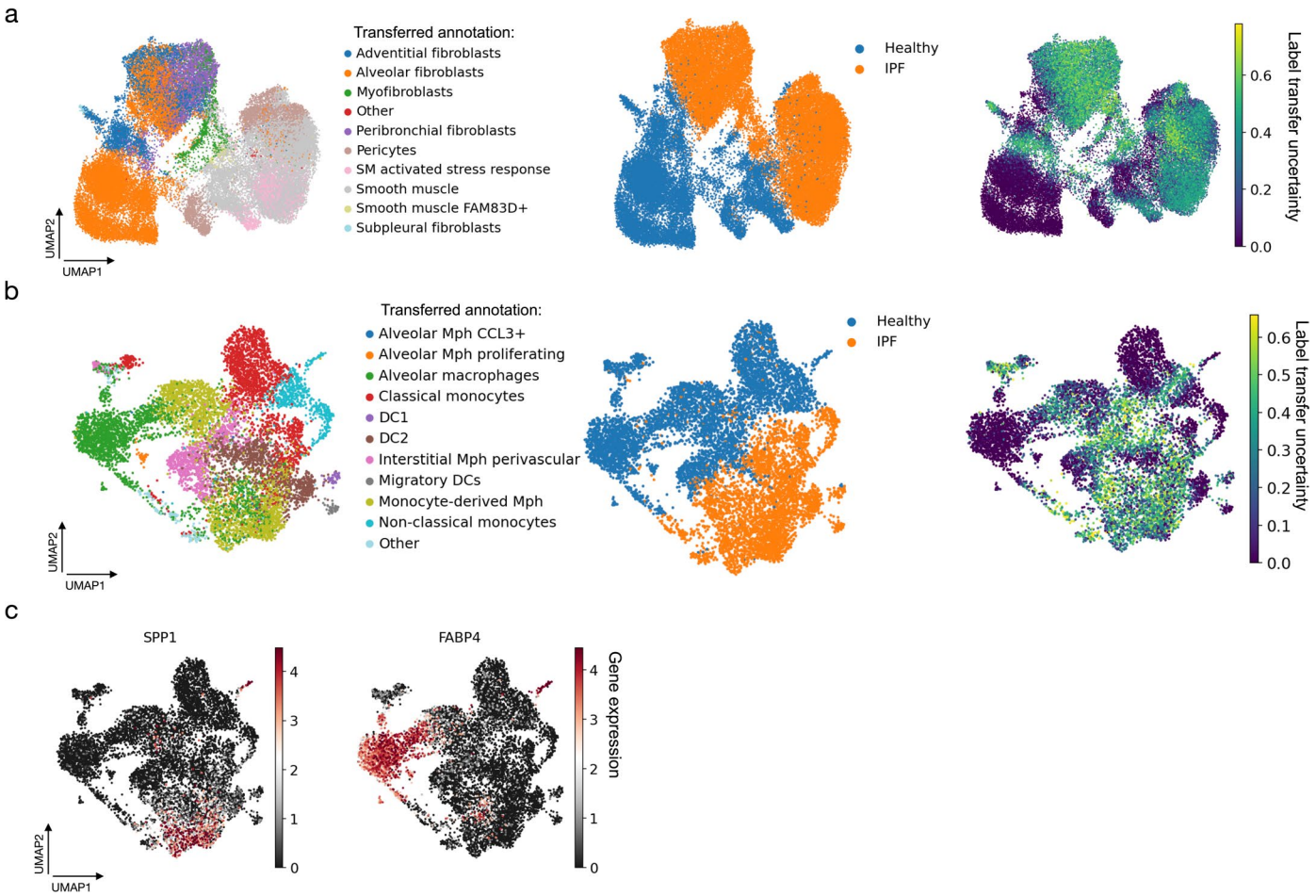

Extended Data Fig. 9 | Transferred labels and matching uncertainty for a mapped IPF dataset. a, UMAPs of cells originally labeled as stroma, from a mapped IPF dataset[56] including both healthy and IPF samples. Cells are labeled by annotation transferred from the HLCA core (left), by disease status (middle), and by label transfer uncertainty (right). Cells with labels transferred to fewer than 10 cells were excluded. **b**, same as **a**, but showing cells originally labeled as macrophages. **c**, As **b**, but now colored by expression of *SPP1* and *FABP4*. SM: smooth muscle. Mph: macrophages. DC: dendritic cells.

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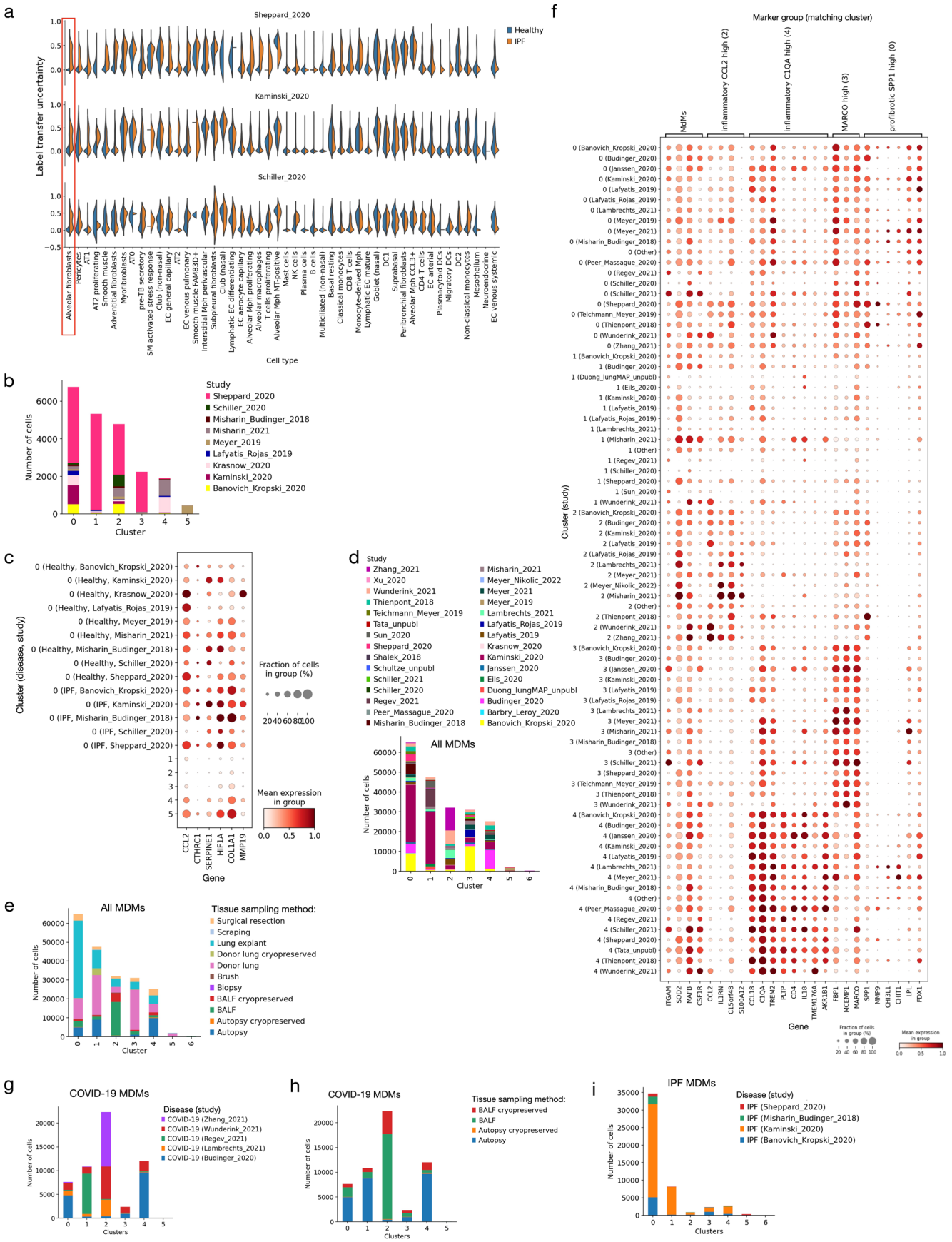

Extended Data Fig. 10 | See next page for caption.

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Extended Data Fig. 10 | Disease-specific cellular states and states shared across diseases in the extended HLCA. a, Label transfer uncertainty shown per cell type, comparing cells from control samples ('healthy', blue) to cells from IPF samples (orange). Results are shown per dataset, only showing datasets that include both control and IPF mapped samples. Alveolar fibroblasts, the cell type chosen for downstream analysis, are boxed in red. AT: alveolar type. DC: dendritic cell. TB: terminal bronchiole. EC: endothelial cell. Mph: macrophage. MT: metallothionein. NK: natural killer. SM: smooth muscle. **b**, Composition of alveolar fibroblast clusters by study. **c**, Expression of several genes highly expressed in IPF-enriched alveolar fibroblast cluster 0, shown per cluster. Cluster 0 is split into control ('Healthy') and IPF, further subdivided by study. **d**, Composition of monocyte-derived macrophage (MDM) clusters by study. **e**, As **d**, but by tissue sampling method. **f**, Expression of MDM cluster marker genes shown per cluster, with clusters split into studies. Studies with fewer than 200 were grouped into 'Other' for each cluster. **g**, Composition of MDM clusters by study, subsetted to only cells from donors with COVID-19. **h**, As **g**, but by tissue sampling method. **i**, As **g**, but subsetted to cells from donors with IPF. For **c** and **f**, mean expressions were normalized such that the highest mean expression was set to 1 for each gene. BALF: bronchoalveolar lavage fluid. IPF: idiopathic pulmonary fibrosis.

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Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection	No software was used for data collection.
Data analysis	All code is made public on GitHub, as also specified in the manuscript. https://github.com/LungCellAtlas/HLCA_reproducibility. Software versions used: R 4.1.1 (covariate modeling) edgeR: 3.28.1 lme4: 1.1-27.1 LDSC: 1.0.1 Limma: 3.46.0, R 4.0.3 (GSEA) Scanpy: 1.9.1 scArches: 0.3.5 scIB: 0.1.1 scikit-learn: 0.24.1 scvi-tools (scANVI): 0.8.1

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Portfolio guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A description of any restrictions on data availability
- For clinical datasets or third party data, please ensure that the statement adheres to our policy

There are no restrictions on data availability. The HLCA is fully public.
Data Availability Statement
The HLCA (raw and normalized counts, integrated embedding, cell type annotations and clinical and technical metadata) is publicly available and can be downloaded via cellxgene:
https://cellxgene.cziscience.com/collections/6f6d381a-7701-4781-935c-db10d30de293
The HLCA core reference model and embedding for mapping of new data to the HLCA can moreover be found on Zenodo, doi: 10.5281/zenodo.7599104.

The original, published datasets that were included in the HLCA can also be accessed under GEO accession numbers GSE135893, GSE143868, GSE128033, GSE121611, GSE134174, GSE150674, GSE151928, GSE136831, GSE128169, GSE171668, GSE132771, GSE126030, GSE161382, GSE155249, GSE135851, GSE145926, GSE178360, EGA study IDs EGAS00001004082, EGAS00001004344, EGAD00001005064, EGAD00001005065, and under urls https://www.synapse.org/#!Synapse:syn21041850, https://data.humancellatlas.org/explore/projects/c4077b3c-5c98-4d26-a614-246d12c2e5d7, https://www.ncbi.nlm.nih.gov/projects/gap/cgi-bin/study.cgi?study_id=phs001750.v1.p1, https://www.nupulmonary.org/covid-19-ms2/?ds=full&meta=SampleName, https://figshare.com/articles/dataset/Single-cell_RNA-Seq_of_human_primary_lung_and_bronchial_epithelium_cells/11981034/1, https://covid19.lambrechtslab.org/downloads/Allcells.counts.rds, https://s3.amazonaws.com/dp-lab-data-public/lung-development-cancer-progression/PATIENT_LUNG_ADENOCARCINOMA_ANNOTATED.h5, https://github.com/theislab/2020_Mayr, https://static-content.springer.com/esm/art%3A10.1038%2Fs41586-018-0449-8/MediaObjects/41586_2018_449_MOESM4_ESM.zip, http://blueprint.lambrechtslab.org/#/099de49a-cd68-4db1-82c1-cc7acd3c6d14/*/welcome, https://www.covid19cellatlas.org/index.patient.html (see also Supplementary Data Table 1).
GWAS summary statistics of COPD(GWAS catalog ID: GCST007692, dbGaP accession number: phs000179.v6.p2), IPF, and of lung adenocarcinoma(GWAS catalog ID: GCST004748, dbGaP accession number: phs001273.v3.p2) were made available on dbGap upon request. Summary statistics of lung function (GWAS catalog ID: GCST007429), of asthma (GWAS catalog ID: GCST010043), and of depression (used as negative control, GWAS catalog ID: GCST005902) were publicly available.

Human research participants

Policy information about studies involving human research participants and Sex and Gender in Research.

Reporting on sex and gender	For each of the previously unpublished datasets included in the paper, the methods state whether sex was self-reported or assigned based on a medic's report. Disaggregated data of subjects' sex is available in the publicly available human lung cell atlas, which includes per-sample (and per-cell) metadata. Information about sex can moreover be found in Supplementary Data table 2, which includes per-sample metadata. Consent was obtained for obtaining and sharing patient data for each study, as indicated in the methods. Effects of sex on cell type transcriptomes were modeled, for which results are shown in figure 4, as well as in Supplementary data table 8 and 9. Overall male/female proportions among subjects included in the HLCA core are shown in figure 2a and specified in the caption (60% male, 40% female).
Population characteristics	This information can be found in Supplementary Data table 2 (sex, age, BMI, smoking history, lung disease). Statistics for the HLCA core are moreover stated in the text: "These datasets include samples from 107 individuals, with diversity in age, sex, ethnicity (harmonized as detailed in Methods), BMI, and smoking status (fig. 2a)." and in the caption of figure 2a: "Donors show diversity in ethnicity (harmonized metadata proportions 65% European, 14% African, 2% Admixed American, 2% mixed, 2% Asian, 0.4% Pacific Islander, 14% unannotated, see Methods)". Further notes on the encoding of ethnicity can be found in the methods: "Ethnicity metadata was based on self-reported ethnicity for live donors, or retrieved from medical records or assigned by the organ procurement team in case of organ donors, as collected in the individual studies. For donor ethnicity, the following categories of self-reported ethnicity were used during metadata collection: black, white, latino, asian, pacific islander, and mixed. To conform to pre-existing 1000 genomes ancestry “superpopulations”, these self-reported ethnicity categories were then harmonized with the superpopulation categories as follows: black was categorized as African, white as European, latino as admixed American, while keeping the category “Asian” (merging superpopulations “East Asians” and “South Asians'' as this granularity was missing from the collected self-reported ethnicity data), and keeping “Pacific Islander”, as this category did not correspond to any of the superpopulations but does constitute a separate population in HANCESTRO. We refer to the resulting categories as “harmonized ethnicity”. Both self-reported ethnicity as collected and harmonized ethnicity per donor are detailed in Supplementary Data Table 2."
Recruitment	Recruitment was done in individual studies as published, recruitment for unpublished data: Banovich_Kropski_2020: Primary tissue was obtained from the Donor Network of Arizona or Tennessee Donor Services. All samples were collected from declined organ donors who were also consented for research. Only lungs with no known lung disease were used in this study.

Barbry_unpubl: Our IPF volunteers patients were selected from a prospective cohort of 180 IPF patients.
Patients involved in this paper respected all these Inclusion and non-inclusion criteria.
Inclusion criteria
Age > 18 years
Diagnosis of idiopathic pulmonary fibrosis made less than 5 years ago on scannographic and/or histological criteria and validated in an interstitial pathology consultation meeting according to the ATS/ERS/JRS/ALAT 2018 recommendations (Raghu G, Remy-Jardin M, Meyers JL, Richeldi L, Ryerson CJ, Lederer DJ, et al. Diagnosis of idiopathic pulmonary fibrosis: an official ATS/ERS/JRS/ALAT clinical practice guideline. Am J Respir Crit Care Med 2018;198:e44-e68.).
GAP Index 1 or 2 (Ley, B. et al. A Multidimensional Index and Staging System for Idiopathic Pulmonary Fibrosis. Ann Intern Med 156, 684-U658 (2012))
FVC > 50% of theoretical
DLCO > 35% of theoretical
Non-smoker (active or passive) or ex-smoker of less than 20 pack-years and stopped for more than 5 years
No current acute pathology at inclusion
No symptoms suggestive of a progressive pathology being diagnosed
Patient with a chest CT scan in the year prior to inclusion
Woman of childbearing age using effective contraception
Patient with written consent
Non-inclusion criteria:
Recent ENT or bronchial infection (< 6 weeks)
Long-term systemic corticosteroid therapy regardless of the reason for prescription
Systemic corticosteroid therapy in the previous 3 months
Patient on long-term oxygen therapy
Chronic cardiovascular, neuro-psychic or metabolic pathology in progress, clinically significant or not controlled during the last 6 months
Other associated chronic respiratory pathology (COPD, asbestosis, bronchiectasis, etc.)
Patient on anti-platelet agents or other anticoagulant at risk of bleeding during sampling
Patient with a history of cancer in the previous 5 years, excluding basal cell disease
Patient with a history of clinically significant (i.e. recurrent or loss of consciousness) vagal discomfort
History of allergy or intolerance to xylocaine and/or propofol
History of significant epistaxis (i.e. recurrent epistaxis of any amount or at least one severe epistaxis)
Patient at risk of difficult intubation according to the criteria of the SFAR 2006 expert conference*.
Relationship between volunteer and investigator
Patient not socially insured
Mental disability
Pregnant woman (a urine test will be carried out for all women of childbearing age) or nursing mother
Vulnerable person (person deprived of administrative and legal freedom).
In addition to the respect of all these inclusion criteria, a nasal swab analysis was made for each patient at the beginning of the procedure and analyzed for viruses (tested for 22 pathogens (RespiFinder® 2Smart). Virus: Influenza A, Influenza B, Influenza A(H1N1)pdm09, RSV-A, RSV-B, Human Metapneumovirus, Rhinovirus/Enterovirus, Adenovirus, Parainfluenza-1, Parainfluenza-2, Parainfluenza-3, Parainfluenza-4, Bocavirus, Coronavirus NL63/HKU1, Coronavirus OC43, Coronavirus 229E, SARS-CoV-2, MERS CoV, Bacteria: Mycoplasma pneumoniae, Chlamydophila pneumoniae, Legionella pneumophila, Bordetella pertussis). None of the patients selected had inhaled treatment.

Duong_HuBMAP_unpubl: The dataset includes a single donor: an organ donor who was a 37 year-old black male with a history of marihuana, with no lung disease.

Jain_Misharin_2021: Healthy volunteers were recruited to match a cohort of patients with cystic fibrosis for the ongoing study at Northwestern University (PI Manu Jain). In both studies Dr. Misharin did not influence participant recruitment and did not introduce biases in sample selection.

Misharin_2020: Donor Lungs. Samples were collected in an opportunistic manner, based on sample availability and organ allocation to Northwestern Lung Transplant center. One donor was rejected for organ transplant. For the other donor, samples were collected during donor lung for size reduction during lung transplantation.

Nawijn_2021: Subjects and methods
Recruitment was performed through advertisements in the local newspaper.
Inclusion criteria:
• Age between 18 and 45 years old.
• Smoking history ≤2 packyears and no smoking during the 6 months before inclusion
• No history of asthma.
• No use of inhaled corticosteroids or β2-agonists for a period longer than 1 month in their lifetime and not during the 6 weeks before inclusion.
• No symptoms of wheeze, nocturnal dyspnea, or bronchial hyperresponsiveness.
• PC20 methacholine > 8 mg/ml, FEV1/FVC > 70% and FEV1 > 80% predicted.
Exclusion criteria
• FEV1 <1.2 L,
• Subjects must be able to adhere to the study visit schedule and other protocol requirements.
• A subject is not eligible to enter and participate if he has not signed and dated a written informed consent form prior to participation in the study.
• A subject is not eligible to enter and participate if he does not agree that we inform his general practitioner.
• Upper respiratory tract infection (e.g. colds), within 6 weeks.
• Serious acute infections (such as hepatitis, pneumonia or pyelonephritis) in the previous 3 months.
• Signs or symptoms of severe, progressive or uncontrolled renal, hepatic, hematologic, endocrine, pulmonary, cardiac, neurologic or cerebral disease.
• Malignancy within the past 5 years (except for squamous or basal cell carcinoma of the skin that has been treated with no

evidence of recurrence).
• Known recent substance abuse (drug or alcohol).
• Females of childbearing potential without an efficient contraception unless they meet the following definition of post-menopausal: 12 months of natural (spontaneous) amenorrhea or 6 months of spontaneous amenorrhea with serum FSH >40 mIU/mL or the use of one or more of the following acceptable methods of contraception:
a) Surgical sterilization (e.g. bilateral tubal ligation, hysterectomy).
b) Hormonal contraception (implantable, patch, oral, injectable).
c) Barrier methods of contraception: condom or occlusive cap (diaphragm or cervical/vault caps) with spermicidal foam/gel/cream/suppository.
d) Continuous abstinence.

Schiller_2021: Non-involved tissue from lung tumor resections was used. All fresh tissues from patients in a given timeframe without any specific selection criteria were included. Only patients with obvious chronic lung disease as comorbidity based on their lung function parameters prior to tumor resection were excluded.

Schultze_unpubl: Patients undergoing lung tumor resections. At Hannover Medical School, MHH, patients with lung cancer were recruited in the course of their operation, i.e. surgical tumor resection according to the ethical vote of the German Centre for Lung Research (DZL), ethical vote 7414 and data safety guidelines. There was no further bias in patients recruitment since the samples were collected as fresh native tissue following surgical tumor resection and availability of "surplus" adjacent non-malignant lung tissue, which was resected in parallel to the tumor tissue.

Tata_unpubl: Transplant donor tissues were collected from individuals that died from accidental death. Lungs were screened by PCR and antigen testing to exclude HIV, HCV, Burkholderia and other respiratory viruses. Sub-transplant quality transplant donor tissues were collected from individuals that died from accidental death.

Ethics oversight

Banovich_Kropski_2020: Vanderbilt IRB nos. 060165 and 171657 and Western IRB no. 20181836
Barbry_unpubl: CHU Nice, registered at clinicaltrials under reference NCT04529993.
Duong_HUBMaP_unpubl: brindl.urmc.rochester.edu/
Jain_Misharin_2021: Protocol was approved by Northwestern University IRB (STU00214826)
Misharin_2020: Protocol was approved by Northwestern University IRB (STU00212120).
Nawijn_2021: University Medical Center Groningen Institutional Review Board (ABR number NL69765.042.19)
Schiller_2021: local ethics committee of the Ludwig-Maximilians University of Munich, Germany (EK 333-10 and 382-10).
Schultze_unpubl: ethical approval of Hannover Medical School Nr. 7414, 2017
Tata_unpubl: Duke University Institutional Review Board (Pro00082379) and the University of North Carolina Biomedical Institutional Review board (03-1396).

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☒ Life sciences ☐ Behavioural & social sciences ☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	All large, publicly available single-cell lung datasets were included in the HLCA where available. For the HLCA core, 10X data of non-diseased lung tissue was included. For the atlas extension, disease data was included as well, from any UMI-based single cell protocol. Furthermore, data from groups who offered to share unpublished data (data generation included in the methods, data included in the -publicly available- HLCA was included. No other data was generated for the HLCA.
Data exclusions	No data were excluded from the analysis. For the HLCA core, only data of control (i.e. non-diseased) tissue was used, as the HLCA core serves as the control/healthy reference. All other data was included in the extended HLCA.
Replication	No replication experiments were done. One of the goals of building the HLCA, i.e. pooling data across studies, is to enable checking reproducibility of findings across studies.
Randomization	There were no different experimental groups, such as treated versus control, in the newly generated HLCA data.
Blinding	As there were no different experimental groups, there was also no blinding.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
☒	☐ Antibodies
☒	☐ Eukaryotic cell lines
☒	☐ Palaeontology and archaeology
☒	☐ Animals and other organisms
☐	☒ Clinical data
☒	☐ Dual use research of concern

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging

Clinical data

Policy information about clinical studies

All manuscripts should comply with the ICMJE guidelines for publication of clinical research and a completed CONSORT checklist must be included with all submissions.

Clinical trial registration	NCT04529993
Study protocol	https://clinicaltrials.gov/ct2/show/study/NCT04529993
Data collection	Participants recruited by the Pneumology Unit of the Nice University Hospital were sampled between the 1st and the 15th of December 2020. The full procedure is detailed in https://www.clinicaltrials.gov/ct2/show/NCT04529993. Nasal and tracheobronchial samples were collected from IPF patients after obtention of their informed consent, following a protocol approved by CHU Nice. The data was derived from the clinical trial registered at clinicaltrials under reference NCT04529993. This study was described as an "interventional study" instead of an "observational study" because the participants were volunteers and all assigned to a specific bronchoscopy not related to routine medical care. Participants were prospectively assigned to a procedure (bronchoscopy) according to a specific protocol to assess our ability to sample the airway. No other procedures were included in this study. "Participants recruited by the Pneumology Unit of the Nice University Hospital were sampled between the 1st and the 15th of December 2020. The full procedure is detailed in https://www.clinicaltrials.gov/ct2/show/NCT04529993".
Outcomes	As this study did not involve any treatments are tracking of patients, just a single biopsy from volunteers, "outcomes" were not tracked in this study.

Endotyping asthma: new insights into key pathogenic mechanisms in a complex, heterogeneous disease

Gary P Anderson

Clinical asthma is very widely assumed to be the net result of excessive inflammation driven by aberrant T-helper-2 (Th2) immunity that leads to inflamed, remodelled airways and then functional derangement that, in turn, causes symptoms. This notion of disease is actually poorly supported by data, and there are substantial discrepancies and very poor correlation between inflammation, damage, functional impairment, and degree of symptoms. Furthermore, this problem is compounded by the poor understanding of the heterogeneity of clinical disease. Failure to recognise and discover the underlying mechanisms of these major variants or endotypes of asthma is, arguably, the major intellectual limitation to progress at present. Fortunately, both clinical research and animal models are very well suited to dissecting the cellular and molecular basis of disease endotypes. This approach is already suggesting entirely novel pathways to disease—eg, alternative macrophage specification, steroid refractory innate immunity, the interleukin-17–regulatory T-cell axis, epidermal growth factor receptor co-amplification, and Th2-mimicking but non-T-cell, interleukins 18 and 33 dependent processes that can offer unexpected therapeutic opportunities for specific patient endotypes.

Lancet 2008; 372: 1107–19

See Editorial page 1009

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Introduction

In recent years, the morbidity and mortality of asthma have decreased, probably as a result of improved management. Some evidence suggests that the relentless rise in disease incidence and prevalence is now also reaching a plateau.[1] However, although contemporary treatment approaches are indisputably effective, many patients have substantial residual disease and some, with very severe asthma, respond suboptimally even to high-dose oral steroids (figure 1).[2–6] Furthermore, asthma—the most common serious chronic lung disease afflicting around 150 million people worldwide—remains both unpreventable and incurable.[7] Despite decades of intensive research, little progress in identification of new treatments has been made since the introduction of inhaled β_2 adrenoceptor selective agonists (1969) and inhaled glucocorticosteroids (1974).

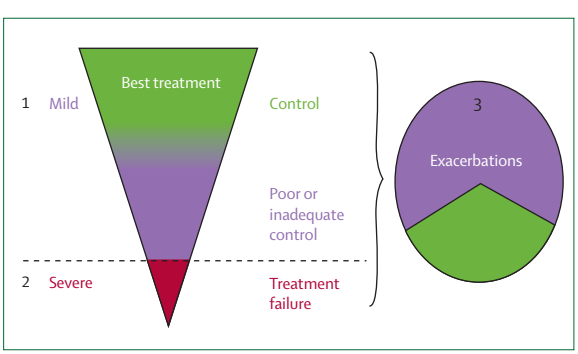

Figure 1: **Residual disease burden in optimally treated asthma**
The diagram shows that as severity increases (purple), the number of afflicted individuals decreases (represented as an inverse triangle). Exacerbations are represented as a pie chart. Severe asthma (red triangle), which is defined as failure of optimised treatment, is rare and estimated at about 5% of all disease. Notional best possible treatment (green) shows (1) residual disease in patients with moderate disease, (2) refractory severe asthma, and (3) breakthrough acute exacerbations. The purple and red regions represent the targets for future therapies. Th2-directed therapies will most probably compete with present therapies for mild asthma.

This Review aims to advance the argument that the way in which we think about the pathogenesis of asthma is flawed (or incomplete), which in turn is preventing the discovery of better treatments, preventions, and cures. Clear evidence now suggests that asthma is a heterogeneous and genetically complex disease (>100 genes have already been implicated) that cannot be explained by one mechanism alone. To order this heterogeneity and the volume and complexity of clinical and basic research data, the new notion of disease endotypes (panel 1), identifying definable subpopulations of asthma with discrete pathogenic pathways, is introduced and a conceptual framework to model endotypes is presented.

This Review is structured into four sections: weaknesses of the current T-helper-2 (Th2)-inflammation

Search strategy and selection criteria

National Center for Biotechnology Information (NCBI) public domain databases, Pubmed, and OMIM (On-line mendelian inheritance in man) were searched by combining the key term "asthma" sequentially with each of the following descriptors: "phenotype", "subtype", "factor analysis", "principal component analysis", "cluster", "clade", "classification", "pattern recognition", "severe", "severity", "refractory", "complex disease", "heterogeneity", "variant", "inflammation", "sputum", "biopsy", "eosinophil", "neutrophil", "lymphocyte", "Th2", "IL4", "IL13", "GATA3", "TBET", "macrophage", "immunity", "immunity-adaptive", "immunity-innate", "Treg", "IL17", "IL23", "biomarker", "exhaled", "condensate", "exacerbation", "virus", "COPD", "atopy", "epidemiology", "genome-wide scan", "candidate gene", "ontology", "childhood", "remission", "lung function", "FEV1 decline", "airway reactivity", "bronchial hyperreactivity", "airway smooth muscle", "inhomogeneity", " resolution", " repair", "resolving", " lipoxin", "obesity", "comorbidity", "perception", and "dyspnea". Very recent papers within the past 3 years have been preferentially selected. Human asthma candidate genes, or mouse genes with human homologues, identified in the primary search from gene profiling studies, linkage studies, or from basic and clinical biology were assigned to endotype component categories on the basis of gene ontogeny (GO) categories, OMIM, and published work proximity to relevant processes with the iHoP (Information hyperlinked over Protein), search tools, and biological plausibility.

***Panel 1:* What is an endotype?**

Endotype—a contraction of endophenotype—is a subtype of disease defined functionally and pathologically by a molecular mechanism or by treatment response. Asthma, like many chronic disorders, is a heterogeneous and genetically complex disease, meaning that many genes (>100 have been identified) are likely to contribute, variably, to its different manifestations. Asthma is likely to have several specific endotypes associated with distinct clinical features, divergent underlying molecular causes, and distinct treatment responses.

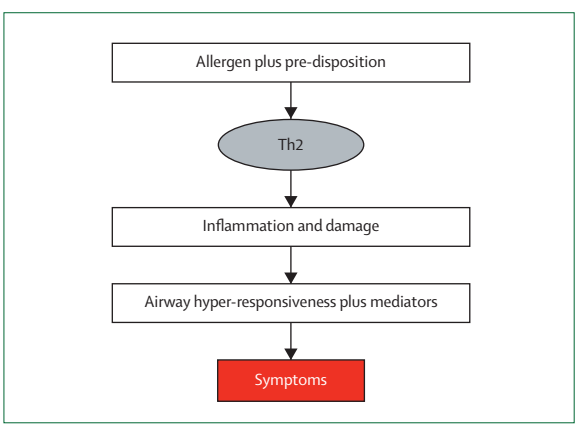

***Figure 2:* Linear representation of the Th2-inflammation hypothesis**
The diagram shows the most common view of asthma, which is that allergen exposure in sensitive individuals induces Th2 immune deviation. Th2 immunity drives eosinophilic inflammation and tissue damage, resulting in airway hyper-responsiveness and mediator release. These, in turn, cause symptoms. The hypothesis is simple but does not accord well with current evidence and cannot explain the heterogeneity of asthma.

hypothesis; asthma heterogeneity in terms of ontogeny, clinical phenotypes, and molecular patterns; asthma endotypes; and novel mechanisms, with particular emphasis on alternative macrophage specification programmes and the role of innate immunity as determinants of more severe and steroid-refractory asthma endotypes. By understanding asthma endotypes, and their molecular determinants, effective therapies, and possibly cures, can be developed that are highly effective in targeted patient subgroups.

Limitations of the Th2-inflammation hypothesis

Since the mid-1990s, asthma research has been propelled forward by innovations stemming from the Th2-inflammation hypothesis, providing a molecular framework for understanding the well known associations of atopy or IgE and eosinophilic lung inflammation with asthma. A helper T-cell population induced by interleukin 4 is able to produce a panel of cytokines—such as interleukin 4 or 13 (causing B-cell IgE production, mucus secretion, and fibrosis), interleukin 5 (causing eosinophilic inflammation and damage), and interleukin 9 (promoting mast cell growth)—which induce traits associated with classic asthma (figure 2). The Th2-inflammation hypothesis[8,9] coincided with the rise of genetically modified mouse technology and molecular profiling methods. Thus, lung Th2 immunity is now understood in fine molecular detail: from the nature of antigen, through the co-stimulation topology of antigen-presenting dendritic cells, to the language of transcription factors and chromatin reshaping that controls gene programmes governing the emergence and persistence of Th2-biased lymphocytes and their trafficking patterns in vivo.[10–12]

Th2 immunity is undoubtedly important in some asthma endotypes. But even from its inception, concerns have arisen about whether the Th2-inflammation hypothesis would lead to improved treatments.[8,9] These concerns have now been heightened. The main reasons for questioning the Th2-inflammation hypothesis is that it cannot explain why airway hyper-responsiveness and tissue remodelling are not clearly linked to inflammation; why existing T-cell immunosuppressives and new Th2-targeted treatments, which often worked well in Th2 disease models, have no or marginal effectiveness in the clinic; why many patients have recurrent exacerbations; why substantial residual disease remains when anti-inflammatory therapy is optimised; why asthma shares some genetic risk factors with chronic obstructive pulmonary disease (COPD); and why some patients have severe asthma. Moreover, the Th2-inflammation model cannot account for the substantial clinical and molecular heterogeneity that has now been unequivocally documented in human asthma. Therefore, this intellectual framework needs to be revised. Indeed, that the entry criteria for patients into almost all clinical trials for asthma does not reflect the pattern of actual asthma in the community is remarkable.[13]

Several problems exist. If T cells were fundamentally important, T-cell inhibitors should be very effective treatments; however, T-cell-directed therapies have uniformly failed in clinical trials.[14] Th2 immunity is fundamental to atopy, but although atopy is a risk factor for asthma in populations, it has poor sensitivity and specificity as a predictor of disease. Eosinophilic inflammation is the Th2 driven trait that most consistently tracks with disease activity, exacerbation susceptibility, treatment responses, and as a useful biomarker to guide treatment.[15–17] However, airway inflammation is much the same between non-asthmatic atopics, allergic rhinitics, and atopic asthmatics.[18] An almost identical pattern of response occurs after allergen challenge in people allergic to house-dust mites with and without asthma.[19–21] The Th2-inflammation model predicts that eosinophilic inflammation should drive airway hyper-responsiveness, but no clear relation exists;[22,23] furthermore, population studies show atopy and airway hyper-responsiveness are not concordant.[24] Neutrophils, mast cell infiltration of

airway smooth muscle, intensity of inflammation, and inflammation of airway smooth muscle, might discriminate between inflammation in atopy versus that in asthma, but the sensitivity and specificity of these putative co-determinants has not been formally proven.[25,26] Lung Th2 cytokines are found as often in atopy as in asthma, and interferon γ (a Th1 cytokine) is actually upregulated in human asthma together with interleukins 4 and 5 in sputum but not in blood.[27]

T-cell immunosuppressive drugs (eg, ciclosporine and methotrexate) have measurable but very weak effects in asthma, and immunosuppressive therapy after allograft transplantation does not prevent asthma or allergy in children and adolescents.[28] Furthermore, glucocorticosteroids (and β_2 agonists), which are highly effective in atopic asthma with eosinophilic inflammation, paradoxically consolidate and intensify Th2 immunity and increase IgE.[29] At the molecular level, steroids preferentially suppress interleukin 12 and T-bet (negative regulators of Th2 immunity) and spare STAT6 (which induces Th2 genes).[30,31] Steroids also dampen expression of T-bet, the transcription factor controlling Th1 (but not Th2) immunity.[32] Steroid sensitivity wanes in severe asthma; however, genetic manipulation to enhance steroid effectiveness does not suppress experimental asthma and instead favours Th2 immunity.[33] In children, steroids worsen Th2 immunity and increase IgE, which has been related to the genetic association of the low affinity IgE receptor (FCER2) with risk of severe exacerbations.[34] Considered together with the weak effects of therapies of anti-interleukins 4 and 13 (eg, pitrakinra) and anti-interleukin 5 (eg, mepolizumab) that are in trials,[35,36] it is reasonable to suggest that steroids exert their beneficial effects in asthma at loci other than Th2 immunity. Steroids suppress the end effects of Th2 immunity but consolidate the underlying aberration.

Despite these caveats, Th2 immunity is clinically important, specifically for childhood asthma with atopy and mild allergic adult asthma, and Th2 directed therapy will be effective in some asthma endotypes.[36] However, present treatments are effective in mild atopic asthma (figure 1), and Th2-directed therapies are unlikely to ameliorate the residual disease burden of more severe disease.

Heterogeneity of clinical asthma and treatment responses

Asthma continues to elude specific definition and can therefore currently only be characterised in functional terms (panel 2). Although evidence has suggested the pronounced heterogeneity of asthma for decades, little interest has focused on understanding its basis. Most asthma trials and research protocols have used inclusion criteria—typically predicted forced expiratory volume in 1 second (FEV_1), degree of reversibility, inflammation, eosinophilia, and often IgE, because they can be measured objectively and accord well with disease notions. However, because asthma is heterogeneous, these criteria have resulted in patients being selected to trial new asthma drugs who are not representative of asthma in general practice.[13] Advances in asthma epidemiology including longitudinal outcomes studies, population genetics, and molecular profiling methods; application of statistical methods, such as clustering and principal component techniques, to show distinct asthma patterns and subsets; and compelling new clinical research, have all contributed to a reawakening of interest in understanding disease heterogeneity.

Paediatricians have defined three major patterns of wheezing in infants that have also been assessed for their effect on subsequent persistent asthma in adulthood: transient infant wheeze, non-atopic wheezing in toddlers, and IgE-mediated wheeze or asthma. A fourth category has also been introduced: late-onset childhood asthma.[38] Evidence now suggests that transdermal sensitisation associated with polymorphism in filagrin, a molecule important in maintaining cutaneous integrity, rather than aeroallergen links asthma risk with atopy and atopic dermatitis.[39,40]

Panel 2: Definitions of asthma and indices of severity

The first systematic attempts to define asthma were made in the 1970s but, despite decades of effort, there is still no specific definition of, or validated diagnostic algorithm for, the disease. Instead, asthma is defined functionally as an inflammatory disorder linked to hyper-responsiveness that causes symptoms:

"Asthma is a chronic inflammatory disorder of the airways in which many cells and cellular elements play a role. The chronic inflammation is associated with airway hyperresponsiveness that leads to recurrent episodes of wheezing, breathlessness, chest tightness, and coughing, particularly at night or in the early morning. These episodes are usually associated with widespread, but variable, airflow obstruction within the lung that is often reversible either spontaneously or with treatment."[37]

Similarly, no current methods exist to objectively define disease severity other than by functional criteria:

- day-time symptoms
- limitations of activity
- nocturnal symptoms and wakening
- need for rescue/reliever drugs
- lung function as percentage predicted
- exacerbations
- treatment responses (success or failure to improve or control these factors)[37]

These functional or operational criteria are, however, practical, pragmatic, and very well thought out because they are simple, work in the context of global asthma guidelines, and allow the disease to be managed even in health-care systems with constrained resources.

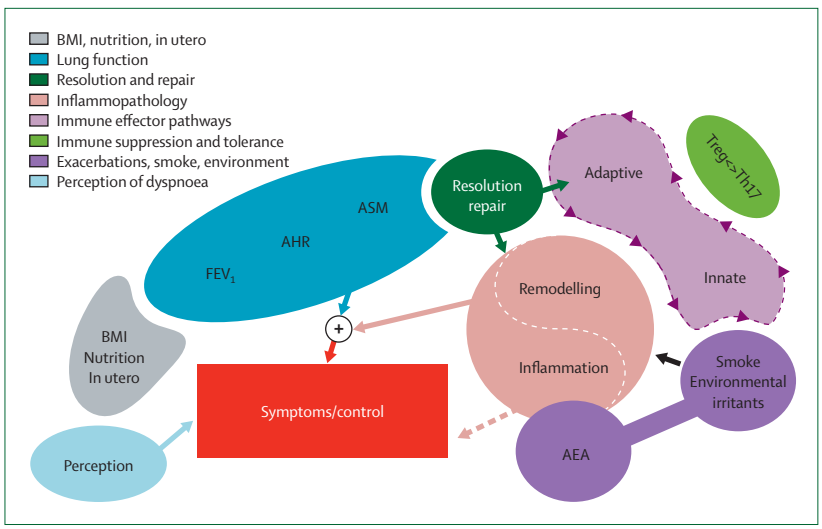

Figure 3: **Open framework asthma endotype model**
The diagram represents processes that are known to be involved in the diverse presentations of asthma as colour-coded elements. Distinct endotypes arise through the variable interplay of these components. In the model, asthma symptoms almost always arise through the interaction of altered lung function (forced expiratory volume in 1 second [FEV_1], airway hyper-responsiveness [AHR], and airway smooth muscle [ASM]) and immunopathologies (remodelling/inflammation) but these symptoms arise and interact independently. Modifying process such as body-mass index (BMI), diet, and in utero exposures; CNS sensitivity to dyspnoea (perception); acute asthma exacerbations (AEA); cigarette smoke (smoke) and environmental irritants are shown impinging on lung function on remodelling and inflammation. Both divisions of the immune system, (adaptive and innate), are shown closely interconnected (arrowed dotted line) and regulated by immune tolerance (Treg) in equilibrium with Th17 immune activation. Defective healing is represented by resolution/repair. Immunity, together with smoke and environmental contaminants, and also exacerbations, are shown interacting with inflammation and remodelling, but no assumption is made that remodelling is dependent on inflammation. Because asthma is defined by symptoms, defects in lung function are essential, and their necessary interaction with other components is indicated by +. Candidate genes linked to these processes are listed in the table.

Wheeze in early life in the absence of atopic sensitisation almost invariably resolves to normal lung function by 13 years of age, whereas atopic sensitisation, particularly before 3 years of age, is associated with a much higher chance of reduced lung function and worsened airway hyper-responsiveness,[41] which occur despite the airway biopsy pathology of non-atopic asthma being almost indistinguishable from atopic asthma in children.[42] This finding suggests that very early intervention with steroids would be beneficial, but this is not the case,[43] perhaps because steroids actually worsen Th2 immune deviation.[29] Long-term follow-up studies also suggest that up to half of asthma in adolescence or early adult life represents a relapse of previously quiescent disease.[44] Asthma affects more boys than girls in childhood, but more women in later life.[45] At the cellular and molecular level, children who are predisposed to life-long asthma might have substantial changes early in disease, including oxidative stress and acetylation,[46,47] IRAK-M (a negative regulator of innate immunity),[48,49] epidermal growth factor (EGF), interleukin 6, and prostaglandin E2.[50]

The heterogeneity of asthmatic inflammation is well documented; Golash first suggested sputum eosinophilia as a hallmark of disease in the 1890s. Woolcock and Peat[24] noted that airway hyper-responsiveness was unimodally distributed in the general population and only partially overlapped with atopy, precluding atopy as a cause of all airway hyper-responsiveness. Wardlaw and colleagues[25] have reported the absence of a clear relation between intensity of inflammation (eosinophils) and severity of asthma. They also noted that only airway smooth muscle infiltration by mast cells differentiated the airway pathology of eosinophilic bronchitis from asthma[25,26] and that this trait, not airway remodelling, is associated with airway hyper-responsiveness.[51]

More recently, Simpson and colleagues[52] have identified distinct inflammatory endotypes in sputum samples from patients with clinical asthma, forming the basis of a simple classification schema: (1) eosinophilic; (2) neutrophilic; (3) mixed (ie, both neutrophils and eosinophils found); and (4) paucigranuloctic (few or no granulocytes in the sputum).

Since these investigators noted evidence of upregulated toll-like receptors, they have specifically linked neutrophilic asthma to innate immunity.[53] Haldar and Pavord[54] have independently replicated the identification of neutrophil variant asthma (which has been suggested for many years). These heterogeneous inflammatory patterns are entirely consistent with the identification of a distinct cytokine profile in patients with asthma.[55] Similarly, application of factor analysis to molecular genetics studies tends to segregate, rather than cluster, atopic and asthmatic disease traits.[56] Segregation of inflammatory subtypes by non-invasive exhaled breath methods is not yet possible.[57]

The biology of very severe asthma is almost certainly distinct from milder forms of disease, and severe asthma is heterogeneous.[14] Severity should be viewed as a separately regulated biology rather than as one end of the spectrum of disease or the result of an inexorably progressive process. Results from long-term epidemiological studies in asthma have shown that the severity grade of asthma tends to be established early in life, and disease seldom progresses to a more severe grade.[58] Two major subdivisions of severe asthma have been proposed on the basis of discordant inflammatory patterns.[59,60] Furthermore, Pavord and colleagues[61] have reported severe asthma in the absence of eosinophilic inflammation and, by inference, Th2 immunity. Brasier and co-workers[55] applied mathematical pattern-recognition methods to compare cytokine patterns in bronchoavleolar lavage from 43 patients with mild or moderate asthma with 43 patients with severe disease, and noted four distinct profiles to predict methacholine responsiveness. However, few molecular mechanisms exist that unequivocally distinguish severe asthma. Possibilities include CREB (cyclic AMP response element binding protein 1), which regulates gene expression in responses to increases in cyclic AMP;[62] RIP-2 (receptor-interacting serine-threonine kinase 2), an intermediate in toll-like receptor signalling;[63] reduced generation of pro-healing lipoxin A4; or anti-inflammatory interleukin 10.[64,65]

Combination therapy (bronchodilator plus inhaled glucocorticsteroid) achieves good outcomes for many, but not all, patients with mild to moderately severe asthma and can reduce the rate of severe exacerbations by up to a third (figure 1). Other therapies have also proven to be of measurable, but lesser and variable benefit—eg, cysteinyl-leukotriene receptor antagonists and anti-IgE monoclonal antibodies.[2–6] Other therapeutic options, such as selective phosphodiesterase isozyme inhibitors, selective chemokine and cytokine antagonists, vaccines, and T-cell-directed or general immunosuppressives, are ineffective or of marginal benefit.[66] Primary prevention has not proven possible so far, since a meta-analysis shows that avoidance of house-dust mites is not effective.[7] The β_2 adrenoceptor is polymorphic and its Arg16 variant might adversely affect response to regular short-acting drugs but not long-acting β agonists.[67] About 15% of patients with asthma respond well to leucotriene antagonists and, although urinary concentrations of leucotriene do not predict responses, polymorphism in 5 lipoxygenase (ALOX) and the receptor (CYSLTR2) can establish drug sensitivity.[68,69] Steroid responses are very heterogeneous in childhood and adult asthma.[31,70]

An asthma endotype model

In view of the manifest problems with the Th2-inflammation model and the pronounced heterogeneity of asthma, how is the disease to be modelled and how will new treatments be found? Gibson's four inflammatory patterns provide one simple and useful model.[52] A more extensive rational approach is to assess components of disease that could be considered in the definition of endotypes. The asthma endotype model shown in figure 3 maps inter-relations between clinical determinants that are known to be important in the manifestation and expression of asthma across its diverse patterns and severities. The table lists candidate genes linked to endotype determinants. The model is non-linear: it does not make a-priori assumption about the weight of a specific factor—ie, inflammation—to expression of disease. Some of the evidence base for the model is summarised below.

Airway smooth muscle

Airway narrowing, which is largely caused by contraction of airway smooth muscle, is the mechanism that can be most clearly linked to symptoms, as can be inferred from the additional benefit of combining long-acting bronchodilators with steroids and the remarkably effective benefits of killing muscle by bronchial thermoplasty in severe disease.[71,72] More smooth muscle exists in airways of asthmatic patients, but alone it might not be a cause;[51] modelling studies suggest that increased bulk might even protect against excessive closure. Airway smooth muscle in asthmatic patients also secretes inflammatory cytokines including stem-cell factor (or kit-ligand), a mast cell growth and activation protein that is now also implicated in dendritic cell activation.[73] Increased mast cell number and degranulation have been suggested as pathological indicators of changed function.[25,51]

Specific molecular changes affecting contractility—such as changes in myosin light chain kinase isoforms or cross talk between signalling G proteins—remain controversial,

	Genetic	Biology
Lung function: basal FEV_1, airway hyperresponsiveness, airway smooth muscle	*EDN1, ADAM33, B2ADR, CREB, CCR5, COL29A1, CSTA, CYSLTR1, CYSLTR2, EP2, FCER2, GSTM, HNMT, KCNS1, LELP1, MMP, MUC7, MLCK, NK2R, PDGFRA, PLA2, PLAU, PTGDR, PTGER, PTGIR, TBX21, VDR*	CREB, GSNOR, NOS, NR3C1
Immunity	*FLG, IL17F, TGFβ, IL6, RORα, RORγ, BDNF,* chemokines, *CD14, CD40, CD86, DPP10, FCER2, FLG, HLA-G, ICOS, IGHG, IL12B, IL2, IL4, IL6, IL9, IL10, IL13, IL16, IL17, IL18, IL27, IL33, IRAKM, ITK, MICB, MMP, MRP1, MUC1, NOD, PHF11, PLA2, PPARG, PTGDR, PTGER, RIP2, RUNX1, SFTPC, SOCS, SPP1, STAT6, TBX21, TIM1, VDR, VEGFR*	CREB, HCK, IL23, IL33, LYN, NFATc, NOS, NR3C1, PTEN, RIP, ROR, SHIP, SHP, TSLP
Inflammation and remodelling	*EDN1, ADAM33, IL17A, IL17F, NRF2, SOD, CREB, VDR, CAT,* chemokines, *COL29A1, CSTA, DPP10, ECP, EP2, FYN, GSTM, IGHG, IL2, IL5, IL9, IL13, IL17, IL18, IL33, PLA2, PLAU, SOCS, STAT6, TNFA, UTG, VEGFR*	AMCase, ARG, C3AR1, c-kit, C3\5, EGFR, CSF2(=GM-CSF), HCK, HMGB1, LYN, NOS, NR3C1, NRF2, PTEN, RAGE, RIP, SCF, SHIP, SHP, SOD, TIMP, TSLP.
Resolution and repair	*VDR, LEP*	IL-10, FAS, NR3C1, RAGE, TIMP, Lipoxin *A4* (15LOX, 5LOX), presqualene phosphates
Exacerbations, smoke, and environmental irritants	*VDR, AOAH, CAT, CYP24A1, GSNOR, HLA-G, IL12B, IL2, IL6, IL12, IL17, IL23, IL33, IRAKM, MMP, MRP1, NOD, SFTPC, UTG, NRF2*	CD200, EGFR, IFN, NR3C1, SOD, TIMP
BMI and nutrition	*ADRB2, FABP, NR3C1, VDR, FABP4, NR3C1*	
Perception	*KCNS1, GAD65*	
Genes of unknown function from linkage	*DCNP1, GCLM, ORMDL3, SCGB3A2*	

Candidate genes selected from linkage and microarray studies (genetic) or from the known biology of established disease processes (biology) are shown as putative determinants of asthma endotype trait elements (see also figure 3). Definitions of these genes can be found via the NCBI website OMIN database (http://www.ncbi.nlm.nih.gov/sites/entrez).

Table: **Putative contribution of asthma candidate genes to endotype elements**

and microarray profiling and genetic linkage studies have not suggested strong candidates for changed function.[74] However, increased rate of shortening has been shown, and although the specific mechanism of this effect is disputed, faster or excessive shorting will probably be the major functional defect causing airway narrowing and symptoms.[75] Clinical asthma is unlikely to occur in almost all cases without the contribution of airway smooth muscle. All known bronchodilators are functional antagonists, or direct pharmacological antagonists of contractile mediators, and they are not fundamentally able to prevent contraction to intense stimuli.

Airway hyper-responsiveness and dynamic inhomogeneity

Alexander and Paddock first described airway hyper-responsiveness to systemic pilocarpine in 1921.[76] People with asthma often show pronounced airway hyper-responsiveness with loss of plateau that is orders of magnitude greater than in healthy controls. Airway smooth muscle responds differently to inflammation in patients with asthma, with substantial variation in degree and site of bronchoconstriction during the late-phase rise in inflammation that follows antigen challenge.[77,78] This heterogeneity of constriction has been linked directly to the loss of bronchoprotective response to deep inspiration,[79] which might indicate a defect in responses of airway smooth muscles to cyclic stretch, leading to risk of catastrophic bronchoconstriction.[80] Dynamic hyperinflation caused by patchy constriction of large airways is also thought to contribute greatly to airway hyper-responsiveness.[81] This dynamic inhomogeneity has been directly recorded by tomography (with hyperpolarited 3He-MRI or CT).

Steroids are able to reverse this defect only in the mildest disease.[82] Airway hyper-responsiveness can be worsened by inflammation, but the main component is inherited separately.[83] Palmer and colleagues[84] noted that serum IgE concentrations, blood eosinophil counts, and airways responsiveness to inhaled agonist were inherited separately in human beings. Inflammation actually correlates poorly with airway hyper-responsiveness. Perhaps the greatest misunderstanding in basic (mouse) and applied asthma research is that intrinsic (inherited) and antigen/inflammation-induced airway hyper-responsiveness arise from different mechanisms.[85] This notion is particularly ironic in asthma models in mice because they clearly show that basal airway reactivity is a heritable trait, whereas the small and transient labile component indicates changes in access of agonists to airways smooth muscle that is secondary to inflammation.[84,85]

FEV_1 decline and fixed obstruction risk

Long-term outcome studies show that basal FEV_1 is set early in life and few patients with asthma have excessive rates of decrease.[58,86,87] However, some patients—particularly those with adult onset asthma, smokers, and those with persistent uncontrolled eosinophilic inflammation, airway hyper-responsiveness, or with inherited polymorphisms in *ADAM33*—can have rapid decline and progress to fixed obstruction.[88] Some evidence suggests that inhaled steroid use can reduce an excessive decline, but this notion is controversial.[87] Linkage studies have shown that inheritance of FEV_1 has no known genetic determinants in common with asthma severity or symptom score.[89] Bisgaard and colleagues[90] have noted that the rate of lung function decline and airway hyper-responsiveness in children is associated with lower lung function, delayed use of steroids for symptomatic disease, smoking, and positive allergic skin-prick test,[90] which accords with Grol and co-workers' earlier work.[87] In childhood, lower lung function and lower increase in FEV_1 predicts worse airway hyper-responsiveness in adulthood,[91] but the molecular basis for this finding remains unknown.

Immunity, inflammation and remodelling, resolution, and repair

The interplay of immunity, inflammation, and remodelling has been a central theme in asthma research for decades. Eosinophilic inflammation is the trait that is best linked to symptoms and treatment responses, but alone it is not enough to cause asthma, which is absent in atopy, eosinophilic bronchitis, and Crohn's disease (for which airway inflammation also occur).[92] Similarly, atopic rhinitis with allergen exposure produces pathological changes similar to asthma in airways and cytokine release without asthma symptoms,[21] and anti-interleukin-5 antibodies seem to have some benefit but only in a patient subset with a high eosinophil load. Lung eosinophilia is a useful biomarker to titrate steroid responsiveness, and decreasing eosinophils reduce the risk of exacerbations. Inflammation is heterogeneous in asthma. Steroids have little effect in neutrophilic asthma, and basal eosinophilia predicts steroid effect.[93] Airway pathological changes are established early in life as basement membrane pseudo-thickening and angiogenesis are evident in children with asthma (and in atopic children without asthma).[94] Similar early changes probably occur in lung nerves that undergo remarkable plastic changes in asthma, which could partly relate to increased cough. Good evidence also suggests that resolution processes are defective in asthma. Resolvin E1 (18R-trihydroeicosapentaenoic acid) suppresses experimental asthma in vivo,[95] and pro-resolving lipoxins are diminished as asthma severity worsens.[96] Production of interleukin 10, which exerts an inflammation-suppression effect under some conditions, is also dampened.[65]

Exacerbations

Acute asthma exacerbations—usually but not uniformly caused by rhinovirus infection—are a major cause of morbidity in asthma. In severe asthma, five risk factors have been identified for recurrent exacerbations: severe nasal sinus disease, gastro-oesophageal reflux, recurrent

respiratory infections, psychological affective disorders, and obstructive sleep apnoea.[97] Rhinovirus infection affects lower airways even in healthy people, causing narrowing and inflammation.[98] Eosinophilia is a known risk factor for asthma exacerbations.[16] Patients with asthma do not have more frequent infections but rather more intense reactions. This finding has been linked to interplay of the EGF receptor (EGFR) with matrix metalloproteases and external regulated kinase (ERK) signalling,[99] and primary or acquired interferon α and λ deficiency.[100,101] Conventional plasmacytoid-dendritic cells govern lung viral immunity. The dendritic cell growth factor FLT3 induces conventional plasmacytoid and plasmacytoid-dendritic cells, and suppresses respiratory syncytial virus infection;[11] however, little is known about variations across endotypes or the contribution of inhibitory pathways such as CD200.[97,102]

Body-mass index, nutrition, and obesity

Thomas Platts-Mills is credited with proposing that watching television was the cause of the asthma epidemic in the 1980s.[103] Analysis of 20 016 children (aged 6–7 years) showed that high bodyweight, salty diet, and time spent watching television were independent risk factors for asthma.[104] This finding is supported by a prospective multiple logistic regression analysis of 932 children in Boston.[105] Body-mass index affects treatment outcome.[106] Vitamin D deficiency, due to less sun exposure and indoor inactivity, has been proposed as an asthma cause.[107] Vitamin D receptor expression in the lung is needed for inflammation and expression of experimental asthma, suggesting that vitamin D might have a stronger effect on host defense than inflammation does.[108,109] Importantly, both the β_2 adrenoceptor and glucocorticosteroid receptor, which are closely associated with asthma, are also obesity candidate genes. Furthermore, asthma risk almost certainly begins in utero. Prenatal stress, smoke, and exposure to air pollutants all increase asthma risk.[110–112] Asthma is defined by symptoms, and patients who have poor perception of the severity of their disease (under-perceivers) and patients who suffer overt symptoms but show only small changes in lung function (over-perceivers) have been described. Because dyspnoea is perceived in the anterior insula and amygdala,[113] perception modifying neuronal mechanisms will probably emerge in coming years.

Asthma–COPD overlap

Many asthmatics smoke, which worsens their disease and impairs steroids responses. Lapperre and colleagues[114] used factor analysis statistics in 117 patients with COPD by measuring lung function, DLCO (the single-breath diffusing lung capacity for carbon monoxide), PC_{20} MeCh (the concentration of inhaled methacholine causing a 20% fall in FEV_1), total IgE, exhaled nitric oxide, and differential cell counts in induced sputum. They noted

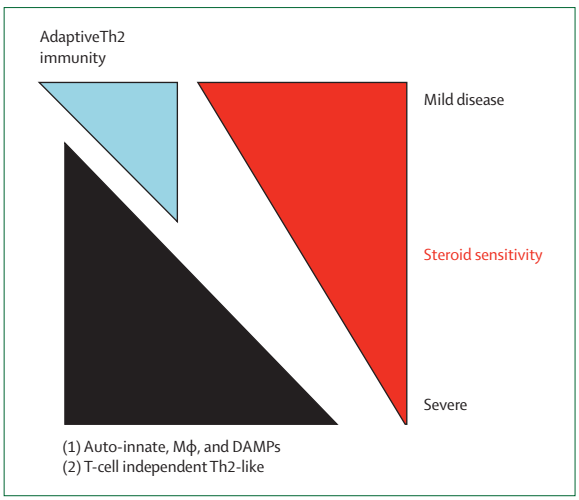

Figure 4: **The unexpected importance of innate immunity in asthma severity and reduced steroid sensitivity**
Innate immunity is associated with more severe disease and steroid insensitivity. The diagram shows that as disease severity worsens and becomes less steroid sensitive (red), the contribution of innate immunity increases (black). Classic Th2 immunity is shown in blue. Two novel processes are represented: (1) Auto-innate immunity is shown activating macrophages (tissue is also activated), particularly by factors released from damaged tissue (DAMPs) that trigger innate immunity locally; and (2) T-cell independent processes mediated by innate immunity that resemble Th2 immunity. MΦ=macrophage.

that airflow inflammation and inflammation of the airways, and systemic features commonly associated with asthma (eg, IgE and eosinophils) were separate and predominantly independent contributors to COPD.[114] These data are entirely consistent with genome-wide scans of COPD susceptibility and severity, and underscore the certainty that shared co-determinates of asthma and COPD exist in some patients.[115–118] Interleukin-13 promoter polymorphism is associated with adverse effects of smoke on lung function,[119] and smoking, airway hyper-responsiveness, and eosinophils interact positively for respiratory symptoms.[120] As in asthma, lung eosinophilia predicts smokers who will benefit from steroids; smoking greatly impedes the activity of steroids.[121,122] The newly discovered intermediate pendrin, like EGF, induces mucin in both asthma and COPD.[123] Furthermore, the interplay of interleukin 1β and tumour growth factor β—recently identified as a cause of small airways disease in COPD—could be a cause of fixed airflow restriction in asthma.[124] Additionally, vitamin D biology, immune defense mediated by serpins and collectins, dysregulation of oxidative stress and apoptosis with decreased clearance of senescent cells, and secondary necrosis and impaired repair capacity are probably co-determinants of asthma and COPD.

Novel disease mechanisms

Asthma has a heritable component that is estimated to be between 36% and 94%. More than 100 plausible candidate genes have been suggested, but each has a

very low attributable risk (<5%), with often poor replication[125] or lack of plausible biology or functional single nucleotide polymorphisms. Of this very large information set, two broad new notions are emerging: auto-innate immunity driven by tissue damage and Th2-like responses that occur without T cells (figure 4).

Until recently, innate immunity was thought of only as a rapid front-line defender against infections. It works by triggering host defence after recognition of pathogen associated molecular patterns (PAMPs) on invading pathogens. However, endogenous ligands released from damage associated molecular patterns (DAMPs—ie, ligands such as heat shock proteins, RAGE ligands, and HMGB1) are very able activators of innate immunity. Like PAMPs, DAMPS often signal via the toll-like receptor system and its characteristic MyD88 transduction pathway to directly promote inflammation. Innate immunity is intrinsically resistant to steroids,[40] which could help to explain why as asthma severity worsens, steroid sensitivity decreases (the molecular basis for this notion is an amalgam of processes including HDAC nitrosylation, MAP kinase phosphatase induction, and inhibitory signalling from altered matrix). This notion has a profound implication: wherever DAMPs and innate immunity contribute to disease, the process will be intrinsically steroid insensitive, which might be why more severe asthma endotypes with established tissue damage are steroid resistant. Furthermore, the main source of ligands to drive this auto-innate immunity is damaged tissue, which is normally removed by macrophages. However, as oxidative stress worsens (with severity or smoking) the ability of macrophages to recognise and remove effete cells decreases, leading to much greater so-called spill of DAMPs.[126,127]

A second major emerging idea is that there are mechanisms to induce Th2-like effects in the absence of Th2 cells, which is probably the reason why interleukin 13 is so widely associated with highly divergent asthma endotypes. In both cases, the existence of these pathways most probably is an indicator of phylogenetic evolution in that ancient innate immunity, and many of its effector cytokines, predate the evolution of lymphocytes and glucocorticosteroids by million of years. Molecular dissection of these processes is suggesting new intervention points for asthma endotypes.

Interleukin 17 came to attention as an indirect mediator of sustained neutrophilic inflammation.[128,129] Evidence suggests that interleukin 17 is a mediator of neutrophil variant and severe neutrophilic asthma endotypes.[130] A defined T-cell subset—Th17—has been identified, but interleukin-17 family cytokine production is not constrained to T cells. Th17 differentiation is dependent on the retinoid receptors RORα and RORγ (implicating diet and lipid metabolism),[131] and signalling intermediates STAT3 and STAT4.[132] Interleukin 17 is induced in human beings by interleukin 23, which is related to interleukin 12 (a negative regulator of Th2 immunity), especially in the context of interleukin 1β, interleukin 6, and tumour growth factor β. This mixture is important because tumour growth factor β and interleukin 6 exert counter-balancing control of the regulatory T cells (Tregs) that suppress inflammation, indicating a fine balance between suppression and disease.[130,133,134]

Dendritic cells are poised at the interface between innate and adaptive immunity, and are essential for both the induction and maintenance of allergic inflammation. Coactivation of dendritic cells by antigen in the presence of stem cell factor—a potent mast cell growth factor—induces sustained interleukin-6 production, triggering concurrent Th2 and Th17 induction via the c-kit receptor.[135] Because stem cell factor is also implicated in mast cell infiltration of airways smooth muscle, this axis predicts the usefulness of imatinib mesylate (Gleevec, or related compounds), which has potent c-kit receptor kinase blocking activity in some asthma endotypes, especially when mast cells are concurrently implicated.

Viral bronchiolitis in infancy, especially with respiratory syncytial virus, is a risk factor for persistent asthma. In rodents, similar exposure leads to life-long inflammation and phenotypic alteration of the lung. Kim and colleagues[136] have discovered that activation of macrophages by CD1d expressing natural killer T (NKT) cells induces production of interleukin 13 and its pathological sequelae entirely independently of adaptive immunity. This mechanism operates in both human asthma and COPD,[136] and defines an adaptive immunity (ie, T cell) independent endotype. Smoke directly and substantially worsens viral inflammation and remodelling.[137,138]

Interleukin-18 polymorphisms have been replicated in several genetic linkage studies. This cytokine is known to induce IgE by causing NKT cells to upregulate the co-stimulation molecule CD40 and interleukin 4. Interleukin 18, which potently induces interferon γ, has been implicated in processes relevant to neutrophilic and mixed inflammatory patterns because exogenous interleukin 18 triggers bystander memory cells—the types of cells that would reflect past viral infections—to release not only interferon γ, causing neutrophilia, but also interleukin 13, inducing airway remodelling.[139] This mechanism might relate to asthma endotypes in which recurrent infection drives accelerated lung function decline or which show extensive neutrophilia. However, the role of NKT cells in asthma, where they have been linked to allergic (via interleukin 4) and neutrophilic (via interleukin 17) endotypes, is controversial.

Interleukin 33 is an interleukin-1-like cytokine that was identified initially as the ligand to an orphan receptor called T1/ST2, which is preferentially expressed on Th2 cells. However, interleukin 33 induces airway hyper-responsiveness and goblet hyperplasia and

eosinophilic inflammation dependent on ST2 binding and transduction via the innate immunity transducer, MyD88, concurrently with induction of interleukins 4, 5, and 13. These effects occur in $Rag^{-/-}$ mice that do not have a functional adaptive immune system. These data suggest that Th2 mimicking pathological changes can be induced entirely in the absence of an adaptive immune system defining a second adaptive immunity independent endotype.[140]

Rhinovirus infection is the main cause of asthma exacerbations. Epidemiological studies have shown that patients with asthma do not have more frequent infections but rather more severe inflammation. Such patients also overexpress the EGFR. Liu and colleagues[99] have identified a new mechanism governing the intensity of inflammatory responses to rhinovirus. Rhinovirus binds to ICAM-1, inducing upregulation of EGF and triggering an inflammatory response in the infected cell. In the presence of raised matrix metalloproteinase, excessive EGF is cleaved from the cell surface and binds to unregulated EGFR, sending a signal via ERKs that synergises with the direct response to rhinovirus, greatly enhancing inflammation.[99] This mechanism is an example of how an asthma candidate gene (matrix metalloproteinase) with weak attributable risk can exert a stronger effect in an altered disease context. Because EGFR also mediates mucus induction, these results suggest the use of EGFR-tyrosone kinase inhibitors (eg, gefitinib or erlotinib) or antihuman epidermal growth factor receptor 2 family antibodies or blockers in some asthma endotypes. Whether EGFR affects the macrophage CD200 pathway, which limits viral inflammation, is not known.[102]

The role of macrophages in asthma has probably been greatly underestimated, and this cell linage is increasingly researched as the role of innate immunity emerges. Macrophages do not always undergo classic activation, but might adopt alternative phenotypes[141,142] associated with hallmark features such as induction of AMCases (chitinases), which are linked to asthma severity.[143] Alternatively, specified macrophages are the main candidates for asthma endotypes that do not need T cells.

Much of the evidence that alternatively specified macrophages can achieve these effects comes from genetically manipulated mice. SHIP-1 is a negative regulator (ie, turns off) inflammatory cytokine and surface receptor signalling. SHIP-1 deficiency causes spontaneous asthma in mice with a Th2-like pattern,[144–146] including AMCases induction.[143] Similarly, mice with activated *Hck*—a *Src* family kinase—develop an aggressive T-cell- independent eosinophilic lung inflammation associated with progressive airway fibrosis.[145–147] *Lyn* is related to *Hck*. A profound and multi-trait severe asthma syndrome develops in *Lyn*-deficient mice who display hyper-IgE, enhanced bronchoconstriction, mast cell, and eosinophil degranulation; very persistent inflammation associated with deficient apoptosis; and Th2-like cytokines together with enhanced interferon γ.[148]

Biochemically, alternative macrophage activation has been linked to tumour growth factor β and interleukin 13.[141] Because these responses can occur in the complete absence of T cells, interleukin 13 probably exerts its effect via the type II interleukin-4 receptor.[149] These findings might explain why interleukin 13 is a candidate gene in both asthma and COPD.[150]

Summary and implications

This Review has developed the argument that the Th2-inflammation hypothesis, although useful, is not adequate to understand the substantial heterogeneity of asthma. The first iteration of an open-frame asthma endotype model has been presented and discussed in the context of entirely novel disease pathways, many of which are independent of adaptive immunity. The role of innate immunity, which is intrinsically insensitive to steroids and can be driven by tissue damage, has been emphasised. There are important implications. Definition of asthma endotypes opens the possibility of much more precise disease classification and definition of biomarkers that meet formal diagnostic or prognostic criteria. From analysis of several asthma candidates across many endotype components, effective future therapies will be used as an adjunct to existing medicines or will be combinations of activities, since few known candidates affect enough crucial endotype components to be effective in their own right. Some asthma endotypes will be reclassified as orphan diseases. Because the inclusion criteria and endpoints for clinical trials directed at some novel endotypes are likely to be unvalidated, we will probably increasingly use adaptive clinical trial methods to identify responding patient endotypes. In adaptive clinical trials, responsive patients (and non-responding patients) are identified during the course of the trial itself (allowing the trial design to be adapted while running) rather than during retrospective statistical analysis after the trial has closed. Specific definition of asthma endotypes should also spur and redirect basic research to discover the mechanisms and highly innovative ideas that are required to improve asthma therapy and ultimately prevent or cure the disorder or disorders.

Conflict of interest statement

I declare that I have no specific conflict of interest with the material presented in this Review. Currently, or within the past 3 years, I have received consultancy, travel, and speaker fees from AstraZenceca Pharmaceuticals in relation to β-agonist steroid combination products. I have received consultancy fees from Roche Pharmaceuticals in relation to review of preclinical investigational concepts and compounds. My laboratory has several full-time staff who undertake fee-for-service testing of compounds in preclinical animal models of asthma and COPD, for which the surplus arising is used to support basic research. The University of Melbourne has licensed patented intellectual property arising partly from research in my laboratory on GM-CSF to MorphoSys AG, Germany, for the treatment of chronic inflammatory disorders including lung disease.

Acknowledgments

The author's research is funded by the National Health and Medical Research Council of Australia (NHMRC) via project and programme grants.

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Chronic lung diseases: prospects for regeneration and repair

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Treatment outcomes with COPD and IPF are suboptimal. Better understanding of the diseases, such as targetable repair mechanisms, may generate novel therapies, and earlier diagnosis and treatment is needed to stop or even reverse disease progression. https://bit.ly/2Ga8J1g

Cite this article as: Barnes PJ, Anderson GP, Fagerås M, *et al*. Chronic lung diseases: prospects for regeneration and repair. *Eur Respir Rev* 2021; 30: 200213 [https://doi.org/10.1183/16000617.0213-2020].

ABSTRACT COPD and idiopathic pulmonary fibrosis (IPF) together represent a considerable unmet medical need, and advances in their treatment lag well behind those of other chronic conditions. Both diseases involve maladaptive repair mechanisms leading to progressive and irreversible damage. However, our understanding of the complex underlying disease mechanisms is incomplete; with current diagnostic approaches, COPD and IPF are often discovered at an advanced stage and existing definitions of COPD and IPF can be misleading. To halt or reverse disease progression and achieve lung regeneration, there is a need for earlier identification and treatment of these diseases. A precision medicine approach to treatment is also important, involving the recognition of disease subtypes, or endotypes, according to underlying disease mechanisms, rather than the current "one-size-fits-all" approach. This review is based on discussions at a meeting involving 38 leading global experts in chronic lung disease mechanisms, and describes advances in the understanding of the pathology and molecular mechanisms of COPD and IPF to identify potential targets for reversing disease degeneration and promoting tissue repair and lung regeneration. We also discuss limitations of existing disease measures, technical advances in understanding disease pathology, and novel methods for targeted drug delivery.

Introduction

COPD and idiopathic pulmonary fibrosis (IPF) both represent a substantial unmet clinical need. COPD has become the third leading cause of death globally [1], and IPF has a median survival of ∼3 years after diagnosis, with survival rates comparable to some aggressive cancers [2], and no observed improvement in survival from 2000 to 2012 [3].

In this article, we discuss the current limitations of treatment for COPD and IPF, and potential future strategies with a focus on disease subtypes and lung regeneration and repair. This article is based on discussions at a meeting organised by the authors on 28–29 November 2018 in Gothenburg, Sweden, and supported by AstraZeneca, involving leading global experts in obstructive lung disease who discussed the latest innovations and issues in chronic lung disease (the participants are listed in the Acknowledgements section).

Provenance: Submitted article, peer reviewed

Received: 6 July 2020 | Accepted: 17 Aug 2020

COPD

COPD is a largely preventable and treatable disease characterised by persistent airflow limitation and respiratory symptoms due to chronic inflammation, which causes structural changes, such as fibrosis of the small airways and alveolar wall destruction (emphysema) [1, 4]. Early pathological changes occur in the small airways, with associated inflammation, wall thickening, peribronchiolar fibrosis and loss of terminal and transitional bronchioles and associated vasculature, before the onset of emphysema [5–9]. These early and irreversible destructive events do not initially affect the lung function parameters usually used to define COPD (forced expiratory volume in 1 s (FEV_1)/forced vital capacity ratio), making early detection difficult [10]. Smoking is a key risk factor for COPD; however, nonsmokers can also develop COPD (especially in low- and middle-income countries) and many smokers do not develop COPD [11], indicating a role of genetic risk, epigenetics and other environmental factors in its development [12]. The most documented genetic risk factor for COPD is α_1-antitrypsin deficiency that represents a specific subtype of COPD (endotype), although different phenotypes exist within this subtype that appear to be caused by variations in other factors, such as tumour necrosis factor (TNF)-α [13]. Autoimmunity and aberrant immunity (suppression of host defence mechanisms and dysfunction of innate immunity) may also contribute to disease progression, especially as the disease advances [14–16].

IPF

IPF is commonly described as a specific form of chronic, progressive, fibrosing, interstitial pneumonia of unknown cause, usually occurring in the elderly [17]. IPF incidence appears to be increasing: the diagnosis of IPF in the UK has increased from approximately 20 per 100 000 patients in 2000 to nearly 40 per 100 000 patients in 2012 [3, 18]. IPF arises from repetitive micro-injuries to the bronchial and alveolar epithelium, which, along with immune system dysregulation [19, 20], results in progressive scarring and the destruction of lung structures [18, 21, 22]. The aetiology of IPF is unknown [3], but smoking is a risk factor and may influence IPF onset [23, 24]. Major genetic risk factors, such as the mucin 5B gene and defective telomerase, have been identified, pointing to future genetic stratification [25, 26]. Of note, pulmonary emphysema and IPF can co-exist in the same patient as a distinct entity termed combined pulmonary fibrosis and emphysema (CPFE) [27]. This condition is characterised by emphysema in the upper lobes and fibrosis in the lower lobes of the lungs [27, 28]. CPFE is estimated to occur in up to 35% of patients with IPF [29].

Limitations of current treatment approaches

Commonly used maintenance treatments in COPD include β_2-agonists, anticholinergics, theophylline and corticosteroids [1]. Such treatments are primarily for improving lung function, reducing symptoms and the risk of exacerbations, and improving exercise tolerance and health status. To date, no disease-modifying treatments are available [30]. This is in sharp contrast with other chronic inflammatory-based diseases. In rheumatoid arthritis, for example, scientific advances and early treatment with disease-modifying drugs have resulted in the prevention of disease progression in up to 90% of patients [31].

Treatment options for IPF are even more limited and represent a pressing unmet clinical need [32]. Currently, only two antifibrotic drugs are recommended; namely, pirfenidone and the tyrosine kinase inhibitor nintedanib [17]. However, a recent analysis revealed that 40% of patients with confirmed IPF did not receive antifibrotics, reflecting a possible lack of understanding around the diagnosis and management of the disease, and problems with treatment access [33]. Furthermore, although these treatments may be life-extending [34, 35], potential adverse events could negatively impact quality of life [17], such as gastro-intestinal effects and photosensitivity with pirfenidone, and diarrhoea with nintedanib [36, 37].

Future treatment strategies for COPD and IPF

The marked unmet needs in COPD and IPF therapy highlight the need for new treatment strategies that focus on underlying disease endotypes, regeneration and repair.

A change in mindset is required among pulmonologists, regulators and policymakers to redefine perceptions of COPD and IPF. Current treatments have a “magic bullet” approach, where a single drug is intended to treat all forms of disease. However, both COPD and IPF are heterogeneous diseases with several clinical phenotypes [38] that may reflect multiple but, as of yet, mostly unidentified endotypes (subtypes of disease defined functionally and pathologically by molecular mechanism or treatment response) [39–41]. A move to a “complex subtypes” approach, where precision medicine allows COPD or IPF subtype-specific treatment, could be possible with combinations of interventions. Future treatment strategies may target different aspects of these diseases chronologically, or target several disease mechanisms simultaneously, with subsequent treatment withdrawal upon improvement. For new treatment strategies that focus on underlying disease endotypes, it will be crucial to study COPD and IPF at an early stage before confounding factors, comorbidities and disease progression mask subtle differences.

In the same way, both diseases have been described as "irreversible" [4, 42, 43]. However, lung regeneration, disease reversal and even a cure for COPD and IPF are the ultimate goals in disease management; merely slowing disease progression is important but does not completely address the unmet clinical need [44, 45]. Regeneration efforts could focus on activating the endogenous repair capacity of the lungs, and/or adopting exogenous regeneration through tissue engineering, bio-artificial scaffolds or adding healthy progenitor or stem cells to the lungs [46]. Evidence from retinoic acid studies shows that lung regeneration is feasible, at least in rodent models [47, 48]; however, we need a clear understanding of how endogenous repair processes become dysfunctional in the diseased lungs to identify targets for potential treatment strategies.

New targets for lung regeneration are being identified, but many of these may not be druggable *via* conventional approaches using either small-molecule inhibitors/activators or systemic antibodies [49]. New modality treatments are being developed, such as approaches using proteolysis-targeting chimaera (PROTAC), inhaled antisense oligonucleotides, gene editing (CRISPR: clustered regular interspaced short palindromic repeats) or exosomes that will allow us to target all pathways of interest (table 1). Although direct delivery to the target organ, in this case the lungs, is possible with inhaled approaches, new methodologies for delivering treatments are also needed.

Several practical considerations must also be borne in mind. The age and frailty of patient populations with COPD and IPF are likely to be among the greatest challenges to lung regeneration, as well as the existence of comorbid diseases. To achieve significant lung regeneration, it is likely that COPD and IPF

TABLE 1 Examples of emerging techniques to deliver therapy in patients with COPD and idiopathic pulmonary fibrosis (IPF) and their limitations

Technique	Description	Uses and advances made with the technique	Current limitations
PROTAC	Proteolysis-targeting chimaera that uses the cell's ubiquitin–proteasome system to target-specific proteins for degradation	Could induce the degradation of proteins previously considered "undruggable" [50] Highly selective for the target protein, with rapid, effective and prolonged degradation of the target [51] Valuable for mechanisms requiring precise targeting for degradation	Can only target a protein for degradation, not for modification
CRISPR	Can manipulate gene function through gene deletion, correction or replacement; enhancement of gene expression; base editing	Huge potential for target-specific genetic medication for gene therapy in COPD and IPF to target dysregulated genes or pathways (*e.g.* epigenetic changes to genes implicated in mucus hypersecretion in COPD) [52]	Concerns exist around safety and off-target effects; these are under investigation [53]
Inhaled antisense oligonucleotides	Single-stranded DNA or RNA (20–21 base pairs) complementary to the target mRNA	Knocks down the expression of the target gene [54] Can modulate molecules that cannot be targeted using antibodies [54] Inhalation could minimise toxicities associated with systemic exposure of antisense oligonucleotides [54]	Currently in the investigational stage Intracellular delivery to the site of action is a challenge [54]
Exosomes as delivery systems	A potential delivery system for nucleic acid drugs	Potential use in delivering drugs such as antagomirs or miRNA molecules, thanks to their low antigenicity and toxicity [55] Could target particular cell types *In vitro* and *in vivo* studies have shown promise in successfully delivering molecules [56]	Currently in the investigational stage

PROTAC: proteolysis-targeting chimaera; CRISPR: clustered regularly interspaced short palindromic repeats.

treatment will need to be at an earlier stage and in younger patients compared with what currently occurs in clinical practice. This argument is supported by evidence suggesting that early treatment and early smoking cessation have a positive effect on longitudinal lung function and symptoms [57]. COPD is currently diagnosed using spirometry [1], but these changes detected by spirometry occur relatively late in disease progression and are a poor measurement of peripheral airway obstruction in early disease [10]. Redefining COPD based on abnormalities in small airway function, measured using techniques such as magnetic resonance imaging and impulse oscillometry [58, 59], may identify disease earlier than current practises [10, 60] (table 2). Population screening of smokers for COPD could also be a possibility, but screening for IPF less so as it is an uncommon disease and difficult to diagnose [33].

Biomarkers

Biomarkers are central in identifying patient subgroups, phenotypes and endotypes [8, 78, 79]. They are crucial in monitoring and predicting disease progression and predicting responders to treatment [8, 79]. COPD and IPF are highly complex and heterogeneous, and no single biomarker has been identified for clinical applications in either disease [80–82]. Dividing COPD and IPF into endotypes is critical for breaking the diseases down into molecular pathways and disease mechanisms, and for linking molecular mechanisms to clinical features. Treatment targets for specific endotypes could thus be identified and could provide precision treatment to those patients most likely to respond [40, 83]. In the management of cancer, it has long been recognised that genetic mutations can give rise to cancer subtypes that predict prognosis and response to treatment [84]. A similar rationale needs to be applied to COPD and IPF to identify subgroups with distinct disease mechanisms [40, 83].

The Evaluation of COPD Longitudinally to Identify Predictive Surrogate Endpoints (ECLIPSE) and the Genetic Epidemiology of COPD (COPDGene) studies have identified numerous putative biomarkers in COPD. These include protein, cellular and genetic biomarkers associated with COPD characteristics and morbidity (including airflow limitation, emphysema and exacerbation frequency) [85–87]. The analysis of six inflammatory biomarkers (white blood cell count, C-reactive protein, interleukin-6, C-X-C motif chemokine ligand 8, TNF-α and fibrinogen) from patients in ECLIPSE led to the identification of a new COPD phenotype [88].

In IPF, several biomarkers are associated with specific phenotypes [89]. Protein degradation biomarkers and serum biomarkers have been identified that can discriminate between healthy individuals, patients with stable IPF and those with progressive IPF [90]. Four serum proteins have been identified from the metaplastic epithelium that could predict disease progression and mortality; namely, surfactant protein D, matrix metalloproteinase-7, carbohydrate antigen 19–9 and cancer antigen-125 [91]. A gain-of-function variant of the promotor of the mucin 5B (MUC5B) gene is associated with the pathogenesis of IPF [25]; identifying this variation in patients with pre-clinical IPF and targeting MUC5B could enable early diagnosis and prevent the progression of IPF to a state where the lung remodelling is irreversible [25, 92].

Early detection

As we learn more about COPD and IPF endotypes and phenotypes, advances in technology are required to identify these in patients and to allow for the early detection of disease and to monitor disease progression. Reliable tests for small airway function and the ability to quantify disease progression and its links to biomarkers will be essential for advancing our knowledge and for the management of COPD and IPF. Advances in the "-omics" field (for example, genomics, transcriptomics, proteomics, lipidomics and metabolomics) have led to new discoveries and promises to provide insights into endotypes. For instance, it may be possible to use blood samples to detect genomic biomarkers [93], and bioinformatic analysis may identify the activation of particular molecular pathways that could be targeted [94]. Also, breathomics of exhaled breath may help identify COPD phenotypes and provide biomarkers for diagnosis and disease progression [77].

A potential barrier to early, preventative therapies may be that patients who do not feel unwell or who are not experiencing any impact on their quality of life may be reticent about taking medication with associated side-effects. Payers may also be resistant to paying for medication when the current classifications of disease categorise the patient as "at risk" rather than having measurable disease, although it is noteworthy that primary prevention measures do exist in other diseases; for example, the treatment of systemic hypertension and hypercholesterolaemia to prevent cardiovascular diseases [95].

Since late disease is associated with profound structural damage to the lung that is currently irreversible [4, 96], there is great interest in identifying potentially treatable processes much earlier in disease, especially those around regeneration and repair [97–101].

TABLE 2 Examples of new or emerging techniques for studying COPD and idiopathic pulmonary fibrosis (IPF)

Technique	Description	Uses and advances made with the technique	Current limitations
Micro-CT imaging	High-resolution CT imaging	Higher-resolution *versus* standard CT imaging [61] Can reveal structural changes associated with small airway disease [61] Reveals massive loss in number and area of terminal bronchioles in patients with centrilobular emphysematous COPD [6] When partnered with parametric response mapping as an imaging biomarker, micro-CT could identify terminal bronchiole pathology in COPD [62]	Performed on *ex vivo* samples, or explants, rather than on the patient [6, 61, 62]
PET	Molecular imaging; most commonly measuring ^{18}F-FDG uptake	Has been explored as a noninvasive biomarker for pulmonary inflammation [63] Ability to quantify inflammation is under investigation [63]	Validation of imaging approaches required; changes in lung air, blood and water volumes depending on disease severity can cause variations in signals [63]
Gas diffusion MRI	Noble gases such as ^{3}He and ^{129}Xe used to visualise lung structure	Could be used to monitor disease progression and response to therapy [59] Can detect microstructural changes in the lung, even in asymptomatic smokers [59] Quantitative microstructure data obtainable by measuring gas diffusion in alveoli; the technique can differentiate between patients with COPD and healthy individuals [64] Alveolar sizes can be visualised to form a picture of alveolar loss in COPD [64] Provides sensitive and reproducible data on gas exchange impairment in IPF, correlating with spirometry data [65]	Adaption of existing scanners is required [66]
SPECT	Radiotracers used to image the lung, where both airways and blood flow can be imaged	Both the airways and blood flow can be imaged, allowing the detection of comorbidities such as pulmonary embolism [67, 68] Can detect abnormalities in apparently healthy smokers [69]	Only semi-quantitative [69] Not as high resolution as other imaging methods [68] Takes a long time to acquire an image (*e.g.* 45 min) [68]
IOS	Noninvasive measurement of respiratory mechanics in response to pressure oscillations	A reliable tool for investigating proximal and peripheral airways resistance in patients with COPD [70] Peripheral airway resistance and compliance using IOS closely linked to COPD severity and exacerbations [58] Could be used as a screening tool for early-stage COPD [58] Useful for patients who cannot perform spirometry manoeuvres [71]	The minimal clinically important difference in IOS parameters needs to be established

Continued

TABLE 2 Continued

Technique	Description	Uses and advances made with the technique	Current limitations
OCT	A high-resolution optical imaging method	Resolution down to micrometre scale [72] Can be used to accurately measure distal airways [73] Could detect early changes to the distal airways and appears to be more sensitive than CT [72, 73]	Ultrafine bronchoscopy (with sedation) required to reach the distal airways [73]
Multiple-breath nitrogen washout	Noninvasive measurement of residual nitrogen in the airways to detect any abnormalities in gas distribution in the lung	Does not require maximal effort and can be used in a paediatric setting [74] Provides information on abnormalities in the small airways, including terminal bronchioles [75] Can detect abnormalities in early disease [76]	Limited standardisation, which impacts the availability of widely applicable reference values [75]
Breathomics	Exhaled breath analysis to detect changes in volatile organic compounds	Could be used to diagnose COPD and differentiate COPD from asthma [77] May be able to predict disease progression [77] Could help distinguish COPD phenotypes [77]	Results can be confounded by parameters such as medication use, comorbidities, smoking and study site [77]

CT: computed tomography; PET: positron emission tomography; ^{18}F-FDG: ^{18}F-2-fluoro-2-deoxy-D-glucose; MRI: magnetic resonance imaging; SPECT: single-photon emission computed tomography; IOS: impulse oscillometry; OCT: optical coherence tomography.

Dysregulated processes presenting opportunities for regeneration and repair

Cellular senescence

Accelerated ageing and senescence are evident in the lungs of patients with COPD and IPF [102, 103]. This can be brought on by DNA damage, mitochondrial dysfunction, telomere shortening, reduced autophagy and stem cell exhaustion, and involves cell cycle arrest and a secretory profile of inflammatory proteins. This is central to lung development and wound repair. In the healthy individual, once wound repair is complete, senescent cells are removed following apoptosis triggered by infiltrating immune phagocytes. However, if senescent cells are not removed, their abnormal secretory profile can lead to pathological tissue changes [104].

Several steps in the senescence pathway could be targeted to halt accelerated ageing and senescence. Cellular senescence is associated with a loss of anti-ageing molecules, such as certain sirtuins and Klotho [105, 106]. The microRNA miR-34a is increased by activation of the phosphoinosite-3-kinase (PI3K)-mammalian target of rapamycin (mTOR) pathway, and downregulates the expression of sirtuin-1 and sirtuin-6; up-regulation of miR-34a in the lungs and cells of patients with COPD results in loss of sirtuin-1 and -6 [106, 107]. miR-570 is also increased in COPD and is activated by p38 mitogen-activated protein kinase, resulting in the downregulation of sirtuin-1 [108]. Inhibition of miR-34a and miR-570 with antagomirs rescues the loss of sirtuin-1 and sirtuin-6, thereby preventing the induction of senescence [106, 108]; the therapeutic administration of antagomirs, possibly *via* inhaled exosomes [55], could therefore represent a strategy to reverse accelerated ageing [106, 108].

Oxidative stress, *via* increased reactive oxygen species production or decreased antioxidants, is central in driving senescence in COPD through the activation of the PI3K-mTOR pathway [102, 109]. Reactive oxygen species are potentially generated by the mitochondria in response to cigarette smoke [102, 103]. This results in secretion of a senescence-associated secretory phenotype of inflammatory proteins, which include pro-inflammatory cytokines, chemokines, growth factors and proteases and may account for the low-grade inflammation seen in COPD and the affected patient's systemic circulation [105, 110]. The mTOR inhibitor rapamycin prevents senescence and inhibits components of the senescence-associated secretory phenotype *in vitro* in pulmonary artery smooth muscle and pulmonary vascular endothelial cells isolated from patients with COPD [109]. The effective dose of rapamycin was low and did not affect cell

growth rate, suggesting relatively low doses may be sufficient for a therapeutic effect, thereby reducing potential toxicity.

Multiple cell types are affected by senescence in COPD and IPF, including epithelial, endothelial, fibroblast and smooth muscle cells in COPD [108, 109, 111] and epithelial cells and fibroblasts in IPF [104, 112, 113]. The elimination of senescent cells, or senolysis, is another approach to tackle senescence. Experimental models have shown that the senolytic agents dasatinib and quercetin kill senescent cells and improve lung function [114], and a pilot study of these agents in patients with IPF has shown improvements in physical function with an acceptable safety profile over a 3-week period [115]. The senolytic drug navitoclax (ABT-263) has also been found to reverse pulmonary fibrosis and induce apoptosis in myofibroblasts implicated in fibrosis in animal models [99, 100].

Wnt/β-catenin signalling

The Wnt signalling pathway guides cells to certain fates during lung development and maintains tissue homeostasis in adulthood [116]. Wnt/β-catenin signalling is reduced in mouse models of elastase- and cigarette smoke-induced emphysema, which were attenuated upon Wnt activation with improvements observed in alveolar epithelial structure and function [98]. Cells affected by this pathway include alveolar epithelial type II (ATII) cells in the alveolar epithelium, which have self-renewal properties and rely on Wnt/β-catenin signalling to differentiate to ATI cells in response to alveolar epithelial injury [116]. ATI cells cover the majority of the lung surface area (95–97%) and are responsible for gas exchange, a key lung function [117]. The noncanonical Wnt ligand WNT-5A, which is overexpressed in lungs from animal models of COPD and patients with COPD, antagonises the canonical Wnt/β-catenin signalling pathway resulting in the inhibition of murine lung epithelial cell wound healing and transdifferentiation from ATII to ATI cells *in vitro* [118]. Lung-specific overexpression of WNT-5A exacerbated the development of emphysema, and prophylactic inhibition of WNT-5A could recuperate alveolar cell function and attenuate lung pathogenesis in COPD animal models [118]. In addition, activation of canonical Wnt/β-catenin signalling with lithium chloride improved alveolar epithelial structure and function in experimental models of emphysema [98]. The canonical Wnt receptor frizzled-4 (FZD4) facilitates ATII to ATI transdifferentiation. FZD4 expression was reduced in patients with COPD, correlating positively with lung function and negatively with smoking (pack-years) [119]. Cigarette smoke directly downregulated FZD4 *in vivo* and *in vitro*, thereby preventing Wnt/β-catenin signalling and alveolar tissue repair [119].

Interestingly, activated Wnt/β-catenin signalling in IPF leads to an increase in the Wnt target, WNT-1-inducible signalling protein-1 (WISP1), which in turn induces the expression and secretion of profibrotic mediators, contributing to lung fibrosis [120, 121]. Using antibodies to neutralise WISP1, Königshoff *et al.* [121] showed reduced pulmonary fibrosis, implicating WISP1 as a potential therapeutic target in IPF.

Several novel approaches to activating and inhibiting Wnt/β-catenin signalling are now in development and look promising with regard to restoring normal lung function in COPD and IPF [97, 122]. These discoveries point to several approaches that could reinstate cell and tissue homeostasis in COPD and IPF.

Stem cell therapy

The basal stem cells (BSCs) in the cartilaginous airways of the lungs are considered to be multipotent lung progenitor cells [123, 124] and drive homeostasis of the normal epithelium and regeneration following injury [123]. Therefore, they could be a potential regeneration target; targeting their proliferation and directing differentiation and stem cell transplantation/bioengineering [123]. In a study of smokers, reductions in the number and function of BSCs were observed in those with COPD compared with those without COPD [125]. Interestingly, low BSC counts in smokers without COPD were associated with lower lung function than in those with high BSC counts [125], which could represent an early pre-diagnostic stage of COPD. However, BSCs isolated from heavy smokers undergoing lung cancer surgery were found to have an increased proliferate potential *in vitro* compared with never-smokers, whereas ATII cell proliferation decreased [126]. This is in part because BSCs repair damaged DNA by nonhomologous end-joining, which is faster but more error-prone than homologous repair and increases the risk of mutagenesis [126].

Elevated BSCs were observed in the bronchoalveolar lavage fluid from patients with IPF in comparison to healthy individuals [127]. Normally located at the bronchoalveolar duct junction, BSCs were enriched in the alveolar compartment and frequently within fibrotic lesions of patients with IPF [127, 128]. This suggests an unexpected role of BSCs in the pathogenic bronchiolisation of the alveoli in IPF, where bronchial cells appear in this compartment by migration or transdifferentiation [127, 128].

While BSCs theoretically represent an opportunity to reverse COPD- and IPF-associated damage, we need to distinguish between healthy BSCs and those potentially carrying DNA mutations to enhance the positive effects without increasing the risk of pathogenic changes. The interactions between BSCs and immune cells and their role in IPF pathogenesis also need to be understood before BSCs can be considered as therapy.

A population of mesenchymal progenitor cells positive for stage-specific embryonic antigen (SSEA)-4, a cell-surface protein expressed by stem cells, has been identified in the lungs of patients with IPF. These SSEA-4 cells were found to display a pathological gene expression pattern, and their progeny developed a pathological IPF fibroblast phenotype [129]; these cells could be targeted as a therapeutic intervention, although it remains to be seen whether some SSEA-4+ cells are beneficial.

Biological molecules

The vasculature should also not be overlooked when elucidating the mechanisms of lung degeneration and seeking targets for lung regeneration. Retinoic acid is a morphogen that drives tissue regeneration [130, 131] and can induce alveolar regeneration in animal models [47, 48]. In humans, retinoic acid is involved in maintaining the lung microvascular endothelium through up-regulating angiogenesis; in emphysema, expression of the retinoic acid-processing enzyme cytochrome P450 26A1 is elevated in the endothelium, potentially reducing the availability of retinoic acid [132]. It follows that retinoic acid could be a treatment option for lung regeneration; however, early-phase clinical trials of retinoic acid in emphysema have failed to show a clinical benefit [133–135], which underlines the need to understand more about retinoic acid in lung regeneration and whether retinoids can induce lung regeneration.

Hepatocyte growth factor (HGF; also known as scatter factor) promotes airway and bronchoalveolar branching in the developing lung [136, 137], possibly through interaction with vascular endothelial growth factor [138]. HGF also promotes the proliferation and survival of airway epithelial cells [139], plays a role in wound healing [140] and has been shown to improve airspace morphology in emphysema models [139]. Levels of both HGF and vascular endothelial growth factor are reduced in smokers with COPD in comparison to smokers without COPD and nonsmokers, which could contribute to pathogenesis [141], indicating that HGF-enhancing therapy could represent a treatment opportunity for COPD and IPF [142].

Receptor tyrosine kinase pathways have been implicated in aberrant lung remodelling, potentially through growth arrest-specific 6 ligand, TYRO3 protein kinase 3 and Axl [143]. Inhibiting this pathway led to decreased fibrotic responses *in vivo* and *in vitro*, suggesting that targeting the receptor tyrosine kinase pathway could be a promising therapeutic strategy [143].

Granulocyte colony-stimulating factor (G-CSF) has been found to be elevated in the lungs of patients with COPD [101]. Interestingly, deletion of G-CSF in a mouse model of COPD led not only to substantially less inflammation and reduced fibrosis in the lung parenchyma and small airways, but it also reduced systemic inflammation and led to improvements in the comorbidities associated with COPD [101], suggesting that G-CSF is a potential therapeutic target in COPD [101].

Role of the extracellular matrix

The extracellular matrix (ECM) plays a central role in guiding cell behaviour and in tissue repair and remodelling. An *ex vivo* model used bronchial ECM from patients with COPD that was stripped of cells and then repopulated with normal human bronchial cells. The model revealed that the COPD-derived ECM modified the gene expression profile of these healthy cells upon differentiation, altering the activity of mediators involved in regeneration, remodelling, apoptosis, vascularisation and inflammation [144]. Similarly, fibroblasts grown on a stiff matrix resembling a fibrotic ECM, as occurs in IPF, were driven to a myofibroblast phenotype with elevated fibrotic activity, compared with fibroblasts grown on a softer ECM resembling healthy tissue [145]. Such findings emphasise that we need to fully understand the contribution of the ECM in disease and lung regeneration, as the enzymes involved in ECM remodelling could be potential therapeutic targets.

Lessons for the future

Advances in treating obstructive lung diseases such as COPD and IPF have been slow, and improvements in patient outcomes and drug discovery have been poor in respiratory medicine compared with other diseases [146]. Currently, clinical trials require a large number of patients to be assessed over a long period to detect any differences in end-points [146], which could delay results and ultimately slow medical advances. The high costs of such large trials needed to show clinically meaningful effects have discouraged investment in new drug development; many drugs also fail in phase 2 and 3 clinical trials, leading to a rethinking of trial design [146, 147]. Clinical trial design needs to be "smarter". The design should focus on the biology of the disease and the drug mechanism of action, and end-points should be appropriate for

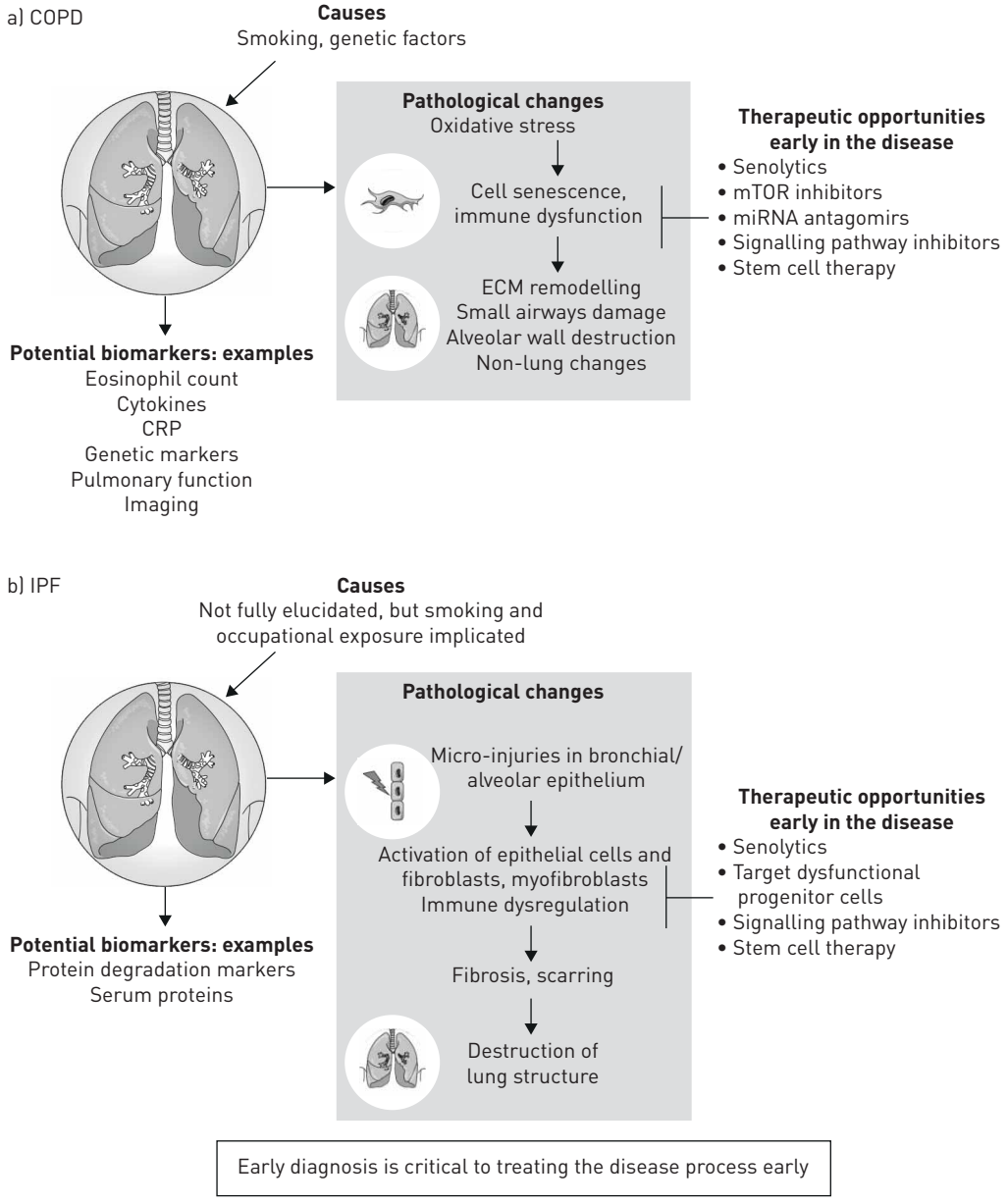

FIGURE 1 Causes, pathogenesis and opportunities for therapeutic intervention in a) COPD and b) idiopathic pulmonary fibrosis (IPF). CRP: C-reactive protein; ECM: extracellular matrix; miRNA: microRNA; mTOR: mammalian target of rapamycin.

the drug's mechanism of action to establish target engagement; improved proof-of-concept or adaptive trials could help rule out ineffective compounds early on to reduce wasted time and costs [146, 148]. If it is anticipated that a biological or clinical effect of the drug would be observed after a certain time, the trial need only last as long as that period. Also, only the subgroup of patients to whom the drug is targeted should be enrolled, even if this results in a relatively small number of patients. Furthermore, any group of patients responding particularly well to a drug should be closely investigated to understand why.

Recognition of the limitations in current therapies for COPD and IPF, which result in a substantial unmet clinical need, points to possible future treatment strategies. For example, a move to precision medicine as opposed to the "magic bullet" approach could lead to therapeutic advances in these highly complex diseases of varying endotypes. In addition, a change in mindset is needed from considering these diseases to be irreversible, to a focus on early diagnosis when reversibility may be possible (figure 1).

The advances in our knowledge of lung degeneration in COPD and IPF raise further questions. For example, is fibrosis a protective mechanism to prevent peripheral airway destruction? Do terminal

bronchioles undergo fibrosis? What is the mechanism for the loss of small airways in early disease? To understand and treat COPD and IPF more effectively, we need clear molecular profiles of the disease; we also need to understand why some areas of the lung are affected and why others are not.

Conclusion

In summary, the considerable body of research into COPD and IPF has yet to translate into improvements in clinical practice. A paradigm shift is required to move the focus to earlier in the disease course, to understand the disease mechanisms more fully, and to measure multiple aspects of the disease. COPD and IPF need to be redefined to better capture the patient populations involved and shift the conceptions about each disease. Novel technologies and the field of "-omics" are providing new insights into COPD and IPF, increasing our ability to predict outcomes and helping to identify new potential therapies to achieve lung regeneration.

Acknowledgements: Meeting attendees: Serge Adnot (Hôpital Henri Mondor & Université Paris Est, Créteil, France), Gary Anderson (University of Melbourne, Melbourne, Australia), Marie-Liesse Asselin-Labat (Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia; Oregon Health and Science University, Portland, OR, USA), Jonathan Baker (Imperial College, London, UK), Peter Barnes (Imperial College, London, UK), Maria Belvisi (AstraZeneca, Gothenburg, Sweden), Francois Xavier Blé (AstraZeneca, Cambridge, UK), Mia Collins (AstraZeneca, Gothenburg, Sweden), James Crapo (National Jewish Health, Denver, CO, USA), Sean Davis (AstraZeneca, Cambridge, UK), Oliver Eickelberg (University of Colorado Denver, Denver, CO, USA), Malin Fagerås (AstraZeneca, Gothenburg, Sweden), Donna Finch (MedImmune Ltd, Cambridge, UK), Hani Gabra (AstraZeneca, Cambridge, UK), Angus Hamblin (AstraZeneca, Gothenburg, Sweden), Petra Hazon (AstraZeneca, Gothenburg, Sweden), Matthew Hind (Royal Brompton Hospital & Imperial College, London, UK), Cory Hogaboam (Cedars-Sinai Medical Center, Los Angeles, CA, USA), Jim Hogg (St Paul's Hospital, Vancouver, Canada), Ellinor Hornberg (AstraZeneca, Gothenburg, Sweden), Rod Hughes (AstraZeneca, Cambridge, UK), Simon Johnson (University of Nottingham, Nottingham, UK), Christina Keen (AstraZeneca, Cambridge, UK), Roland Kolbeck (MedImmune, Gaithersburg, MD, USA), Melanie Königshoff (University of Colorado Denver, Denver, CO, USA), Brian Lipworth (University of Dundee, Dundee, UK), Alex Mackay (AstraZeneca, Gothenburg, Sweden), Toby Maher (Royal Brompton Hospital & Imperial College, London, UK), Anna Malmgren (AstraZeneca, Gothenburg, Sweden), Marc Miravitlles (Hospital Universitari Vall d'Hebron, Barcelona, Spain), Catherine Overed-Sayer (MedImmune Ltd, Cambridge, UK), Edward Piper (AstraZeneca, Cambridge, UK), Adam Platt (AstraZeneca, Cambridge, UK), Ioannis Psallidas (AstraZeneca, Cambridge, UK), Steve Rees (AstraZeneca, Cambridge, UK), Stephen Rennard (AstraZeneca, Cambridge, UK), John Taylor (AstraZeneca, Gothenburg, Sweden), and Jim Wild (University of Sheffield, Sheffield, UK).

Medical writing support (including development of the first draft under author direction) was provided by Sarah Hoyle and Kate Silverthorne (CMC Connect, McCann Health Medical Communications Ltd, Macclesfield, UK) and funded by AstraZeneca (Cambridge, UK), in accordance with Good Publication Practice guidelines.

Conflict of interest: P.J. Barnes reports grants and personal fees from AstraZeneca and Boehringer Ingelheim, and personal fees from Novartis, Teva and Pieris, outside the submitted work. G.P. Anderson served on secondment as Chief Scientist in the Respiratory, Inflammation and Autoimmune division of AstraZeneca in 2016. M. Fagerås is an employee of AstraZeneca. M.G. Belvisi reports personal fees from AstraZeneca, during the conduct of the study.

Support statement: The development of this article was funded by AstraZeneca, Cambridge, UK.

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Interleukin-4 Is Required for the Induction of Lung Th2 Mucosal Immunity

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Aerosol antigen challenge of ovalbumin-sensitized mice induced an eosinophilic airway inflammation that was dependent on interleukin (IL)-5 and $CD4^+$, but not $CD8^+$, T lymphocytes. The involvement of the Th2 phenotype of $CD4^+$ T cells was supported by demonstrating that FACS-sorted purified lung T cells from sensitized, but not control, mice produced IL-4, IL-5, and IL-10 after activation of the CD3/TCR complex. To determine the role of IL-4 in this process, we used mice in which the gene for IL-4 was deleted by homologous recombination. Antigen challenge of IL-4 gene–targeted mice resulted in a marked attenuation of eosinophilic inflammation and IL-5 secretion. To more fully understand the time when IL-4 was involved, we administered a neutralizing anti-IL-4 antibody (11B11) either immediately before antigen challenge or during immunization. Inhibition of IL-4 before antigen challenge had little effect on antigen-induced eosinophil infiltration. However, when 11B11 was administered during immunization, there was a marked reduction in eosinophil infiltration. Cross-linking of the CD3/TCR complex of FACS-sorted lung T cells revealed that only when anti-IL-4 was administered during immunization was there an inhibition of T cell–derived IL-5 and IgE production. These results suggest that IL-4 is central both to the induction of a local Th2 response and to the development of eosinophilic inflammation of the lung. Moreover, we suggest a sequential involvement of IL-4 and IL-5, with IL-4 committing naive T cells to a Th2 phenotype which upon activation by aerosol provocation secrete IL-5, resulting in eosinophil accumulation.

There is now a considerable body of evidence to suggest that infiltration of eosinophilic leukocytes to the airways is important in the pathophysiology of bronchial asthma. Although the precise mechanisms by which these cells accumulate in the airways still remain to be fully elucidated, recent studies have suggested that eosinophil recruitment is dependent on interleukin (IL)-5 derived from $CD4^+$ T cells (1, 2). Murine $CD4^+$ T cells have been divided into two distinct subsets on the basis of their ability to mediate different functions and secrete different cytokine profiles. Th1 cells produce IL-2 and interferon-γ (IFN-γ) and are involved in delayed hypersensitivity responses, whereas Th2 cells produce IL-4, IL-5, and IL-10 and provide help to B cells to produce IgE (3).

(Received in original form November 7, 1994 and in revised form January 31, 1995)

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Abbreviations: Fluorescence activated cell sorter, FACS; interferon-γ, IFN-γ; ovalbumin, OA; vascular cell adhesion molecule-1, VCAM-1; very late activation antigen-4, VLA-4.

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Although the cytokine networks that control the commitment of T cells to either of these subsets is not fully understood, IL-4 has been demonstrated *in vitro* to be important in the switch of naive cells to the Th2 phenotype (4, 5). Moreover, studies performed in IL-4 gene–deleted mice (6) or using selective IL-4 monoclonal antibodies (7) have demonstrated the essential role of this cytokine in the development of Th2 cells and in the induction of an IgE response. However, the exact time when IL-4 generation is required is at present unclear.

In the present study, we have used two separate approaches to address these questions. Experiments were performed either in mice in which the gene for IL-4 was deleted by homologous recombination or by administering a neutralizing antibody to IL-4. Our results indicate that mice lacking the gene for IL-4 fail to develop an IgE response and have a marked attenuation of eosinophil infiltration and lung T-cell IL-5 production. Furthermore, anti-IL-4 applied during immunization, but not immediately before challenge, abrogated eosinophil infiltration and lung T-cell IL-5 production. We conclude that IL-4 is not involved in the mechanisms by which eosinophils adhere and migrate to the airways but plays a central role in switching T cells to an IL-5-producing phenotype.

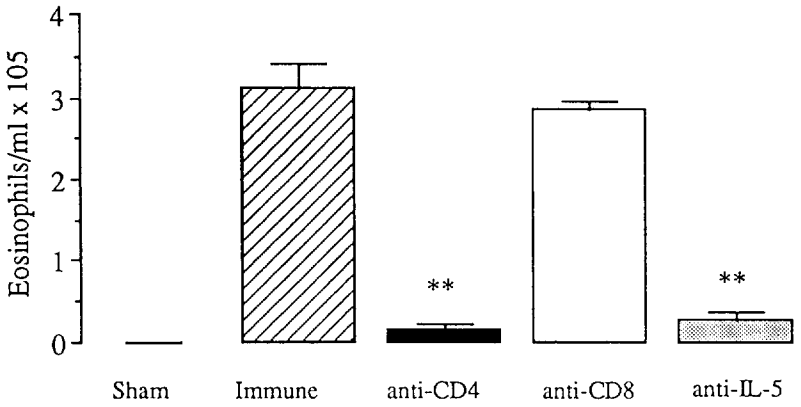

Figure 1. Effect of depletion of $CD4^+$ cells (*closed columns*) or $CD8^+$ cells (*open columns*) or neutralization of IL-5 (*shaded columns*) on antigen-induced eosinophil infiltration in immunized Balb/C mice. Control animals were treated with IgG as the appropriate isotype control antibody (*diagonal shaded columns*). Data are expressed as the mean ± SEM for five to six mice in each group, and statistical significance was determined by a Student's *t* test and indicated by ** for a P value of < 0.01.

Materials and Methods

In Vivo Experiments

Quantification of eosinophil recruitment to the lung. Balb/C mice (20 to 25 g) were immunized intraperitoneally with 10 μg of ovalbumin (OA) (Grade V; Sigma Chemical Co., St. Louis, MO) in 0.2 ml of alum (Serva, Heidelberg, Germany) on day 0 and day 14. Sham immunized mice received two injections of alum alone. Seven days after the last immunization, animals were challenged with an aerosol of OA (50 mg/ml) for 20 min. In the first series of experiments, mice were injected intraperitoneally with either an anti-IL-5 antibody (TRFK-5), an anti-CD4 antibody (GK1.5), or an anti-CD8 antibody (2.43) 24 h before challenge. Seventy-two hours after antigen inhalation, each animal was anesthetized with urethane, the trachea cannulated, and bronchoalveolar lavage performed by five repeated lavages with 0.3 ml of saline injected into the lungs via the trachea. Total cell counts were performed, cytospin preparations were prepared, and a differential count of 200 cells was performed.

Experiments were also performed as described above in either OA-immunized or sham immunized IL-4 gene-deleted mice (6). In another series of experiments, C57/BL6 mice were injected with an antibody against IL-4 (11B11, 5 mg per mouse, intravenously), either 4 h before aerosol antigen exposure or 4 h before the second immunization and 4 h before antigen exposure. Control mice were injected with a rat IgG antibody as the appropriate isotype control.

In Vitro Experiments

Purification of lung T cells. To analyze the lung T-cell cytokine profile, experiments were performed 72 h after antigen challenge. After the five repeated lavages for assessment of the inflammatory cell infiltrate, a further five lavages were performed with 1 ml of sterile phosphate-buffered saline to remove alveolar macrophages. The lungs were then perfused via the right ventricle with 5 ml of phosphate-buffered saline containing 100 U/ml of heparin to remove any blood and intravascular leukocytes. The lungs were then removed and placed into Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 2-mercaptoethanol (50 μM), L-glutamine (2 mM), sodium pyruvate (1 mM), HEPES (10 mM), and gentamycin (50 μg/ml). The lungs were then gently homogenized, and the cell suspension was filtered through a 70-μm filter and lymphocytes enriched over a single-step Ficoll gradient. B cells were depleted from the cell suspension using magnetic sheep anti-mouse Ig beads (Dynabeads, Nycomed, Norway). Cells were then labeled with Thy1.2-FITC (Pharmingen, Palo Alto, CA) and purified by flow cytometry (Becton Dickinson, Mountain View, CA). In all experiments, purity was > 99% $Thy1.2^+$.

Cell culture. Lung T cells were then plated at a concentration of 2×10^5 in 96-well plates coated with an anti-CD3 antibody (2C11, 50 μg/ml). Cells were cultured for 72 h in the presence of human IL-2 (200 U/ml). Supernatants were harvested and cytokine production was determined by enzyme-linked immunosorbent assay as described elsewhere

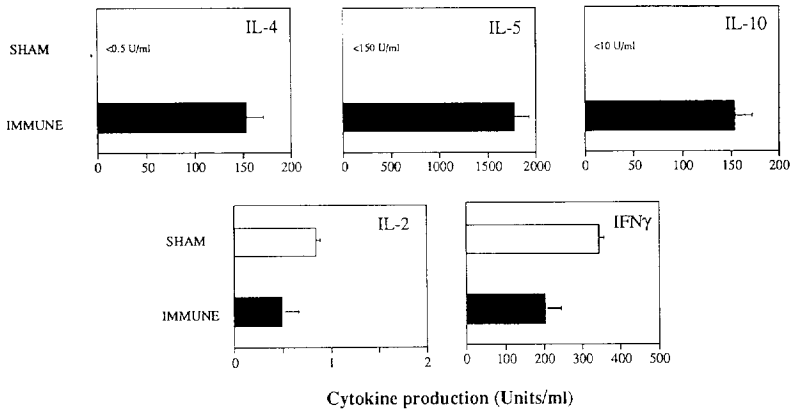

Figure 2. Cytokine production from FACS-sorted lung $Thy1.2^+$ T cells from either sham immunized (*open columns*) or OA-immunized (*closed columns*) mice. Each group represents the mean ± SEM of three cultures obtained from the lungs of four to five mice. Data are expressed as U/ml/2×10^5 cells.

(6). The limits of detection were IL-4, 0.5 U/ml; IL-5, 100 U/ml; IL-10, 20 U/ml; IFN-γ, 50 U/ml; and IL-2, 0.1 U/ml.

Results

OA provocation of immunized, but not sham immunized, mice resulted in a selective eosinophil infiltration. Pretreatment with anti-IL-5 or anti-CD4, but not anti-CD8, antibody inhibited eosinophil infiltration (Figure 1). Fluorescence activated cell sorter (FACS) analysis of mesenteric lymph nodes confirmed that injection of these antibodies induced > 98% depletion of either $CD4^+$ or $CD8^-$ populations (data not shown). These results confirm earlier findings that antigen-induced eosinophil infiltration is IL-5 and $CD4^+$ T cell dependent. These observations are supported by the demonstration that while stimulation of FACS-sorted purified lung T cells from sham immunized mice produced IL-2 and IFN-γ, activation of the CD3/TCR complex of lung T cells from sensitized mice produced IL-4, IL-5, IL-10, and reduced amounts of IL-2 and IFN-γ (Figure 2). In contrast, stimulation of T cells from the mesenteric lymph node of sensitized mice failed to produce Th2 cytokines (data not shown), indicating a local lung Th2 immune response.

Allergen-induced eosinophil infiltration was inhibited in IL-4 gene-deleted mice ($0.60 \pm 0.17 \times 10^5$ cells/ml, $n = 5$) as compared with provocation of immunized IL-4 wild-type mice ($3.15 \pm 0.66 \times 10^5$ cells/ml, $n = 5$, $P < 0.01$) (Figure 3A). There was no significant change in the number of neutrophils, lymphocytes, or macrophages (data not shown). Furthermore, stimulation of FACS-sorted $Thy1.2^+$ cells from the lungs of IL-4 gene-deleted mice not only

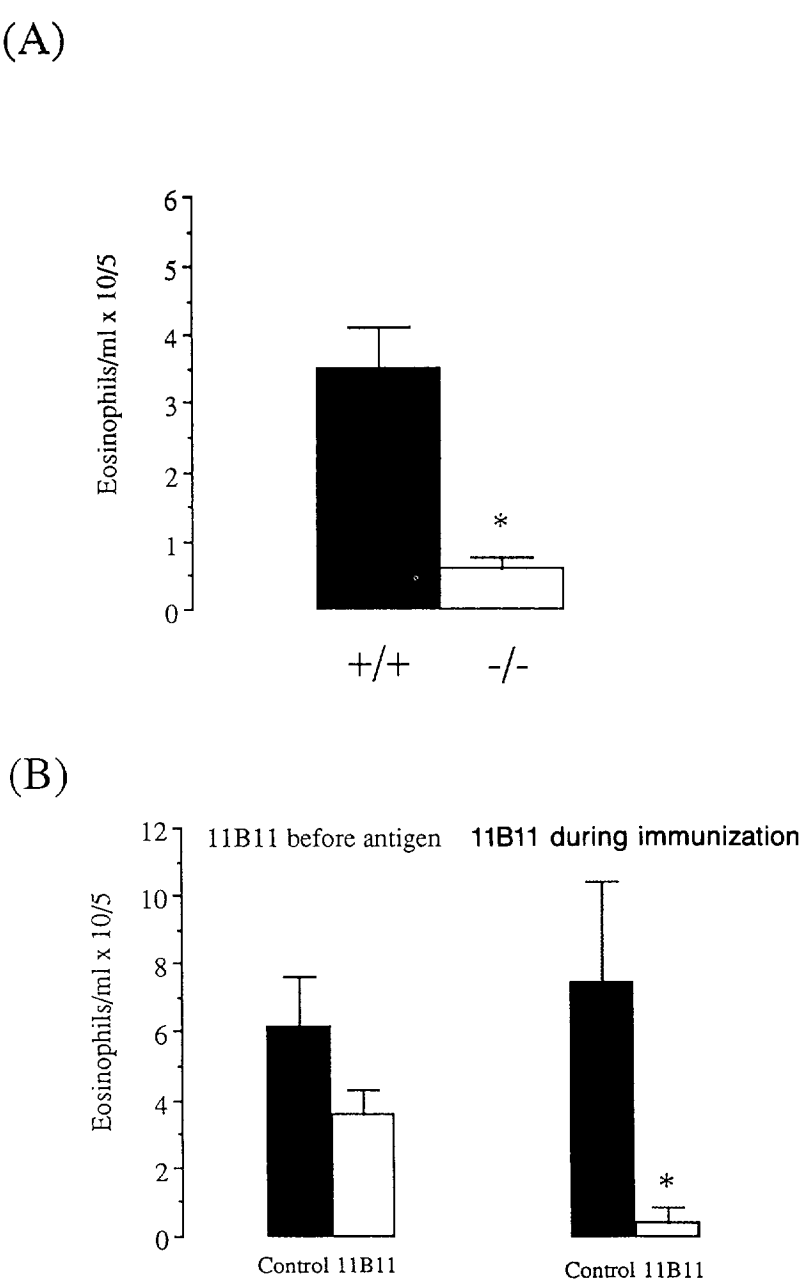

Figure 3. A. Antigen-induced eosinophil accumulation in mice lacking the IL-4 gene (*open columns*) as compared with that of wild-type control mice (*closed columns*). B. Effect of 11B11 on allergen-induced eosinophil infiltration. Mice were injected with 11B11 either 4 h before antigen challenge or 4 h before the second immunization and 4 h before antigen challenge (*open columns*). Control mice were treated with the isotype control antibody (*closed columns*). Data are expressed as the mean number of eosinophils/ml $\times 10^5 \pm$ SEM of three to five mice. Statistical significance (*) was determined by a Student's *t* test, and a value of $P < 0.05$ was considered significant.

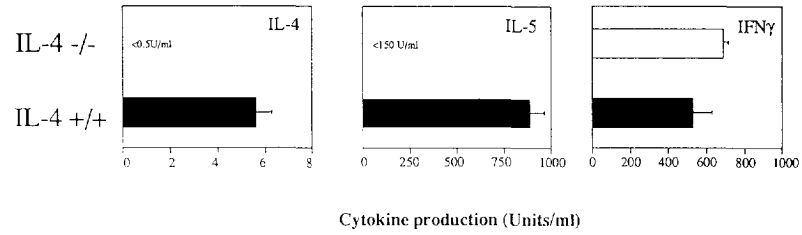

Figure 4. Cytokine production from FACS-sorted lung Thy1.2$^+$ T cells from either immunized IL-4 gene-deleted mice (*open columns*) or immunized IL-4 wild-type control mice (*closed columns*). Each group represents the mean ± SEM of three cultures obtained from the lungs of four to five mice. Data are expressed as U/ml/2 × 10^5 cells.

failed to secrete IL-4 but failed to secrete IL-5 after cross-linking of the CD3/TCR complex (Figure 4). Stimulation of lung T cells from either sham immunized IL-4 gene-deleted or IL-4 wild-type control mice failed to produce IL-4 or IL-5 (data not shown). In addition, serum IgE was undetectable (< 10 ng/ml, $n = 5$) in IL-4 gene-deleted mice as compared with wild-type mice (3,300 ± 1,300 ng/ml, $n = 5$).

To more fully understand when IL-4 contributes to the allergic response, we administered a neutralizing antibody to IL-4 (11B11) either before allergen challenge or both during immunization and before challenge. Injection of 11B11 before allergen challenge failed to significantly inhibit eosinophil infiltration (3.58 ± 0.75 × 10^5 cells/ml, $n = 4$) as compared with control mice (6.12 ± 1.52 × 10^5 cells/ml, $n = 5$, $P = 0.21$) (Figure 3B). In contrast, when 11B11 was injected before the second injection of OA and before allergen provocation, there was > 90% inhibition of eosinophil infiltration (7.43 ± 2.97 × 10^5 cells/ml, $n = 4$ and 0.42 ± 0.42 × 10^5 cells/ml, $n = 3$, respectively; $P < 0.05$) (Figure 3B). Stimulation of the T cells from the lungs of mice that received 11B11 revealed that only when IL-4 was administered during immunization, and not before challenge, was there an inhibition of IL-5 production after activation of the CD3/TCR complex (Figures 5A and 5B). Similarly, 11B11 inhibited serum IgE only when given during immunization (isotype control, 2,200 ± 300 ng/ml; 11B11, 400 ± 100 ng/ml; $n = 3$, $P < 0.05$) and not before challenge (isotype control, 3,000 ± 800 ng/ml; 11B11, 2,100 ± 400 ng/ml; $n = 3$, $P = 0.12$).

Discussion

Analysis of the T-cell subsets present in the BAL fluid of asthmatic individuals has revealed a predominance of the Th2 (IL-4 and IL-5-producing) subset of CD4$^+$ T cells (8). Similarly, biopsy samples from the lungs of allergic individuals has revealed an increase in mRNA for IL-5 (9). These cytokines are believed to be central to the etiology of asthma, as IL-4 is essential for B cells to switch to produce IgE (10, 11), whereas IL-5 is important in eosinophil accumulation (2). This concept is supported by the observation that pretreatment with an anti-IL-5 and an anti-CD4, but not an anti-CD8, antibody inhibited eosinophil infiltration. In the present report, we extend these observations further and demonstrate that T cells obtained from the lungs of immunized, antigen-challenged mice indeed secrete a Th2 profile of cytokines after activation of the CD3/TCR complex. In contrast, sham immunized mice produce IL-2 and IFN-γ and undetectable levels of IL-4, IL-5, and IL-10. Interestingly, stimulation of CD4$^+$ cells from the mesenteric lymph node of immunized mice also failed to produce detectable levels of Th2 cytokines. The production of Th2 cytokines only from lung T cells is most likely a result of the airway aerosol antigen exposure, which would induce both a recruitment (1) and expansion of CD4$^+$ T cells in the lungs.

To determine whether IL-4 has a role to play in the induction of eosinophilic inflammation, studies were performed in mice in which the gene for IL-4 was deleted by homologous recombination (6). Our results demonstrate that mice lacking the IL-4 gene are unable to mount an IgE response and have a marked attenuation of antigen-induced eosinophil recruitment. These data support a previous finding that IL-4 contributes to the development of a peripheral blood eosinophilia after infection with the nematode *Nippostrongylus braziliensis* (6). However, despite the usefulness of such gene-targeted mice, these studies do not address the precise involvement of this cytokine. IL-4 has a number of biologic effects that may contribute to the inhibition of eosinophil infiltration observed in IL-4 gene-deleted mice. IL-4 may facilitate eosinophil accumulation by the upregulation of endothelial vascular cell adhesion molecule-1 (VCAM-1) expression, which binds to very late activation antigen-4 (VLA-4) on the eosinophil (12, 13). Indeed, VCAM-1/VLA-4 interactions have been postulated as a mechanism by which eosinophils can be selectively recruited to the site of inflammation (13, 14). Furthermore, IL-4 transgenic mice have been reported to develop an eosinophilic inflammation of mucosal surfaces (15). However, more recent studies have demonstrated in a similar experimental system to our own, that neutralization of IL-4 *in vivo* failed to have a major inhibitory effect on antigen-induced upregulation of VCAM-1 (16). To investigate these possibilities further, we performed studies with a neutralizing anti-IL-4 antibody. Administration of 11B11, at a dose that has previously been shown to inhibit IL-4-dependent IgE production *in vivo* (7), 4 h before antigen provocation, failed to modify eosinophil accumulation into the lungs. Our data suggest that either IL-4 is not involved or, in the absence of IL-4, other factors can substitute in facilitating eosinophil adherence to the vascular endothelium and transmigration into the airways. These observations are in contrast to a recent report demonstrating that administration of a polyclonal antibody to IL-4 immediately before allergen challenge inhibited the recruitment of eosinophils into the lungs (17). However, the specificity of the antibody used in this study was not reported. This point is particularly critical in interpreting this data, as we have previously observed that some antibodies to IL-4 also cross-react with IL-5 (18), which may provide a possible explanation for this discrepancy.

It has previously been shown that activation of noncommitted naive CD4$^+$ T cells from nonallergic donors secrete

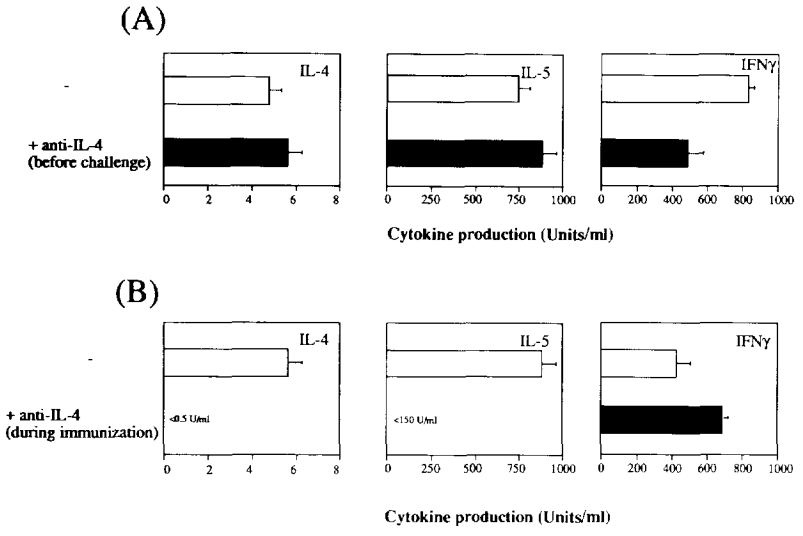

Figure 5. A. Cytokine production from FACS-sorted lung Thy1.2$^+$ T cells from C57/BL immunized mice after treatment with 11B11, 4 h before antigen challenge (*closed columns*) or from immunized mice treated with the isotype IgG control (*open columns*). B. Cytokine production from FACS-sorted lung Thy1.2 $^+$ T cells from C57/BL immunized mice after treatment with 11B11, during immunization and 4 h before antigen challenge (*closed columns*) or from immunized mice treated with the isotype IgG control under the same conditions as described above (*open columns*). Each group represents the mean ± SEM of three cultures obtained from the lungs of three to five mice. Data are expressed as U/ml/2 × 10^5 cells.

mainly IL-2 and must be "primed" or "committed" to either a Th1 (IFN-γ-producing) or Th2 (IL-4-producing) phenotype. IL-4 has been demonstrated *in vitro* to be essential in the commitment of naive CD4$^+$ T cells to the Th2 phenotype after either polyclonal (4, 5) or antigen-specific (19) stimulation. We therefore reasoned that this inhibition of eosinophil accumulation in IL-4 gene-deleted mice may be related to an inhibition of the commitment of naive CD4$^+$ T cells to a Th2 phenotype. Our experiments confirm this possibility, as cross-linking of the TCR/CD3 complex of purified lung T cells from IL-4 gene-deleted mice failed to produce IL-5. Similarly, we found that administration of 11B11 during immunization inhibited both IgE production and the accumulation of eosinophils into the airways and provided essentially the same effect as observed in IL-4 gene-deleted mice. Additionally, cross-linking of the CD3/TCR complex revealed that T cells from mice that had received 11B11 during immunization failed to secrete IL-4 and IL-5 as compared with T cells from isotype control-treated mice, providing an explanation for the suppression of both IgE and eosinophil accumulation into the lungs. In marked contrast, administration of 11B11 before challenge failed to modify the *ex vivo* secretion of cytokines from lung T cells. These results support the observation that 11B11 administered before challenge failed to inhibit eosinophil accumulation. This finding appears to be analogous to IL-4-mediated *in vitro* Th2 cell commitment where IL-4 is required for Th2 commitment, but once these cells are switched (in this case by OA/alum immunization *in vivo*), there is no further requirement for IL-4 in the activation and expression of Th2 cytokines (4, 5).

The initial source of IL-4 required for Th2 cell commitment is at present uncertain. Although mast cells and basophils can produce IL-4 after cross-linking of IgE bound to FcεR1 (20), it is unlikely that an antigen could activate mast cells to secrete IL-4 in the absence of IgE, which, at least in the mouse, is strictly IL-4 dependent. Adoptive transfer of CD4$^+$ T cells and not basophils, mast cells, or monocytes from normal mice to IL-4 gene-deleted mice results in the development of an IgE response after immunization (21), suggesting that CD4$^+$ T cells are the major if not the only source of IL-4 required for induction of IgE. Additionally, it has recently been shown that a small subpopulation of T cells bearing the markers CD4$^+$/NK1.1$^+$ rapidly produce IL-4 mRNA after *in vivo* activation of the CD3/TCR complex (22). These observations suggest that these cells may provide the initial source of IL-4, which can then prime naive CD4$^+$ T cells to the Th2 phenotype. Whether this population of cells function as the source of IL-4 required to switch cells to a IL-5-producing phenotype in our model remains to be investigated.

As both deletion of the IL-4 gene and administration of 11B11 during immunization resulted in an inability to mount an IgE response, the possibility arises that IgE-dependent mechanisms play a pivotal role in the induction of airway eosinophil recruitment. Indeed, IgE-dependent activation of mast cells has been shown to result in the secretion of eosinophilic chemotactic factors (23). However, recent studies have cast doubt on this hypothesis, as mast cell-deficient mice do not show any defect in eosinophil recruitment (24). A second possibility is that IgE via cell surface CD23 captures and internalizes antigen, resulting in enhanced peptide presentation to T cells (25–27). Whether such interactions between IgE bound to CD23-positive cells in the lungs (B cells and dendritic cells) are able to focus antigen to CD4$^+$ T cells and enhance IL-5 production is currently under investigation in our laboratory.

In conclusion, we have demonstrated that immunization and allergen provocation leads to lung T cells switching to a Th2 (IL-4, IL-5, and IL-10-producing) phenotype. Experiments performed in IL-4 gene-deleted mice demonstrate a marked impairment in the induction of this local Th2 immune response and in the development of eosinophilic inflammation of the airways. Moreover, studies performed with neutralizing antibodies to IL-4 and IL-5 suggest a sequential involvement of these cytokines in the recruitment of eosinophils to the lung, with IL-4 secreted during immunization, switching naive CD4$^+$ T cells to a Th2 phenotype, which upon aerosol challenge are activated to secrete IL-5, leading to eosinophil accumulation.

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