



# Allergen provocation tests in respiratory research: building on 50 years of experience

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**Allergen provocation of the airways identifies novel inflammatory pathways and guides our understanding about efficacy of new therapies. Testing of the upper or lower airways may affect the other compartment but cannot be substituted for each other.** <https://bit.ly/3FuT1qt>

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## Abstract

The allergen provocation test is an established model of allergic airway diseases, including asthma and allergic rhinitis, allowing the study of allergen-induced changes in respiratory physiology and inflammatory mechanisms in sensitised individuals as well as their associations. In the upper airways, allergen challenge is focused on the clinical and pathophysiological sequelae of the early allergic response, and is applied both as a diagnostic tool and in research settings. In contrast, bronchial allergen challenge has almost exclusively served as a research tool in specialised research settings with a focus on the late asthmatic response and the underlying type 2 inflammation. The allergen-induced late asthmatic response is also characterised by prolonged airway narrowing, increased nonspecific airway hyperresponsiveness and features of airway remodelling including the small airways, and hence allows the study of several key mechanisms and features of asthma. In line with these characteristics, allergen challenge has served as a valued tool to study the cross-talk of the upper and lower airways and in proof-of-mechanism studies of drug development. In recent years, several new insights into respiratory phenotypes and endotypes including the involvement of the upper and small airways, innovative biomarker sampling methods and detection techniques, refined lung function testing as well as targeted treatment options further shaped the applicability of the allergen provocation test in precision medicine. These topics, along with descriptions of subject populations and safety, in line with the updated Global Initiative for Asthma 2021 document, will be addressed in this review.

## Introduction

For over five decades, the allergen provocation test has served as a gold standard model of allergic mechanisms underlying asthma and allergic rhinitis, and more recently has been used to understand the interactions between both airway compartments [1–4]. As an indirect stimulus, allergen challenge allows



investigation of subsequent changes in respiratory physiology and inflammatory pathways in sensitised individuals and the effect of interventions [5–8].

In the upper airways, nasal allergen challenge (NAC) is presently used both as a diagnostic tool and for research purposes with a focus on the early allergic response, symptom scores and the release of acute pro-inflammatory mediators [5, 6]. In contrast, in the lower airways, while occupational agents are well established as diagnostic and research tools for occupational asthma, challenge with allergen extracts has almost exclusively been used in specialised research settings, focusing on the sequelae associated with the late asthmatic response (LAR), *i.e.* the underlying type 2 (T2) inflammation, prolonged airway narrowing and increased nonallergic airway hyperresponsiveness (AHR): all pathognomonic features of asthma [7, 9–11].

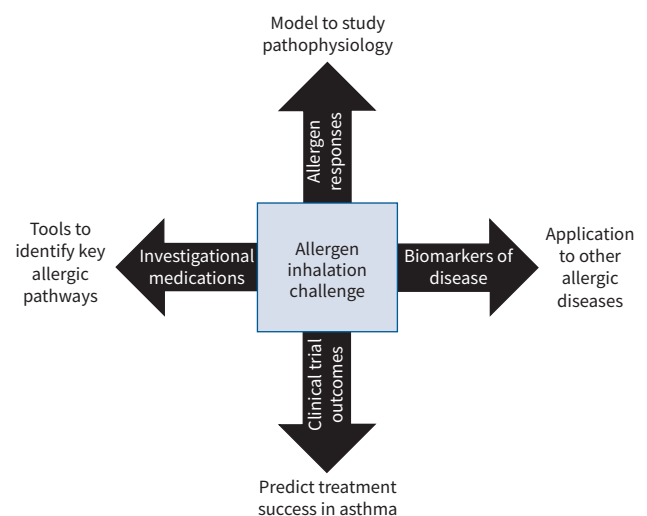
As an extension of the European Respiratory Society/American Thoracic Society Task Force position document on indirect challenges [12] and our previous expert review [7], this review discusses additional frequently used, standardised allergen challenge methods of the airways, and updates existing methodologies according to recent insights about the development of T2 biologics for precision medicine of asthma and allergy, as well as study populations and safety aspects, in line with the Global Initiative for Asthma (GINA) 2021 document [13].

### Bronchoprovocation and drug development

Provoked models of bronchoconstriction and airway inflammation have significantly contributed to our understanding of asthma pathophysiology and the development of targeted drugs [14]. T2 asthma has recently been recognised as a relevant asthma endotype [15]. Through characterisation and standardisation of clinical trial end-points such as the early asthmatic response (EAR), LAR, allergen provocative concentration/dose causing 20% fall ( $PC_{20}/PD_{20}$ ) in forced expiratory volume in 1 s ( $FEV_1$ ), shift in allergen-induced  $PC_{20}/PD_{20}$  methacholine/histamine, and sputum eosinophils and derived biomarkers, allergen bronchoprovocation has been used to assess the potential utility and clinical efficacy of T2 biologics with an overall good predictive value [16–22]. Indeed, a wide variety of therapeutics within many different inflammatory pathways have been tested, including those with broad anti-inflammatory properties, those targeting T2 pathways, bronchodilators and other more specific targets (supplementary material). Through comprehensive testing, efficacy data applicable to other disease indications can be generated (figure 1) [23, 24].

### Allergen bronchoprovocation and asthma mechanisms

More recent investigations have further characterised the inflammatory response following inhaled allergen and included whole-blood RNA biomarker panels that are predictive of the LAR [25]. Identification of different inflammatory phenotypes in sputum during the LAR [26–28] and examination of the kinetics of T2 inflammatory cytokines and chemokines in sputum [11] together with the observed increase in effector cells such as dendritic cells in bone marrow and trafficking of these cells into the airway [29] should help



**FIGURE 1** Applications of allergen bronchoprovocation.

to further elucidate the cellular and molecular mechanisms underlying allergen-induced airway responses and promote the development of targeted treatments for asthma.

### ***T2 biomarker assessments and allergen bronchoprovocation***

During bronchoprovocation, biomarkers can be used to readout various mechanisms involved in the allergic airway response. The allergen-induced airway inflammation can be explored either locally *via* upper airway samplings or lower airway samplings including exhaled air, induced sputum or bronchoscopy, or systemically in peripheral blood [10, 30, 31]. Examination of the lipid metabolism pathway demonstrates enrichment of significant genes and metabolites post-challenge [32], with leukotrienes and prostaglandin D<sub>2</sub> increased [33, 34]. In the case of these lipid mediators, urine sampling has been particularly useful [35–37]. Primarily, T2 inflammatory biomarkers have been shown to be involved in the allergic response, with a main focus on eosinophils and eosinophil-related biomarkers and cytokines, *e.g.* exhaled nitric oxide fraction ( $F_{ENO}$ ), alarmins (interleukin (IL)-25, IL-33, thymic stromal lymphopoietin), IL-4, IL-5 and IL-13 [38–41] as well as eosinophil activation markers [42, 43]. Furthermore, neutrophils [43], basophils and mast cells [44] are altered in response to allergen challenges. More recently, the proportions of other cell types in sputum have been shown to alter following allergen challenge, highlighting the dynamic nature of inflammatory subtypes in asthma [26, 28, 29, 45–48]. In addition, changes in RNA profiles of T2 inflammation have been defined in sputum cells [27, 45, 49]. One should be aware that many of the aforementioned biomarkers are responsive to corticosteroid treatment, which lowers their respective levels [7, 50].

### ***Non-T2 pathways and allergen bronchoprovocation***

The airway response to inhaled allergen is mainly described as a T2 response but non-T2 inflammation is also described. Non-T2 asthma may have different underlying mechanisms and presently there is no consensus on signature biomarkers, but a main hallmark is neutrophilic rather than eosinophilic inflammation [51–53], with increased airway neutrophil numbers and their pro-inflammatory products neutrophil elastase and myeloperoxidase observed after allergen bronchoprovocation [54]. Several other cell types and cytokines, including T-helper (Th)1, Th17, T-regulatory (Treg) cells, and IL-1 $\beta$ , IL-6, IL-8, IL-17, interferon- $\gamma$ , tumour necrosis factor- $\alpha$  and neutrophil extracellular traps, are believed to contribute to mechanisms of non-T2 pathobiology [53]. Allergen bronchoprovocation has documented the activation of a subset of circulating mature neutrophils [55], an increase in Th17 cells and IL-17A in blood [56], an increase in blood Th17/Treg cell ratio specifically in dual responders [57], and a parallel diminished percentage of sputum Treg cells [58] and an increase in IL-6 [59] following allergen bronchoprovocation. Targeted treatment using a single dose of tocilizumab (an IL-6 receptor blocker), however, was not able to prevent allergen-induced bronchoconstriction [60]. Markers of remodelling have also been detected post-allergen challenge, including increased myofibroblasts in endobronchial biopsies [61] and metalloproteinase-9 in sputum [62].

The innate immune system may play an important role in the development of allergen-induced airway inflammation with a number of potential signalling pathways. The epithelial cell-derived alarmins are upregulated after allergen challenge [63, 64], and are widely recognised as important upstream orchestrators of both T1 and T2 inflammation. Epithelial cell triggers can also drive inflammation through innate receptors such as protease-activated receptors (PAR2) and Toll-like receptors including TLR4 and TLR7; intranasal administration of a TLR7 antagonist significantly reduced allergen-induced LAR, demonstrating the potential of these pathways to support the development of asthmatic responses [65, 66]. Bronchial vasculature may be activated after an allergen challenge *via* increased lung homing of endothelial progenitor cells [67]. Apart from the inflammatory pathways, it has also been suggested that airway obstruction could be driven by structural changes within the airways, such as airway smooth muscle hypertrophy [68].

### ***Microbiome***

The human microbiome is an exciting new area of study. Defined as a characteristic microbial community living in a normally well-defined environment with distinct physio-chemical properties, the microbiome interacts with the host immune system and may drive future development of inflammatory and allergic disorders [69, 70]. The pulmonary microbiome consists of microorganisms such as viruses, bacteria and fungi. Although we have some insight about which bacteria are present in health and disease, we are only beginning to learn how the microbiota and disbalance interact with the local and systemic immune system and responses to allergen [71, 72]. Inhaled corticosteroids (ICSs) are known to suppress the microorganisms of the lungs [73], potentially leading to an exponential growth of other organisms resulting in pathology on a macroscopic scale. Allergic diseases show a dramatic increase in prevalence in recent decades, with influencing factors before and in the first years of life, such as breastfeeding, (air

pollution and exposure to (domestic) animals considered to be major contributors. These factors in turn might influence epigenetic mechanisms and thereby increase or decrease the chance of (pulmonary) allergy and altered immune response. Studying interactions of the microbiome with controlled allergen challenges might help in finding medications to stabilise and create a microbial homeostasis of the respiratory system.

### Methodology of allergen bronchoprovocation

Presently, there are three standardised protocols with inhaled allergen [7]. Two protocols use (relatively) high-dose allergen, applying the incremental or titrated step-up method or the single-bolus method. Despite a different approach to achieve EAR, both methods yield comparable allergen-induced airway responses [74–76]. The third protocol is a repeat low-dose allergen bronchoprovocation. All methods are extensively described in our previous expert review [7].

#### High-dose allergen bronchoprovocation

##### Procedure

Titration, relatively high-dose, allergen inhalation tests date back to the 1950s [1, 2, 77, 78] when exact methodological details were frequently lacking. Current standardised methods are based on those originally described by Hargreave and coworkers in 1974 [79], extensively outlined in our previous expert review [7]. Allergen bronchoprovocation is conducted for research purposes and can occasionally lead to severe airflow obstruction and/or systemic allergic response [79], therefore qualified staff experienced in allergen challenge and a safe starting concentration are required. In the titrated protocol, the starting concentration should be at least three doubling concentrations below the allergen predicted  $PC_{20}$  calculated using the skin test (with the same allergen) end-point and  $PC_{20}/PD_{20}$  methacholine/histamine [80] or based on the  $PC_{20}/PD_{20}$  methacholine/histamine only (modified formula) [81, 82]. Incremental allergen concentrations are inhaled at 12–15 min intervals until the EAR has been reached [79]. For within-subject repeatability, any reliable jet [7] or vibrating mesh [83] nebuliser and reproducible inhalation method is acceptable.

In the single-bolus protocol, a titrated allergen challenge is initially performed to identify the cumulative dose required to produce an EAR [74]. Repeat challenges are conducted using this single (cumulative) high dose of allergen by counted deep breath inhalations from a dosimeter. Although shortening the procedure by ~30 min, inhalation of a single high dose of allergen has been reported to produce a greater EAR than the same (cumulative) dose of allergen administered incrementally over 20 to  $\geq 30$  min [74]. Another study did not find such a difference with respect to drop in lung function but showed increased release of cysteinyl leukotrienes in cumulative as opposed to single-dose challenge [84].

#### Airway response measurements

Airway response is measured by  $FEV_1$  every 10–15 min for the first hour post-allergen challenge and every 30 min for the second hour to capture the EAR, then hourly (and in case of a steep decline in  $FEV_1$ , more frequently) up to  $\geq 7$  h to capture the LAR. The EAR can be defined as the maximum percentage fall in  $FEV_1$  during the first hour or the area under the curve (AUC) from 0 to 2 h (or 0 to 3 h) and the LAR as the maximum percentage fall over 3–7 h (or  $>7$  h) or the 3–7 h (or  $>7$  h) AUC. Changes in nonallergic AHR are assessed by measuring the shift in  $PC_{20}/PD_{20}$  methacholine/histamine 24 h after allergen challenge. Isolated EAR investigations can also be assessed and quantitated using the allergen  $PC_{20}/PD_{20}$  dose shift [81, 83, 85]. More sensitive lung function measurements, including impulse oscillometry (IOS), body plethysmography and carbon monoxide diffusion capacity, show more extensive involvement of small airways in dual responders (EAR+LAR) compared with isolated EAR responders (EAR-only) [86, 87]. IOS measurements may help to further differentiate responders based on baseline physiology and to detect initial airway response to allergen (or other stimuli) prior to changes in spirometry, thus supporting safety during the procedure.

#### Repeat testing

The EAR and LAR are reproducible outcomes when the test is properly standardised [9, 88, 89]. For studies addressing the LAR and sequelae, repeat tests should be performed after a washout of at least 2 weeks and strive to use the same dose of allergen administered in the same manner whenever possible. Occasionally an earlier-reached EAR can hinder this approach. To ensure safety and data integrity, subjects should meet asthma stability criteria (symptoms, lung function and  $PC_{20}/PD_{20}$  methacholine/histamine) pre-challenge [7]. For studies addressing only the EAR  $PC_{20}/PD_{20}$ , the appropriate allergen dose should be administered [81] to quantitate the treatment effect. In such studies, an LAR can be prevented by administration of a single moderate-to-high dose of ICS after the EAR [90]. While the EAR method has several logistical advantages such as less time, fewer eligibility criteria and a greater ability to discriminate large changes, it fails to capture the clinically more relevant LAR and sequelae.

### Safety

Incremental allergen inhalations inducing a gradual decline in FEV<sub>1</sub> during the EAR are preferred. Irrespective of the inhalation method, allergen should only be administered in asthmatic subjects where pre-challenge clinical stability is confirmed [7]. Although linear in theory due to geometrical dose increments, the allergen dose–response curve steepens as a 15–20% fall in FEV<sub>1</sub> is approached. The possibility of excessive airway narrowing (*i.e.* a decrease in FEV<sub>1</sub> ≥50%) underscores the requirement for an experienced clinician and medications to treat severe bronchoconstriction or anaphylaxis (bronchodilators, corticosteroids, antihistamines, epinephrine, oxygen and cardiopulmonary resuscitation equipment) to be immediately available. To minimise exposure of laboratory personnel to aerosolised allergen, good ventilation, exhaust hoods and other protective measures are required [7].

### Selection of subjects

Inhaled allergen bronchoprovocation tests have mainly been conducted in subjects with mild, clinically stable asthma in specialised research settings [7] for the purpose of studying mechanisms of allergic airway disease [10] and in the context of clinical trials to support understanding of precision medicine [38]. Current research allergen bronchoprovocation methodologies are not recommended as clinical tests in asthmatic patients, except in rare cases where guidelines of some countries permit these for diagnostic purposes in occupational settings. Neither a safe starting allergen dose [91] nor the safety of allergen-induced bronchoconstriction have been established in patients with more severe asthma, considering such patients would require withholding of their medications with the risk of losing asthma control or have effects of these medications confound interpretation of the test.

Inhalation of a provocative allergen in subjects with mild allergic asthma, while generally safe and well tolerated, can lead to significant acute bronchoconstriction, prolonged airway responses (*i.e.* a clinically relevant exacerbation) and, in rare cases, anaphylaxis [92]. Careful tracking of spirometry and follow-up with availability of medical assistance after the test needs to be applied in all settings. Safety begins with subject selection: individuals with (very) mild well-controlled asthma requiring infrequent use of rescue medication and with no or relatively little resting airflow limitation, *i.e.* baseline FEV<sub>1</sub> ≥70% predicted. Since asthma medications interfere with allergen challenge end-points (table 1), a key inclusion criterion is the requirement for mild allergic asthma with infrequent use of a short-acting bronchodilator (short-acting β-agonist (SABA)) only. However, as per the revised GINA 2021 document, individuals who experience infrequent asthma symptoms are now recommended “as-needed” low-dose ICS associated with rescue medication (*i.e.* bronchodilators) [13]. Subjects should, however, be able to maintain good asthma control and optimal lung function despite fulfilling washout requirements indicated in table 1 [7, 13]. Washout periods are consistent with at least five half-lives of the drug plus an estimate of the duration of drug effect. It is unethical and unsafe to discontinue maintenance medications in more severe asthma to meet these washout requirements for allergen bronchoprovocation.

### Medication washout, withhold and rescue

Clinical trials utilising allergen bronchoprovocation tests are often complex, long lasting and require multiple challenges with adequate washout periods (table 1). The timeframe between two subsequent allergen challenges is 2–3 weeks minimum, and studies typically span the cold and flu season to avoid the pollen seasons in pollen-allergic participants. Lengthy study durations and potential viral exposure could increase the risk of exacerbation, and ICS may be required for subject safety and wellbeing. Situations where an exacerbation with or without the use of ICS occurring within the required washout period should be dealt with on a case-by-case basis with the goal of postponing a scheduled visit until the appropriate washout can be achieved. Subjects requiring oral corticosteroid (OCS) rescue during a bronchoprovocation study should be withdrawn with the focus on regaining asthma control.

After allergen bronchoprovocation, a SABA is usually administered to reverse existing bronchoconstriction such that the FEV<sub>1</sub> returns to within 10% of the pre-challenge value. In the case of a partial reversal, a short-acting muscarinic antagonist (SAMA) and/or low-dose ICS may be added (*e.g.* budesonide 200 µg, 1–2 times during the next 2–3 days post-challenge). Prior to leaving the unit, subjects should be provided with appropriate rescue (*e.g.* SABA±SAMA) and controller (*e.g.* ICS) medications with instructions to use if required, and a 24 h emergency contact, keeping in mind use of these medications could require subsequent tests to be aborted if withhold periods (*i.e.* continuation criteria) cannot be met.

### Low-dose repeated allergen bronchoprovocation

#### Procedure

Repeated low-dose allergen bronchoprovocation has been introduced as a standardised method to mimic natural allergen exposure [93, 94]. Small doses of allergen titrated to cause minimal bronchoconstriction



**TABLE 1** Recommended washout periods for medications and other general measures before and during allergen inhalation challenge testing in clinical research settings

|   | Recommended washout or avoidance period before start of study                                      | Recommended washout or avoidance period during the study   |
|---|--|--|
| <b>Medication</b>   |  |  |
| Immunosuppressants (e.g. methotrexate, dactinomycin, mercaptopurine)  | 4 weeks washout  | Prohibited during study  |
| Systemic corticosteroids (intravenous/ intramuscular/oral)  | 12 weeks washout; up to one burst of OCS in the previous 5 years                                   | Prohibited during study  |
| Inhaled corticosteroids   | 4–6 weeks washout from regular daily dosing  | Permitted if used acutely to rescue after allergen challenge or to stabilise asthma during the study if needed, provided dosing is acute (3–5 days maximum) and 1 week washout is applied before subsequent study visits |
| Nasal corticosteroids   | 4 weeks washout from seasonal dosing   | Permitted if used at a constant dose throughout the study and subject meets all eligibility criteria while dosing  |
| Dermal corticosteroids  | 4 weeks washout from maintenance therapy on large body surfaces (larger than ~10 cm <sup>2</sup> ) | Permitted if infrequent use on small body surface  |
| Allergen immunotherapy for challenging allergen   | Full exclusion   | Prohibited during study  |
| Allergen immunotherapy for nonchallenging allergen  | 12–16 weeks washout  | Permitted if ongoing throughout the study and at least 12–16 weeks on stable oral or subcutaneous dose   |
| SABA (e.g. salbutamol, albuterol, terbutaline)  | No washout required  | Permitted with ≥6 h washout before allergen challenge  |
| SAMA (e.g. ipratropium bromide, oxitropium bromide)   | No washout required  | Permitted with ≥12 h washout before allergen challenge   |
| LABA (e.g. salmeterol, formoterol)  | 2 weeks washout  | Prohibited during study  |
| Ultra-LABA (e.g. abediterol, indacaterol, olodaterol, vilanterol)   | 2 weeks washout  | Prohibited during study  |
| LAMA (e.g. aclidinium bromide, tiotropium bromide, glycopyrronium bromide, umeclidinium bromide)                                      | 2–3 weeks washout  | Prohibited during study  |
| Leukotriene modulators including LTRAs (e.g. montelukast, zafirlukast, pranlukast, zileuton)  | 2 weeks washout  | Prohibited during study  |
| PDE inhibitors (e.g. Daxas, ensifentrine)   | 2 weeks washout  | Prohibited during study  |
| Xanthines (e.g. caffeine, theobromine)  | No washout required  | Permitted with 4–12 h washout before allergen challenge  |
| Cromoglycate/nedocromil   | 2–4 weeks washout  | Prohibited during study  |
| Theophylline  | 4 weeks washout  | Prohibited during study  |
| Oral anti-inflammatories: salicylates (e.g. acetylsalicylic acid), acetaminophen, other NSAIDs (e.g. ibuprofen, naproxen, diclofenac) | No washout required  | Permitted with 7 days washout before allergen challenge  |
| Short-acting antihistamines   | No washout required  | Permitted with 3 days washout before allergen challenge  |
| Intermediate-acting antihistamines  | No washout required  | Permitted with 4 days washout before allergen challenge  |
| Biologics (registered or experimental)  | 3–6 months washout or five half-lives  | Prohibited during study  |
| Living vaccines   | 3 months washout   | Prohibited during study  |
| Other vaccines (e.g. vector, mRNA/DNA vaccines)   | 4 weeks washout  | Prohibited during study  |
| Chemotherapy  | Full exclusion   | Prohibited during study  |
| Investigational medications   | 3–6 months washout or five half-lives  | Prohibited during study  |
| <b>Interfering factor</b>   |  |  |
| Major surgery   | 3–6 months washout   | Subjects with planned major surgery should not be enrolled on the study  |
| Lower respiratory tract viral infection; upper respiratory tract common cold  | 6 weeks; 3 weeks washout   | Allergen challenge should not be conducted within 6 weeks of a lower respiratory tract infection   |
| Strenuous exercise  | 72 h delay   | No strenuous exercise (e.g. marathon) during the study or 1–2 weeks after an allergen challenge test, depending on recovery  |

Continued

TABLE 1 Continued

|   | Recommended washout or avoidance period before start of study | Recommended washout or avoidance period during the study |
|---|---|--|
| Alcohol-containing beverages  | 48–72 h washout   | Prohibited during study                                  |
| Party drugs   | 72 h washout  | Prohibited during study                                  |
| Hard drugs (e.g. heroin, cocaine, amphetamine, LSD, ecstasy)                          | Full exclusion for current or history of use                  | Prohibited during study                                  |
| Increased allergen exposure (e.g. contact with animals, pollen season, moving houses) | 3–4 weeks washout   | Prohibited during study                                  |
| Increased environmental triggers (e.g. cigarette smoke, pollution)                    | 72 h washout  | Prohibited during study                                  |

OCS: oral corticosteroid; SABA: short-acting  $\beta$ -agonist; SAMA: short-acting muscarinic antagonist; LABA: long-acting  $\beta$ -agonist; LAMA: long-acting muscarinic antagonist; LTRA: leukotriene receptor antagonist; PDE: phosphodiesterase; NSAID: non-steroidal anti-inflammatory drug; LSD: lysergic acid diethylamide.

are inhaled once daily over 1–2 weeks [95–101]. The allergen dose is selected from an initial incremental dose challenge (usually as the provocative dose causing an early drop in FEV<sub>1</sub> of 5% (PD<sub>5</sub>)) [95, 98, 100, 101]. Methacholine or histamine challenge to measure airway responsiveness and induced sputum collection are performed before and after the low-dose challenge period. Spirometry recordings as well as  $F_{ENO}$  measurements are performed daily, and subjects record home monitoring of lung function, asthma symptom scores and short-acting  $\beta_2$ -agonist usage. A 2-week washout is recommended between subsequent challenge periods [95, 102]. The procedure generates a distinct and consistent increase in nonspecific AHR [93, 96, 97, 99, 102, 103], as well as increases in airway eosinophils [95, 96, 99, 100, 102, 103] and  $F_{ENO}$  [99–102] that are more pronounced than after a single high-dose allergen bronchoprovocation inducing a dual asthmatic response [95, 100–103]. Notably, the increase in AHR occurs in the relative absence of asthma symptoms.

#### Implementation

Because this bronchoprovocation model induces robust readouts such as increased AHR and many signs of T2 inflammation with minimal bronchoconstriction and symptoms, it has received interest as a valuable tool to investigate early events of importance for the development of symptomatic and possibly persistent asthma. In addition, this model has been useful in evaluating drug effects in mild asthma, particularly for ICS [99, 102, 103]. However, it is not as widely used as high-dose bronchoprovocation, most likely because of the greater number of visits required for the subjects. In the current search to understand how different biological treatments affect asthmatic airway inflammation and related sequelae, it might be of value to use repeated low-dose allergen bronchoprovocation again. The emergence of sensitive omics technologies may offer new opportunities to define mechanisms by measurements in blood, sputum, exhaled breath and urine during the development of the response [104].

#### Segmental allergen bronchoprovocation

##### Procedure

Segmental allergen challenge (SAC) is an invasive, bronchoscopic research procedure which allows direct investigation of the inflammatory response following local allergen instillation [105]. Compared with inhaled bronchoprovocation, SAC allows a more precise dosing with one or more agents, and the procedure has been shown to be safe and easy to control [106]. In both mild and moderate asthma, SAC allows reproducible readouts as well as simultaneous control lavage within individual subjects [107]. A drawback is the requirement of two bronchoscopic procedures: one for allergen instillation and one ~24 h post-challenge for the evaluation of the inflammatory response.

##### Implementation

SAC allows study of direct effects of allergen on the inflammatory airway response within lower lung segments. In addition to biopsy, lavage, wash or brushings for cellular and molecular components, SAC allows visualisation of the airway response with imaging techniques which may substitute the second bronchoscopy [108, 109]. In contrast to inhaled bronchoprovocation, the overall physiological response in the airways may be suppressed as a result of procedural medications and, given the localised administration of allergen, may not evoke a full airway response, which precludes studying the relationship between the allergen-driven inflammation and airway physiology.

### Occupational bronchoprovocation

Specific inhalation challenges (SICs) with occupational agents are well established as useful diagnostic and research tools for occupational asthma [110] and hypersensitivity pneumonitis [111]. The main purpose for which occupational challenges are being used is to establish an aetiological diagnosis in occupational asthma and hypersensitivity pneumonitis, as well as to investigate the pathogenic mechanisms underlying these diseases.

Originally, SIC was an empirical approach in which a patient was asked to reproduce his/her working environment [112]. Currently, SIC is a standardised test, in which the respiratory effects of an agent present in the workplace are assessed ideally under controlled conditions, including the generation of a low and stable concentration of the agent [113]. SIC is considered the reference standard test to confirm occupational asthma and it is essential to include the assessment of airway inflammation in the procedure [113, 114]. Exposure to occupational agents can be produced in various ways, depending on the physical state of the agent suspected of causing occupational asthma [113], and the method of bronchoprovocation is quite different for high-molecular versus low-molecular agents, particularly for safety reasons as the latter can induce an isolated late response. A 2014 European Respiratory Society Task Force consensus statement on SIC in the diagnosis of occupational asthma provides practical recommendations and reviews the interpretation and limitations of these tests [115].

### Nasal allergen challenge

#### Standardisation of NAC

NAC is a simple and safe method used both as a research tool to study allergen-induced pathophysiology and drug interventions in allergic individuals [5, 30], and as a diagnostic tool in clinical practice [116, 117]. Topical application of increasing doses of an allergen extract or compound in solution to the nasal mucosa of sensitised individuals induces an IgE-mediated immune response, which rapidly evokes key symptoms of allergic rhinitis including itching, sneezing, nasal congestion and/or rhinorrhoea. The occurrence and severity of symptoms and underlying mechanisms can be captured by standardised composite symptom scores and nasal patency assessments (table 2).

Ocular symptoms (irritation, itching and tearing) are common during the acute phase [129], while a more generalised response (*e.g.* exanthema) is rare. Pharyngeal symptoms (itching and swelling) can usually be avoided by applying allergen to the nose after the subject has breathed in. In line with the bidirectional cross-talk between both airway compartments, one report suggests NAC may induce both an EAR and/or LAR in subjects with pre-existing AHR [130]. Hence, in these individuals, lung function measurements and therapeutic measures should be considered for safety reasons. Asthma medication can usually be continued, especially if NAC is used for upper airway diagnostic purposes.

While most of the acute-phase symptoms subside within ~15–30 min, nasal congestion may persist for several hours post-NAC. Prolonged congestion serves as a clinical marker of the local late-phase response, and is associated with both upper and lower airway inflammation and AHR. Analytes from the upper airways can be collected using exhalates, nasal lavages, mucosal brushings or curettage, absorbent strips and sponges. For RNA-based analysis, apart from biopsies, minimally invasive scrapings can be used (table 3).

Drugs which could interfere with challenge outcomes (topical and systemic corticosteroids, antihistamines, decongestants, antileukotrienes and topical cromones) need to be stopped. Specific drug washout periods are in line with those for bronchial challenges (table 1), with a special notice that intranasal corticosteroids and antihistamines must be stopped. Topical and systemic decongestants should be avoided 3 days pre-challenge. Standardisation of allergen concentration and major allergen content (including batch reproducibility) remains difficult, largely due to the use of numerous concentration units across different manufacturers, rather than  $\mu\text{g}\cdot\mu\text{L}^{-1}$  major allergen.

#### Bilateral NAC

A European Academy of Allergy and Clinical Immunology Task Force convened and reached consensus on the best practice of NAC for clinical use being carried out as bilateral challenge with a volume of 0.1 mL per nostril applied *via* nasal spray (ideally as two 50  $\mu\text{L}$  actuations) [6]. Single-dose rather than dose-titration challenges were generally recommended. Both subjective (symptom scores or visual analogue scale scores) and objective (peak nasal inspiratory flow, acoustic rhinometry and rhinomanometry) outcomes should be recorded, with defined “strong” thresholds for a positive challenge in either outcome or a combination of both at more modest levels. Clinical indications for NAC are broad, including cases of discordant history and sensitisation results, allergen selection for immunotherapy, and suspected occupational causes [117]. A 4-week post-exposure window was agreed on for testing to seasonal allergens, but difficulty remains concerning acceptable levels of background exposure to perennial allergens when these are being tested.



TABLE 2 Clinical assessments of nasal allergen challenge (NAC) testing

| Method                      | Notes  | References |
|-----------------------------|--|------------|
| Symptom scores              | <p>Lebel composite symptom score/TNSS±ocular symptoms; typically, four domains, each scored 0–3: itch, sneezing, running/secretions, blockage; total 0–12</p> <p>Visual analogue scale, 0–10 cm Likert scale, individual symptoms (blockage, itch, running/secretions, sneezing) or overall score</p> <p>Scored by the participant/patient; simple to use, but subjective (except for sneezes if these are counted by the assessor)</p> <p>Sneezing is primarily a feature of the early-phase response, so this tends to skew the score upwards during the 5–15 min immediately post-challenge</p> <p>Relatively blunt tool for capturing late-phase responses where blockage alone is often the predominant symptom</p> | [118, 119] |
| PNIF (L·min <sup>-1</sup> ) | <p>In-Check nasal inspiratory flow meter; scale 30–370 L·min<sup>-1</sup> (Clement Clarke, Harlow, UK) in combination with a well-fitting anaesthetic face mask</p> <p>Standardised normal range</p> <p>Good intraindividual reproducibility and response to NAC and to decongestants</p> <p>Cheap, quick and easy measurement, but user dependent</p>   | [120–126]  |
| Acoustic rhinometry         | <p>Sound waves are passed into the nostril through a closed tube; they reflect off the nasal passage walls and are then sampled by a microphone and mathematically converted into a graph of the cross-sectional area of the nasal passage against the distance into the nose from the nares</p> <p>Good for demonstrating cross-sectional area (a surrogate for obstruction) of the anterior portion of the nose</p> <p>Responsive to NAC and decongestant use</p> <p>Technical and expensive compared with PNIF with a limitation to the anterior portion of the nose, but user independent</p>  | [127]      |
| Rhinomanometry              | <p>Anterior (or posterior) recording of pressure–flow relationship using one or both nostrils during active breathing cycle (or breath holding if passive rather than active)</p> <p>Response to nasal challenge and to decongestant use</p> <p>Technically demanding method requiring expensive equipment compared with PNIF, difficult to standardise, but user independent</p>  | [127, 128] |

TNSS: total nasal symptom score; PNIF: peak nasal inspiratory flow.

### Exposure rooms

Environmental exposure chambers (EECs) are clinical facilities allowing for controlled exposure of subjects to inhaled substances in an enclosed environment, mimicking natural exposure under tightly regulated conditions [155]. Several different agents have been tested in these chambers, including allergens, chemicals, endotoxins and airborne pollutants. EECs allow several subjects to be studied at the same time, and have been utilised for investigation of the pathophysiology of respiratory allergic diseases and to assess the efficacy of new therapies for allergy and asthma, including allergen-specific immunotherapy [155–158]. There are few EECs available worldwide due to the need for costly and sophisticated equipment, qualified personnel, and validated procedures. However, there is growing interest including from regulatory authorities. Future goals include harmonisation of protocols among centres, standardisation of dosage and reactivity of allergens used, and reduction in costs of these facilities [155, 159].

### Readouts and biomarkers of NAC

Although the NAC and EEC should not be used interchangeably [145], both challenge techniques can be complemented with various clinical readouts and biomarker samplings. Readouts include subjective composite symptoms and visual analogue scale scores, typically covering itching, sneezing, nasal congestion and rhinorrhoea [119]. Objective measures include peak nasal inspiratory flow, acoustic rhinometry and rhinomanometry (table 2).

The upper airways allow several (semi-)invasive samplings for biomarkers to study the kinetics of the immunological mechanisms underlying the acute- and late-phase upper airway response to allergen (table 3). Upper airway biomarkers include volatile products such as nasal nitric oxide [131], although reliability may be an issue [160]. Samples for cytology are collected by nasal lavage, biopsy or brushings/scrapings and soluble mediators from nasal lining fluid, following nasal lavage or direct absorption of nasal fluid with filter strips or sponges. Analytes include early-phase mediators such as histamine, tryptase, chymase, prostaglandin D<sub>2</sub> and leukotrienes [134–136, 161], and T2 chemokines and cytokines, with IL-5, IL-4 and IL-13 elevated during the late phase [119]. Additionally, activation markers, neuropeptides, markers of vascular leakage and local antibodies have also been successfully quantified from upper airway samplings (table 3).

TABLE 3 Changes in upper airway and systemic biomarkers after nasal allergen challenge (NAC)

| Sample source            | Biomarker  | Notes   | Reference  |
|--------------------------|--|---|------------|
| Exhaled air              | nNO  | nNO measured by a chemiluminescence analyser (CLD88sp; Ecomedics, Duernten, Switzerland) at baseline and 7 and 24 h post-NAC; large intersubject variability, although good intrasubject performance, showing significant increase at 24 h post-NAC | [131]      |
| Nasal lavage with saline | Cytology   | Manual or automated counting of eosinophil influx post-NAC; shown to increase post-allergen challenge and differentiate between allergic rhinitic subjects and controls   | [5, 132]   |
| Nasal lining fluid       | Mediators  | Direct sampling with absorbent strips or sponges produces concentrated sample of lower volume   | [119, 133] |
|                          | Tryptase ng·mL <sup>-1</sup>                             | Highly stable, measured by ImmunoCAP (Thermo Fisher Scientific, Waltham, MA, USA); peaks 5–10 min after nasal challenge   | [119, 134] |
|                          | Histamine ng·mL <sup>-1</sup>                            | Less stable than tryptase; elevated during early-phase response; may be second peak during late-phase response  | [135]      |
|                          | Cysteinyl leukotrienes pg·mL <sup>-1</sup>               | Elevated during early-phase response; level correlated with clinical symptom scores after NAC   | [136]      |
|                          | T2 cytokines pg·mL <sup>-1</sup>                         | Measurable by commercial multiplex immunoassay; IL-4, IL-5, IL-13 elevated from 6 h post-grass pollen NAC; inhibited by allergen immunotherapy  | [119, 133] |
|                          | T2 chemokines pg·mL <sup>-1</sup>                        | Eotaxin, RANTES elevated from 4–6 h post-NAC  | [128, 137] |
|                          | Eosinophil activation markers ng·mL <sup>-1</sup>        | ECP (measurable by ImmunoCAP), MBP, EDN; elevated at 6–8 h post-NAC; also reported at 24 h post-NAC   | [138, 139] |
|                          | Neuropeptides pg·mL <sup>-1</sup>                        | Substance P, CGRP, VIP increased during the early-phase reaction  | [140]      |
|                          | Markers of plasma leakage/transudate µg·mL <sup>-1</sup> | α <sub>2</sub> -macroglobulin, albumin elevated in early- and late-phase response to NAC  | [141, 142] |
|                          | Markers of glandular secretion µg·mL <sup>-1</sup>       | Lactoferrin, lysozyme   | [143]      |
|                          | Fluid metabolomics                                       | Differences in metabolic pathways and metabolite levels seen in patients with N-ERD <i>versus</i> controls; yet to be studied in allergic subjects post-NAC   | [144]      |
| Nasal cytology brushing  | Nasal mucosal gene expression                            | Nasal scrapes 8 h post-NAC and EEC show similar patterns of altered expression of mucosal biology and transcriptional regulation genes; nasal ACE2 receptor expression reduced after NAC  | [145–147]  |
| Nasal curettage          | Cytology   | Nasal curettage 24 h post-NAC show increases in mucosal ILC2 cells  | [148]      |
| Nasal biopsy             | Cytology   | Compartmental assessment of NAC-induced inflammatory cells  | [149–153]  |
| Peripheral blood         | Mediators  | Serum cytokines (IL-5, IL-13) increased at 8 h post-NAC and EEC   | [145]      |
|                          | Allergen-specific T-cells                                | Assayed by flow cytometry; levels increased after both EEC and NAC exposures  | [145]      |
|                          | Basophils  | IgE-dependent basophil activation <i>in vitro</i> increased following cat NAC   | [154]      |

nNO: nasal nitric oxide; T2: type 2; IL: interleukin; ECP: eosinophil cationic protein; MBP: major basic protein; EDN: eosinophil-derived neurotoxin; CGRP: calcitonin gene-related peptide; VIP: vasoactive intestinal peptide; N-ERD: nonsteroidal anti-inflammatory drug-exacerbated respiratory disease; EEC: environmental exposure chamber; ACE2: angiotensin-converting enzyme 2; ILC2: innate lymphoid cell type 2.

Nasal brushings/scrapings provide material for PCR and transcriptomics [145–147], and biopsy followed by immunohistochemical staining and/or *in situ* hybridisation has previously demonstrated influx of T2-associated cells and cytokines [162, 163]. Finally, systemic markers including cytokines, antibodies and allergen-specific T-cells have been measured in blood [145].

Due to upregulation of inflammatory mechanisms, in a crossover clinical study, a washout period of at least 2 weeks is required between repeat NAC [145, 164].

### United airways

#### *Bidirectional communication between the upper and lower airways*

Asthma and rhinitis are common disorders that often coexist, representing respiratory manifestations of the same (systemic) inflammatory process [165], referred to as “one airway disease” within the context of the “united airways concept” [166]. Apart from shared pathophysiology, there is ample evidence of cross-talk between both airway compartments, *i.e.* challenging one compartment induces inflammatory responses both locally and also within the other compartment [167, 168]. NAC induces upregulation of inflammatory mechanisms and eosinophilia in both the upper and lower airways in nonasthmatic subjects with seasonal allergic rhinitis [3, 169]. Conversely, allergen instillation into a lung segment of nonasthmatic subjects with allergic rhinitis induces both an inflammatory response locally, systemically (in blood) and in the upper airway mucosa [4]. Consequently, international guidelines advocate proactive diagnosis and

treatment of both airway compartments to achieve optimal disease control [13, 170]. Despite similarities between both airway compartments and in airway responses, data derived from NAC cannot substitute for data acquired from a bronchoprovocation test.

### Allergen extracts

#### General information

Allergen extracts are marketed for clinical testing of IgE-mediated allergic disease and in many countries package monographs do not include the inhaled route into the lower airways. Hence, studies incorporating allergen bronchoprovocation may require regulatory approval and the associated oversight, including reporting of serious adverse reactions. For regulatory purposes, a serious adverse reaction to allergen bronchoprovocation can be defined as a fall in FEV<sub>1</sub> of >50% from baseline, requiring treatment with ICS or OCS and relievers, or anaphylaxis or hospitalisation.

Manufacturing of allergen extracts is regulated by organisations including the Center for Biologics Evaluation and Research and the Committee for Medicinal Products for Human Use. Some extracts are standardised by comparing the potency (concentration) to a reference, while nonstandardised extracts are simply based on protein nitrogen content or weight per volume without establishing a relationship between concentration and biological activity. Both standardised and nonstandardised extracts have become increasingly difficult to source.

#### Selection of extracts

Allergen extracts should be sourced from a reliable supplier prepared according to Good Manufacturing Practice [171]. The magnitude and characteristics of allergic asthmatic responses may vary according to the type of allergen, and the same allergen extract, batch and dose should be used for an individual subject throughout a clinical trial. Natural exposure to indoors allergens such as house dust mite is thought to prime a more marked airway eosinophilia than seasonal allergens in atopic subjects with or without asthma [98]. High-dose allergen bronchoprovocation was shown to induce a stronger LAR with house dust mite compared with grass pollen [172, 173], yet in a different study, similar inflammatory patterns have been shown [26]. Proteolytic activity in some extracts such as house dust mite may influence airway responses [174].

#### Conclusions and take-home messages

Allergen exposure to the airways is a common environmental trigger of asthma and allergic rhinitis. Allergen bronchoprovocation is an effective model of asthma for evaluation of the efficacy of various treatment options along the T2 pathway with the ability to link noninvasive biomarkers to measures of physiology. The test is primarily a research tool and the methods described here are not appropriate for common clinical practice. In contrast, bronchoprovocation with occupational agents is a useful diagnostic test that can be conducted for clinical purposes. NAC can be also used as a diagnostic test for allergy and occupational substances, as well as a research tool.

Challenging the upper or lower airway compartment may affect the other compartment, thus NACs may have implications for safety in subjects with inadequately controlled asthma. Despite this united airway response, upper and lower airway tests cannot be substituted for each other. When used in a controlled setting, allergen provocation of the airways identifies novel inflammatory pathways, and guides our understanding about the efficacy of new therapies and precision medicine.

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