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Published in:
Pulmonary Pharmacology & Therapeutics

DOI:
10.1016/j.pupt.2018.01.004

Publication date:
2018

REALISING THE POTENTIAL OF VARIOUS INHALED AIRWAY CHALLENGE AGENTS THROUGH IMPROVED DELIVERY TO THE LUNGS

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Competing interests statement

DS reports grants and personal fees from Almirall, AstraZeneca, Boehringer Ingelheim, Chiesi, GlaxoSmithKline, Glenmark, Johnson and Johnson, Merck, NAPP, Novartis, Pfizer, Takeda, Teva, Therevance and Verona, and personal fees from Genentech and Skyepharma, outside the submitted work. HWF reports that his employer receives royalties on the sales of the Genuair inhaler from AstraZeneca and royalties on the sales of the Novolizer inhaler from MEDA, outside the submitted work. HWF has a patent PCT/NL2004/000427 licensed to PureIMS, which involves the inhaler technology used for administration of the dry powder adenosine formulation. MvdB reports grants paid to the University from Astra Zeneca, TEVA, GSK and Chiesi, outside the submitted work. GWC was not affiliated with hVivo Ltd at the time of initial writing of the manuscript and declares that his involvement is purely on a scientific basis. AJL, GWC, CPP and BF declare that they have no competing interests.

Author's information: AJL was holder of the C.W. Maplethorpe Postdoctoral Fellowship of the University of London during the writing of this work.

Abstract

Inhaled airway challenges provoke bronchoconstriction in susceptible subjects and are a pivotal tool in the diagnosis and monitoring of obstructive lung diseases, both in the clinic and in the development of new respiratory medicines. This article reviews the main challenge agents that are in use today (methacholine, mannitol, adenosine, allergens, endotoxin) and emphasises the importance of controlling how these agents are administered. There is a danger that the optimal value of these challenge agents may not be realised due to suboptimal inhaled delivery; thus considerations for effective and reproducible challenge delivery are provided. This article seeks to increase awareness of the importance of precise delivery of inhaled agents used to challenge the airways for diagnosis and research, and is intended as a stepping stone towards much-needed standardisation and harmonisation in the administration of inhaled airway challenge agents.

Key words: bronchial challenge test, bronchial hyperresponsiveness, asthma, drug delivery, delivery method optimization, delivery method standardization

1. Introduction

Inhaled airway challenges are a key tool in the study and diagnosis of obstructive lung diseases. Bronchial challenge tests that measure bronchial hyperresponsiveness (BHR) of the airways have established applications in the clinic, where they are used to rule out or confirm a diagnosis of asthma (1,2). Inhaled airway challenges can also be used to study disease mechanisms and symptoms other than BHR, either by varying the outcome measure (e.g. inflammation measured by exhaled nitric oxide or inflammatory cell count) or the stimulus (e.g. allergen or endotoxin). The various airway challenges thereby allow the monitoring of disease activity and effectiveness of treatments (3), and they can provide a robust disease model in early phase clinical trials (4,5). Given the reliance on inhaled airway challenges in respiratory medicine, there is surprisingly little standardisation of techniques or guidance regarding the administration of different test agents. In this article we consider various challenge agents and discuss the importance of standardisation and harmonisation of their administration methods.

Historically, bronchial challenge tests have been developed to measure BHR by means of spirometry and the change in forced expiratory volume in one second (FEV_1) is still considered the primary outcome measure in the recently published technical standard on methacholine challenge testing (6). However, whether FEV_1 is the most appropriate outcome measure is subject of debate and a recent study points out that the change in effective specific airway conductance (sG_{eff}) measured with body plethysmography actually has a much larger diagnostic value than FEV_1 for the challenge agent methacholine (7). Other techniques that can be used to measure airway function after provocation include forced or impulse oscillometry (airway resistance) (8) and multiple breath nitrogen washout (ventilation heterogeneity) (9). The relative value of these techniques in challenge testing is beyond the

scope of this paper. In this review we will refer to the outcome measure in a general way in appreciation of this on-going discussion.

2. Inhaled airway challenge agents

Bronchial challenge testing can be performed with a wide range of stimuli, with selection of a particular agent depending on the aim of the test. BHR can be measured using stimuli that have either a direct effect on airway smooth muscle (ASM) (e.g. methacholine or histamine) or an indirect effect where the inhaled agent stimulates inflammatory or neuronal cells (e.g. mannitol, bradykinin or AMP) (10). Furthermore, certain agents can be used that trigger other (patho)physiological mechanisms in the airways (e.g. endotoxin-induced inflammation or allergen-induced responses in allergic subjects), possibly accompanied by BHR in susceptible subjects.

2.1 Direct challenge agents

The most commonly used stimulus in bronchial challenge testing is the direct-acting stimulus methacholine, a synthetic analogue of the neurotransmitter acetylcholine that acts as an agonist on muscarinic M_3 receptors on ASM cells. Histamine, an agonist for the histamine H_1 receptors on ASM cells, can also be used, although this compound is associated with more systemic side effects such as flushing and headache due to vasodilation (11). When a direct-acting stimulus is used the test generally has a high sensitivity for asthma, meaning that the majority of asthma patients will respond to this stimulus, and the responsiveness increases with the severity of lung disease (1). However, the specificity is poor since healthy subjects also respond when the dose is high enough; they are just less sensitive and less reactive to the stimulus than asthmatic subjects (**Figure 1**). Even though cut-off values for healthy and hyperresponsive individuals have been agreed upon (1,6,11), these values can still be

considered as quite arbitrary due to the many factors affecting lung deposition of the stimulus, such as the patient's breathing pattern or the presence of emphysema, inflammation, mucus deposition and/or oedema. Moreover, it is becoming clear that methacholine can miss newly diagnosed asthmatic subjects whose symptoms are mild and whose lung function is excellent, but who demonstrate asthma in terms of significant exercise-induced bronchoconstriction (12). However, the use of a different predefined threshold value for the outcome measure in methacholine challenge as compared to exercise-induced bronchoconstriction (*i.e.*, 20% reduction in FEV_1 in the former versus 10–15% reduction in FEV_1 in the latter) may explain, at least in part, such discrepancy. Additionally, testing with methacholine does not allow for absolute differentiation between patients with asthma or COPD, or indeed other diseases such as allergic rhinitis (13–16).

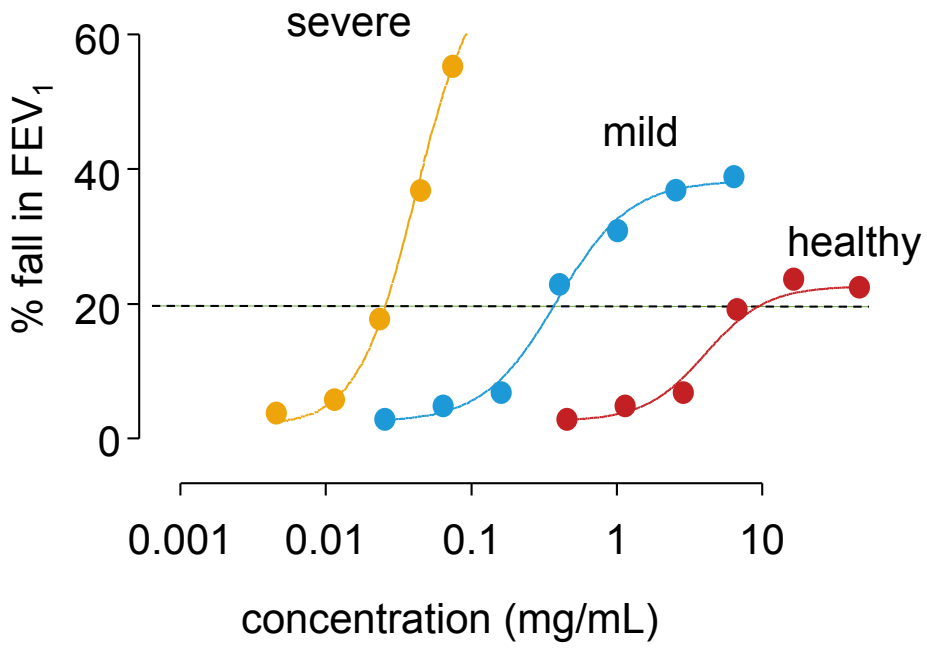

Figure 1: Dose-response curves to inhaled methacholine in a healthy, mild-asthmatic, and severe-asthmatic subject, showing both the leftward shift of the curve (hypersensitivity) and steeper slope (hyperreactivity) that characterise BHR. Reproduced with permission from the European Respiratory Society (1,117).

2.2 Indirect challenge agents

In the search for stimuli that produce responses through mechanisms that better reflect the underlying disease pathology, indirect challenges have been introduced, which exert their effects on intermediary cells involved in the asthmatic response, rather than acting directly on ASM. Most indirect stimuli evoke a heterogeneous response by affecting multiple pathophysiological pathways (3). Especially in the 1980s and 1990s many different potential indirect stimuli have been investigated, which have been reviewed comprehensively by Van Schoor et al., first in 2000 (10), and subsequently updated in 2005 by the same authors (17). In 2003, a European Respiratory Society (ERS) Task Force published their recommendations on the use of indirect stimuli in diagnosis and monitoring of asthma (3).

Indirect stimuli can be sub-classified as physical or pharmacological stimuli. Physical stimuli induce airways obstruction without acting on specific receptors, exemplified by exercise-induced bronchoconstriction or that induced by "fog" challenges with distilled water or hypotonic aerosols (18,19). Exercise induces dehydration of the airway epithelium, resulting in an increased osmolarity of the airway lumen and subsequent release of mediators from mast cells and activation of sensory nerves (20). This process is mimicked during challenge with hyperosmolar aerosols (21,22). Pharmacological stimuli induce airways obstruction secondary to the activation of intermediary cell types, such as inflammatory, epithelial, or neuronal cells, or combinations of these. The effects of indirect agents depend on the specific cells and receptors involved (10), but many of the stimuli used are known to activate sensory nerves, for example bradykinin, sulphur dioxide and adenosine (reviewed in (23)). Some indirect stimuli are endogenous compounds known to be released during airways obstruction, such as adenosine, AMP, tachykinins and bradykinin (24–31). Another group of indirect-acting stimuli is comprised of sulphur-containing compounds, which originated from the

observation that sulphur dioxide, a common air pollutant, and sulphites used as preservatives in food processing, may induce bronchoconstriction in susceptible subjects through activation of sensory neuronal pathways (32,33). However, lack of reproducible test outcomes has led to discontinuation of studying several of these stimuli (sulphur dioxide, sodium metabisulphite, bradykinin and tachykinins) and focus has predominantly shifted to the most easily and widely applicable indirect stimuli, mannitol (physical) and AMP/adenosine (pharmacological).

2.3 Allergen challenge

The preceding "non-specific" bronchial challenge tests are targeted to mechanisms that are thought to be intrinsic to the underlying hyperresponsive state of the airways in subjects with asthma. In contrast, so-called "specific" airway challenges can be used to assess the airway responsiveness to sensitising agents, such as aeroallergens or occupational agents. In allergic subjects, following sensitisation to an allergen, minute quantities of that allergen are sufficient to cause an immediate IgE-mediated early asthmatic response (EAR). In approximately 50% of positive allergen challenges a recurrence of airflow obstruction occurs between 3 to 8 hours after allergen exposure, the so-called late asthmatic response (LAR) (34), which is associated with airway inflammation and in some patients can be associated with an increase in BHR to agents like methacholine (35–37). Often-used outcome measures for inhaled allergen challenge are a >15% decrease in FEV_1, >50% decrease in specific airway conductance, or >100% increase in specific airway resistance compared to baseline (38). Additional outcome measures can be the change in non-specific BHR to e.g. methacholine, or the occurrence of airway inflammation expressed as increase in sputum eosinophils or exhaled nitric oxide (38). Inhaled allergen challenge has also been widely used as a model to assess the efficacy of novel therapeutic interventions (39–49).

2.4 LPS challenge

Another interesting application of the inhaled airway challenge test concept is provocation with lipopolysaccharide (LPS), also called endotoxin, a major component of the outer membrane of Gram-negative bacteria that induces fever and inflammation upon systemic exposure (50). Aerosolised endotoxins are present ubiquitously in the environment in concentrations that do not elicit immune responses. However, in certain aerosols, like tobacco smoke and organic dusts, concentrations can be high enough to induce responses in the lungs (51). Inhalation of endotoxin has been associated with lung inflammation, most notably neutrophilia (52–54). It can further lead to bronchial obstruction in people with asthma or other forms of BHR (55,56). Interestingly, it has recently been reported that LPS can elicit BHR through a mechanism involving cholinergic transmission (57).

Airway challenge with a nebulised LPS solution has been used mainly to study neutrophilic inflammatory processes in the lungs and has been developed into a challenge model to investigate the effect of novel anti-inflammatory drugs under development for the treatment of diseases that are associated with neutrophil infiltration into the lungs, particularly COPD and severe asthma, as this challenge is not sensitive to treatment with glucocorticosteroids (5,58–61). This model has also been used for early proof-of-concept and dose-ranging studies for novel drugs for the treatment of respiratory diseases in healthy volunteers (58,62).

3. General considerations for challenge delivery to the lungs

Despite the wide array of challenge test agents, there are relatively few methods used for their administration. Hence various aspects need to be considered that apply to airway challenge methodology in general, irrespective of the agent that is used.

3.1 Delivered dose

Inhaling a challenge agent usually leads to a dose-dependent response in the lungs. For challenges that induce BHR, the minimal dose required to obtain a response, as well as the slope of the dose-response curve, varies amongst patients and defines the severity of their BHR (1). The aim of such bronchial challenge tests is to induce a pre-defined degree of bronchoconstriction without risking a response that is too severe, meaning that the dose of the stimulus requires titration. For this reason, ascending dosing protocols have been developed in which the stimulus is administered by nebulisation of ascending (usually doubling) concentrations or doses, after each of which the lung function is measured (1,6,11,63,64). The concentration or dose is gradually increased and the test continues until a predefined threshold value for the outcome measure is obtained. The test result is negative when the threshold value is not reached after administration of the top dose.

This methodology has been initially developed for direct stimuli (65), but indirect-acting stimuli are generally administered following similar dosing protocols (3), although different concentrations (or doses) may be used to accommodate differences in potency, as shown in **Figure 2**. For AMP for example, a 16-fold lower potency has been reported compared to methacholine (66). Allergen exposure should be increased gradually either by extending the duration of the exposure or increasing the concentration in order to prevent severe acute reaction (38). For the same safety reason, allergen dose increments should have longer time intervals (10–15 min) compared to direct or indirect challenge methods (<5 min). Due to their specific mode of action, as little as a few ng can suffice to elicit the airway response. Endotoxin challenge on the other hand is usually administered by nebulisation of a fixed dose in the μg-range.

It is clear that due to these differences in dosimetry, the various challenges require different methods for administration. However, currently no universally standardised methodology is used in the majority of cases to deliver these different agents. It is important to realise that this may have important implications that are now largely neglected by the field.

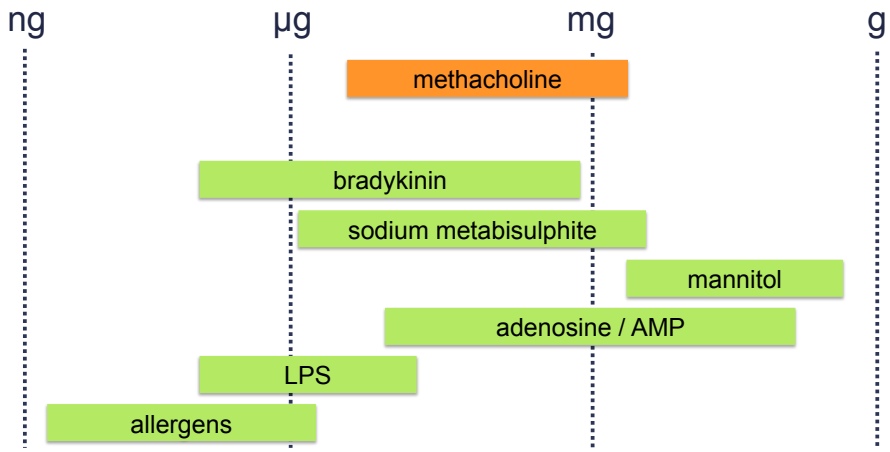

Figure 2: Dose ranges of various inhaled airway challenge agents. Allergens and LPS can also be expressed in biological units and endotoxin units respectively.

3.2 Administration by nebulisation

Most challenge agents are administered by nebulisation, except for mannitol and an investigational formulation of adenosine that are both administered as a dry powder (see below). Preparing nebuliser formulations can be very straightforward, which is the main reason why nebulisers are often used for off-label or investigational drugs and non-medicinal compounds, like many of the agents used for challenge testing. However, it has to be carefully evaluated whether the formulation affects nebuliser performance (in terms of droplet size and output rate) and, in the case of more complex molecules, whether the nebulisation process leads to degradation of the agent (e.g. allergens of biological origin).

Two standardised dosing protocols have been published for administration of methacholine by nebulisation (6,11). The two methods have different pros and cons, and the choice of method has been left to the preference of individual investigators/clinicians. The dosimeter method involves five deep and slow inhalations, which allows for accurate quantification of the administered dose, thereby making this method suitable for studies that require administration of an exact dose, such as LPS challenge studies or allergen challenges using a bolus dose. It has been claimed, however, that such deep breaths have bronchoprotective and bronchodilatory effects per se (67–69), which may therefore interfere with an accurate interpretation of the test result. In the newest technical standard on methacholine challenge testing a deep-breath method is therefore not recommended (6). The other method is the tidal breathing method in which the stimulus is inhaled during a specified time of calm, tidal breathing, although the patient's inhalation flow rate is generally not controlled. This more shallow way of inhaling does not evoke bronchoprotective and bronchodilatory mechanisms, but could result in different deposition patterns of the aerosolised challenge compared to the five-breath dosimeter method, since penetration of the aerosol into the more distal airways is dependent on the mixing of old and new air in the lungs. Moreover, the total amount that is inhaled depends on the inspiratory cycle of the subject, but also on the output rate of the nebuliser. In the first universal (American Thoracic Society) guidelines on methacholine challenge testing, the output rate had therefore been standardised at 0.13 mL/min (based on the output rate of the Wright nebuliser that was commonly used for challenge testing), regardless of the nebuliser that was used (11). However, adjusting the jet pressure to obtain this output rate may have detrimental effects on the droplet size distribution. As an example, this has been shown for the SideStream nebuliser (Philips Respironics), where the median mass aerodynamic diameter (MMAD) increased from 5.1 μm to 8.5 μm when the jet pressure was reduced from 1.5 bar (manufacturer's specifications) to 0.5 bar to reach the required

output rate of 0.13 mL/min (70). To prevent such unforeseen changes, a better strategy is to control the total administered volume of the solution containing the challenge agent by altering the nebulisation time rather than using an output rate of the nebuliser outside of the manufacturer's specifications, which is now discussed in the recently published European Respiratory Society technical standard on methacholine challenge testing (6).

In addition to the effect of jet pressure on droplet size, the type of compound and its concentration may affect the droplet size distribution as well. For AMP, which has a dose range exceeding that of methacholine, it has been shown that the increased viscosity of the more concentrated solutions resulted in a large shift in the aerosol droplet size distribution (70). In this study, a decrease in MMAD of almost 50% was measured at the highest AMP concentration (320 mg/mL) compared to saline and the lowest AMP concentrations. These results indicate that applying methods developed for a certain stimulus and a certain device cannot simply be used for other compounds or in other situations without verifying their suitability.

A third factor that should be accounted for is the evaporation of solvent (water) during nebulisation. The driving force for evaporation is saturation of the outgoing air with solvent (71), which leads to an increase in concentration of the remainder of the solution in the nebuliser cup (70,72). Importantly, evaporative water losses lead to an overestimation of the administered dose when the output rate is measured gravimetrically (6). Evaporation is an endothermic effect and the energy needed for this process is drawn from the solution, resulting in a temperature drop in the nebuliser solution that in turn may affect the output rate of the nebuliser (72). Calibration of the nebuliser output rate should therefore be performed under precise operating conditions. Newer jet nebulisers and especially vibrating mesh

nebulisers exhibit much lower amounts of evaporative loss (6), but still it is preferable to measure output rate by means of filter measurements (collection of the active compound) rather than gravimetrically. Such data should either be provided by the manufacturer or can be obtained in a pharmaceutical lab specialised in inhaled drug delivery.

In contrast to methacholine, there are no universal standardised protocols for other challenge agents, although recommendations have been made for similar ascending administration protocols (3).

3.3 Aerosol deposition and distribution in the lungs

For optimal efficacy and discriminatory power, inhaled medical aerosols should achieve maximal delivery to, and deposition in the target area in the lungs. Bronchial challenges that measure bronchoconstriction should be targeted to the proximal part of the bronchial tree, where the effects of ASM contraction are most pronounced. This means that the requirements for aerosol particle size are quite easily met, since an MMAD of roughly 3 to 5 μm should generally suffice, especially at tidal breathing. Aerosols with particles in this size range have the additional benefit of increased deposition efficacy, resulting in a higher total lung dose compared to particles smaller than 1.5 μm (73). It has indeed been found that aerosols with MMADs of 3 and 5 μm result in a lower methacholine PC_{20} compared to those with an MMAD of 1 μm (74), which can be attributed to a combination of greater lung deposited dose and targeting to the proximal airways.

It could be reasoned that challenges that act on inflammatory processes should be targeted more distally, as inflammation occurs throughout the lungs. However, investigations into the effects of particle (droplet) size on airway responsiveness to AMP have thus far been

inconclusive due to a high number of non-responders to small-particle AMP, which could be explained by a higher exhaled fraction or the discrepancy between deposition in the peripheral airways and an outcome measure of the more central airways (FEV_1) (75). For allergens, significant effects of particle size on the response to cat and mite allergen have been found, with larger particles (around 10 μm) being more effective in inducing the immediate response (76,77). A study investigating the effect of particle size on responses to endotoxin found a greater inflammatory response at the bronchial and systemic level when challenged with larger particles, although it could not be concluded whether this was due to regional distribution differences or the higher total lung dose (78).

3.4 Patient-related factors affecting aerosol deposition

Patient-related factors, such as the size and morphology of the oropharynx and bronchial tree, and the severity of lung disease can also affect aerosol deposition patterns (79). Additionally, inhalation flow rate has an important influence on the site of deposition (80). A higher flow rate shifts deposition to the higher airways at the cost of peripheral deposition. The patient's flow rate should therefore be adjusted to the type of delivery device being used to prevent loss of aerosol through deposition in the throat. Newer delivery systems that provide electronic control over the inhalation flow rate and volume (e.g. APS Pro system, see section 4.1, and AKITA) can provide better control over the delivered dose and deposition in the lungs (81–83).

Current medication use of patients undergoing a bronchial challenge has to be accounted for as well, since these treatments are intended to reduce or prevent the symptoms evoked during the challenge. To prevent possible confounding effects on the test outcome, lung medications have to be withheld for a specified time prior to execution of the test. The duration of

withholding is dependent on the mechanism of action of the drug and ranges from a few hours for short-acting beta-agonists up to a few days for long-acting anticholinergics or even weeks for ICS (depending on the challenge agent and the aim of the challenge test).

4. Optimisation and standardisation of challenge delivery per agent

Both optimisation and standardisation of challenge methods by different agents are urgently needed, in order to address the issues identified in the preceding section and make scientific progress towards more precise and rigorously controlled diagnostic procedures. Some efforts have already been undertaken in this regard, for example with the mannitol test (Aridol/Osmohale; see section 4.2).

4.1 Methacholine

Soon after publication of the first universal guidelines for methacholine challenge in 2000, which recommend both the dosimeter method and tidal breathing method, studies began to appear that investigated the comparability of the two dosing protocols. The first study reported similar results (geometric mean PC_{20} 1.8 mg/mL for tidal breathing vs. 1.6 mg/mL for dosimeter). However, the authors compared different dosing protocols (twofold vs. fourfold increases in concentration for tidal breathing and dosimeter respectively) (84). Cockcroft et al. addressed this disparity by comparing identical dosing regimens (doubling concentrations) and found that the tidal breathing method, which exposes the subject to twice as much aerosol at each concentration, resulted in a PC_{20} that was 1.6 (PC_{20} <1 mg/mL) to 2.1-fold (PC_{20} >1 mg/mL) lower compared to the dosimeter method (85). This difference between subjects with mild and severe hyperresponsiveness has been suggested to be explained by bronchodilator and/or bronchoprotective effects of the inhalation manoeuvre adopted in the dosimeter method (85,86). Prieto et al. reported a difference of 0.78 doubling

concentrations, with dosimeter values being higher than tidal breathing values, but found similar values for slope and level of plateau of the dose-response curve (87). Acknowledging the difference in administered volume, they performed another study in which they administered the same volume of challenge. Still an average difference in PC_{20} of 0.9 doubling concentrations was reported (88). However, when looking at the individual subjects it can be seen that this difference was mainly caused by a higher number of non-responders when using the dosimeter method (**Figure 3**). This may actually indicate that in some subjects with (mild) asthma the bronchodilatory effect of deep inhalations can indeed effectively counteract any bronchoconstriction induced by methacholine, as suggested by Cockcroft et al. (85). Indeed deep-breath methods have now been excluded from the new technical standard on methacholine challenge for this reason (6).

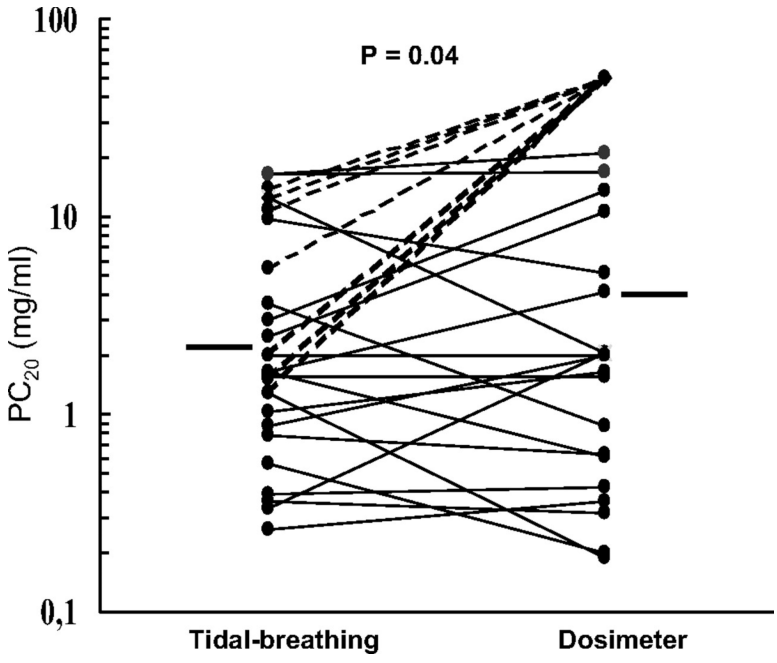

Figure 3: Comparison of tidal breathing and dosimeter methacholine PC_{20} in 27 subjects with suspected asthma. The dashed lines indicate seven subjects in whom no dosimeter PC_{20} was obtained. Reproduced with permission from (82).

More recent investigations into methacholine challenge method optimisation focused on the delivery systems used, as the nebulisers recommended in the first guidelines (11) had become obsolete (89). The Aerosol Provocation System (APS) Pro (CareFusion) is especially noteworthy in this respect. The APS Pro system is a computer-controlled nebuliser system specifically developed for bronchial challenge testing with methacholine and can be integrated with other CareFusion systems for spirometry, impulse oscillometry, respiratory resistance and body plethysmography depending on the outcome measure of choice. The dosimeter method is the preferred method, although the system can also be used for the tidal breathing method. The PD_{20} from a pulse of aerosol of a single methacholine concentration was found to correlate well with the PC_{20} obtained with a standard dosimeter test (90). *In vitro* studies indicate that these new delivery systems often have a higher output rate than the systems recommended in the first guidelines, thus a faster delivery of the challenge, which should be controlled for in terms of exposure time (70,89).

The plethora of available nebuliser systems (with variable output rates) introduces new concerns regarding equivalence of the test results obtained with different systems. Interestingly, it has been found that no differences between test systems are found when the PD_{20} is calculated (91,92) instead of the PC_{20} (91,93), which has led to the recommendation to use the PD_{20} as the end-point in the new technical standard (6). On a different note, using more efficient nebulisers also introduces a risk of extreme individual responses to methacholine aerosols. Patients who are highly responsive to methacholine may experience large drops in FEV_1 when the full dose is presented to them in a shorter time, which should be accounted for by thorough safety assessment of using these more efficient nebulisers.

4.2 Mannitol

Bronchial challenge with mannitol has been developed to overcome technical difficulties (i.e. the need for filters and scales to determine the administered volume) encountered with bronchial challenge tests using hypertonic saline (20,94,95). This agent has been shown to cause contraction of ASM through release of inflammatory mediators such as leukotriene E_4 and prostaglandin D_2, which are thought to be released from mast cells (96). BHR measured in response to inhaled mannitol is dependent on the presence of inflammation and can be reduced by ICS treatment (97,98). The low sensitivity (59.8%) of the mannitol test compared with the clinical assessment determined in more than 500 subjects was attributed to ICS use by 75% of the diagnosed asthmatics in this study, as this value greatly improved when ICS-users were excluded from analysis (to 88.7%) (95). This finding supports the concept that mannitol responsiveness can be used to monitor ICS effectiveness (97,98) and highlights the growing appreciation that indirect challenge tests are useful for diagnosis and monitoring treatment of current asthma. Mannitol responsiveness – expressed as the provocative dose that causes a 15% decrease in FEV_1 (PD_{15}) – has been found to correlate well with responsiveness to bronchial challenge tests with other physical stimuli or AMP (99,100). Bronchial challenge testing with mannitol may therefore be of particular use in diagnosis of asthma in elite athletes, who require an official diagnosis of asthma, but whose bronchoconstriction is hard to induce by exercise in a laboratory setting (101).

The mannitol challenge test is registered with various regulatory authorities worldwide and is currently the only fully standardised challenge method. The mannitol formulation consists of a spray-dried powder with an MMAD of around 3.5 μm that is inhaled with a simple capsule inhaler device. Benefits of this mannitol test are that it comes in a standardised kit that does not need any special equipment, and that it is relatively easy to perform. However, a

drawback of mannitol challenge is the deep inhalation-dependent modality of powder administration, which has been suggested to counteract bronchoconstriction as discussed in section 4.1. Moreover, the quantity of powder that needs to be inhaled is large in comparison to other agents, up to a cumulative dose of 635 mg, as a consequence of its mechanism of action (i.e. increasing the osmolarity of the lung lining fluid). This in combination with the low resistance device, and hence a high inspiratory flow rate, can result in cough through a mechanical cough reflex due to oropharyngeal deposition of the mannitol (102). In a phase III study investigating the safety and efficacy of inhaled mannitol as a bronchial challenge test, cough occurred in 535 of 592 (of whom 91 were non-asthmatic) subjects. In some cases, cough was so severe that the test had to be delayed (one in seven subjects), or even ended prematurely (one in 100 subjects) (95). Although cough does not occur exclusively in subjects with asthma, it has been demonstrated that cough in response to inhaled mannitol is associated with asthma (103), which would be interesting to elucidate further. To which extent the occurrence of (severe) cough is diagnostic for asthma and to which extent it is due to oropharyngeal deposition of mannitol could be investigated by provoking subjects with the same mannitol formulation, but using a high-resistance inhaler device and controlled slow inhalation to minimise throat deposition.

4.3 Adenosine

Other efforts towards optimisation have been undertaken with inhaled adenosine. Adenosine and its precursor AMP have been the subject of a considerable amount of research in respiratory medicine since the early 1980s. Adenosine, a purine nucleoside involved in many biological processes, is considered a pro-inflammatory mediator in asthma (104) as it is thought to induce mast cell degranulation, a process mediated through the A_{2b} receptor, leading to contraction of ASM and most notably airway eosinophilia (105,106). More

recently a role for A_1 receptors has been implicated in the contraction of ASM from subjects with asthma induced by adenosine (107), suggesting that adenosine triggers bronchoconstriction through both inflammatory and neuronal pathways.

Historically, AMP has been used instead of adenosine because of its much higher aqueous solubility, which is required for nebulisation, and it is generally assumed that AMP is converted in vivo to adenosine instantaneously by endonucleotidases when it comes in contact with lung lining fluid (10). However, because of the above mentioned effects of high AMP concentrations on aerosol particle size produced by nebulisation (70), an adenosine dry powder challenge test has been developed that consists of simple spray-dried formulations containing pure adenosine or adenosine diluted with lactose, which so far have only been administered with an investigational inhaler device. With this inhaler, the entire dose range of adenosine (0.04–80 mg) was consistently delivered in the first proof-of-concept studies that have been performed with this formulation (108–110). So far, these studies justify the chosen dose range for adenosine and indicate that the response rate and thus diagnosis of asthma can be improved by the administration of the higher doses that are possible with the powder formulation. These findings now have to be complemented by studies in healthy subjects and in subjects with lung diseases other than asthma to determine the specificity and sensitivity of this test. Since the test concerns a powder for inhalation, any effects on bronchoconstriction of the deep-inhalation dependent administration should also be considered for this adenosine challenge test.

4.4 Occupational agents and allergens

For occupational agents, the suspected causative agent should be delivered in the same conditions that it is found in the workplace in terms of physical and chemical properties in

relevant concentrations (38). It is now recognised that there are a large variety of occupational agents and allergens and therefore a handbook has recently been prepared that summarises the delivery methods for the most commonly used agents (see online supplement to (38)). This handbook provides an excellent start towards harmonisation of specific inhaled challenges, although it could benefit from inclusion of recommendations on nebulisers for those agents that are administered following a tidal breathing or dosimeter method.

Allergens are administered in very low doses compared to the nonspecific bronchial challenges. Nebulisation can therefore in general be considered a suitable administration method, provided the stability of the agent is checked during storage and upon administration, particularly for more complex molecules (e.g. antigens). Chemical stability issues upon storage arise when an agent is sensitive to degradation reactions (e.g. oxidation, hydrolysis), as these occur faster in aqueous conditions than in the dry state. Additionally, stability can become an issue when the stresses induced by the nebulisation process itself may damage the material(s) (e.g. proteins) in the formulation (111,112).

4.5 LPS

LPS is very stable and can withstand high temperatures and strong shear forces. It can be kept in solution for up to a month. However, LPS adheres readily and strongly to surfaces such as glass, for example to the vial in which it is stored. Extra care (e.g. rigorous vortexing) should therefore be taken in the preparation of the nebuliser solution. Additionally, endotoxins from different sources can have a different biological activity (potency). Studies report the use of different sources of LPS and different doses, ranging from 0.5 to 100 μg (5,52–54,59–62). However, expressing dose in units of weight has little value because of the different potencies. Variability in the dose delivered to patients is further increased by differences in

administration method. Both dosimeter and tidal breathing methods have been used to date and the differences between devices and inhalation manoeuvres inevitably result in differences in the delivered and deposited doses.

The lack of control of dose in terms of potency, in addition to differences in administration methods, complicates the comparison of studies performed over a period of about 30 years. Studies have been performed in healthy (5,51–54,58–62,113) and diseased subjects (55,56,114,115), in smokers (116) and non-smokers, but the potency of the LPS was often not reported. Although a promising disease model, standardisation of the dosing protocol and administration method should be established before LPS challenge can be accepted as a validated tool to be used more widely in drug development studies. Other issues that need to be addressed are the lack of dose-response studies performed in humans and uncertainty regarding why some people do not respond to LPS inhalation. To study the latter issues, it is imperative to know the exact dose of LPS that is delivered and its potency. The first steps forward should be to decide on a preferred administration method (i.e. slow deep inhalation) and performing a potency measurement of the LPS in the nebuliser solution to be used for administration.

5. Conclusions

Inhaled airway challenges are versatile tests that are relatively easy and cheap to perform. Classical bronchial challenge tests that assess BHR have proven their value in excluding or confirming a suspected diagnosis of asthma and have been shown to be useful for monitoring the disease and effectiveness of therapy. These tests can thus help in providing more accurate information to patient and prescriber as to how to treat an individual patient. As such, bronchial challenge tests can help in improving the individual patient's health through better

treatment of their disease. In research and development, inhaled airway challenge can be applied even more widely, from studying disease mechanisms to investigating the effectiveness of new drugs. Careful selection of the challenge agent may provide significant benefits, in terms of both selecting suitable subjects (e.g. using response to a discriminatory challenge as an inclusion criterion) and addressing the research question. There is also a lot to be gained through optimisation and reporting of challenge test posology, especially to ensure the comparability of studies performed by different laboratories. In general all compounds described so far would strongly benefit from the development of defined inhalation systems that provide a reproducible and reliable deposition in the lungs and further standardisation of administration protocols as has been done for mannitol (**Table 1**). Improved delivery may also open doors for revisiting some challenge agents that have been used in the past, but were abandoned due to lack of reproducibility. Creating a "tool box" of well-characterised challenge agents with tailored delivery systems would provide a valuable tool for studying and discriminating different airway diseases, but also for investigating mechanisms and novel treatments for affecting BHR.

Table 1: Opportunities to improve the application of bronchial challenge testing

Issue	Need
Delivery method optimisation	Control (and quantification) of delivered dose by optimising the production and administration of challenge agent aerosols with suitable aerodynamic size distributions. This will require the tailoring of delivery methods for each individual challenge agent.
Standardisation	International consensus on best practice. Inter-lab comparisons to verify the reproducibility of standard methods. Reporting of dose characterisation/validation for all research studies.
Specific issues for mannitol	Investigate the relative contributions of throat deposition and increased airway sensitivity to the occurrence of cough in asthma.
Specific issues for adenosine	Compare responsiveness to dry powder adenosine challenge to responsiveness to nebulised AMP including the response of healthy subjects and patients with lung diseases other than asthma. Determination of the specificity and sensitivity of dry powder adenosine bronchial challenge in patients with asthma.
Specific issues for allergens	Extension of existing guidelines (37) to include recommendations on nebulisers for those agents that are administered following a tidal breathing or dosimeter method. Verification of chemical stability and tolerance to nebulisation on a case-by-case basis.
Specific issues for LPS	Control of test agent potency and reproducibility of delivery (dose and lung distribution).

References

1. Sterk PJ, Fabbri LM, Quanjer PH, Cockcroft DW, O'Byrne PM, Anderson SD, Juniper EF, Malo J-L. Airway Responsiveness: Standardized challenge testing with pharmacological, physical and sensitizing stimuli in adults. Eur Respir J. 1993; 6(Suppl. 16):53–83.
2. Cockcroft DW. Direct challenge tests: Airway hyperresponsiveness in asthma: its measurement and clinical significance. Chest. 2010; 138(2 Suppl):18S – 24S.
3. Joos GF, O'Connor B. Indirect airway challenges. Eur Respir J. 2003;21(6):1050–68.
4. Boulet LP, Gauvreau G, Boulay ME, O'Byrne P, Cockcroft DW. The allergen bronchoprovocation model: An important tool for the investigation of new asthma anti-inflammatory therapies. Allergy. 2007;62(10):1101–10.
5. Kitz R, Rose MA, Placzek K, Schulze J, Zielen S, Schubert R. LPS inhalation challenge: A new tool to characterize the inflammatory response in humans. Med Microbiol Immunol. 2008;197(1):13–9.
6. Coates AL, Wanger J, Cockcroft DW, Culver BH, Carlsen K-H, Diamant Z, Gauvreau G, Hall GL, Hallstrand TS, Horvath I, De Jongh FHC, Joos G, Kaminsky DA, Laube BL, Leuppi JD, Sterk PJ. ERS technical standard on bronchial challenge testing: general considerations and performance of methacholine challenge tests. Eur Respir J. 2017;49:1601526.
7. Kraemer R, Smith H-J, Sigrist T, Giger G, Keller R, Frey M. Diagnostic accuracy of methacholine challenge tests assessing airway hyperreactivity in asthmatic patients - a multifunctional approach. Respir Res. 2016;17(1):154.
8. Oostveen E, MacLeod D, Lorino H, Farre R, Hantos Z, Desager K, Marchal F. The forced oscillation technique in clinical practice: methodology, recommendations and future developments. Eur Respir J. 2003;22(6):1026–41.

9. Robinson PD, Latzin P, Verbanck S, Hall GL, Horsley A, Gappa M, Thamrin C, Arets HGM, Aurora P, Fuchs SI, King GG, Lum S, Macleod K, Paiva M, Pillow JJ, Ranganathan S, Ratjen F, Singer F, Sonnappa S, Stocks J, Subbarao P, Thompson BR, Gustafsson PM. Consensus statement for inert gas washout measurement using multiple- and single- breath tests. Eur Respir J. 2013;41(3):507–22.
10. Van Schoor J, Joos GF, Pauwels RA. Indirect bronchial hyperresponsiveness in asthma: mechanisms, pharmacology and implications for clinical research. Eur Respir J. 2000;16(3):514–33.
11. Crapo RO, Casaburi R, Coates AL, Enright PL, Hankinson JL, Irvin CG, MacIntyre NR, McKay RT, Wanger JS, Anderson SD, Cockcroft DW, Fish JE, Sterk PJ. American Thoracic Society Guidelines for Methacholine and Exercise Challenge Testing — 1999. Am J Respir Crit Care Med. 2000;161:309–29.
12. Anderson SD, Charlton B, Weiler JM, Nichols S, Spector SL, Pearlman DS. Comparison of mannitol and methacholine to predict exercise-induced bronchoconstriction and a clinical diagnosis of asthma. Respir Res. 2009;10:4.
13. Yan K, Salome CM, Woolcock AJ. Prevalence and nature of bronchial hyperresponsiveness in subjects with chronic obstructive pulmonary disease. Am Rev Respir Dis. 1985;132(1):25–9.
14. Ramsdale EH, Hargreave FE. Differences in airway responsiveness in asthma and chronic airflow obstruction. Med Clin North Am. 1990;74:741–51.
15. Fabbri LM, Romagnoli M, Corbetta L, Casoni G, Busljetic K, Turato G, Ligabue G, Ciaccia A, Saetta M, Papi A. Differences in airway inflammation in patients with fixed airflow obstruction due to asthma or chronic obstructive pulmonary disease. Am J Respir Crit Care Med. 2003; 167(3):418–24.

16. Prieto L, López M, Bertó J, Peris A. Modification of concentration-response curves to inhaled methacholine after the pollen season in subjects with pollen induced rhinitis. Thorax. 1994;50:711–3.
17. Van Schoor J, Pauwels R, Joos GF. Indirect bronchial hyper-responsiveness: the coming of age of a specific group of bronchial challenges. Clin Exp Allergy. 2005;35(3):250–61.
18. Allegra L, Bianco S. Non-specific broncho-reactivity obtained with an ultrasonic aerosol of distilled water. Eur J Respir Dis Suppl. 1980;106:41–9.
19. Simone P, Robuschi M, Vaghi A, Fasano W, Bianco S. Prevention of fog-induced bronchospasm by high doses of ipratropium bromide in asthmatics. Respiration. 1986;50(suppl 2):209–11.
20. Anderson SD. “Indirect” challenges from science to clinical practice. Eur Clin Respir J. 2016;3:31096.
21. Smith CM, Anderson SD. Inhalation provocation using nonisotonic aerosols tests. J Allergy Clin Immunol. 1989;84:781–90.
22. Rodwell LT, Anderson SD, Du Toit J, Seale JP. Nedocromil sodium inhibits the airway response to hyperosmolar challenge in patients with asthma. Am Rev Respir Dis. 1992;146(5 Pt 1):1149–55.
23. Spina D, Page CP. Airway sensory nerves in asthma - Targets for therapy? Pulm Pharmacol. 1996;9(1):1–18.
24. Cushley MJ, Tattersfield AE, Holgate ST. Inhaled adenosine and guanosine on airway resistance in normal and asthmatic subjects. Br J Clin Pharmacol. 1983;15:161–5.
25. Caruso M, Holgate ST, Polosa R. Adenosine signalling in airways. Curr Opin Pharmacol. 2006;6(3):251–6.

26. Brown RA, Spina D, Page CP. Adenosine receptors and asthma. Br J Pharmacol. 2008;153 Suppl(December 2007):S446–56.
27. Fuller RW, Dixon CM, Cuss FM, Barnes PJ. Bradykinin-induced bronchoconstriction in humans. Mode of action. Am Rev Respir Dis. 1987;135(1):176–80.
28. Ichinose M, Nakajima N, Takahashi T, Yamauchi H, Inoue H, Takishima T. Protection against bradykinin-induced bronchoconstriction in asthmatic patients by neurokinin receptor antagonist. Lancet. 1992;340(8830):1248–51.
29. Polosa R, Phillips GD, Lai CK, Holgate ST. Contribution of histamine and prostanoids to bronchoconstriction provoked by inhaled bradykinin in atopic asthma. Allergy. 1990;45(8):174–82.
30. Joos G, Pauwels R, van der Straeten M. Effect of inhaled substance P and neurokinin A on the airways of normal and asthmatic subjects. Thorax. 1987;42(10):779–83.
31. Cohen J, Burggraaf J, Schoemaker RC, Sterk PJ, Cohen AF, Diamant Z. Relationship between airway responsiveness to neurokinin A and methacholine in asthma. Pulm Pharmacol Ther. 2005;18(3):171–6.
32. Dixon CM, Ind PW. Inhaled sodium metabisulphite induced bronchoconstriction: inhibition by nedocromil sodium and sodium cromoglycate. Br J Clin Pharmacol. 1990;30(3):371–6.
33. Nichol GM, Alton EW, Nix A, Geddes DM, Chung KF, Barnes PJ. Effect of inhaled furosemide on metabisulfite- and methacholine-induced bronchoconstriction and nasal potential difference in asthmatic subjects. Am Rev Respir Dis. 1990;142(3):576–80.
34. O'Byrne PM, Dolovich J, Hargreave FE. Late asthmatic responses. Am Rev Respir Dis. 1987;138:740–51.

35. Demonchy JGR, Kauffman HF, Venge P, Koëter GH, Jansen HM, Sluiter HJ, de Vries K. Bronchoalveolar eosinophilia during allergen-induced late asthmatic reactions. Am Rev Respir Dis. 1985; 131(3):373–6.

36. Gauvreau GM, Parameswaran KN, Watson RM, O'Byrne PM. Inhaled leukotriene E4, but not leukotriene D4, increased airway inflammatory cells in subjects with atopic asthma. Am J Respir Crit Care Med. 2001;164(8 I):1495–500.

37. Cockcroft DW, Ruffin RE, Dolovich J, Hargreave FE. Allergen-induced increase in non-allergic bronchial reactivity. Clin Allergy. 1977;7(6):503–13.

38. Vandenplas O, Suojalehto H, Aasen TB, Baur X, Burge PS, de Blay F, Fishwick D, Hoyle J, Maestrelli P, Muñoz X, Moscato G, Sastre J, Sigsgaard T, Suuronen K, Walusiak-Skorupa J, Cullinan P. Specific inhalation challenge in the diagnosis of occupational asthma: consensus statement. Eur Respir J . 2014;43(6):1573–87.

39. Gauvreau GM, Doctor J, Watson RM, Jordana M, O'Byrne PM. Effects of inhaled budesonide on allergen-induced airway responses and airway inflammation. Am J Respir Crit Care Med. 1996;154(5):1267–71.

40. Leigh R, Vethanayagam D, Yoshida M, Watson RM, Rerecich T, Inman MD, O'Byrne PM. Effects of montelukast and budesonide on airway responses and airway inflammation in asthma. Am J Respir Crit Care Med. 2002; 166(9):1212–7.

41. Kelly MM, O'Connor TM, Leigh R, Otis J, Gwozd C, Gauvreau GM, Gauldie J, O'Byrne PM. Effects of budesonide and formoterol on allergen-induced airway responses, inflammation, and airway remodeling in asthma. J Allergy Clin Immunol. 2010; 125(2):349–56.

42. Cahn A, Boyce M, Mistry S, Musani N, Rambaran C, Storey J, Ventresca P, Michel O. Randomized trial of allergen-induced asthmatic response in smokers and non-smokers: Effects of inhaled corticosteroids. Clin Exp Allergy. 2015; 45(10):1531–41.

43. Krug N, Hohlfeld JM, Kirsten A-M, Kornmann O, Beeh KM, Kappeler D, Korn S, Ignatenko S, Timmer W, Rogon C, Zeitvogel J, Zhang N, Bille J, Homburg U, Turowska A, Bachert C, Werfel T, Buhl R, Renz J, Garn H, Renz H. Allergen-induced asthmatic responses modified by a GATA3-specific DNAzyme. N Engl J Med. 2015; 372(21):1987–95.
44. Twentyman OP, Finnerty JP, Holgate ST, Harris A, Palmer J. Protection against allergen-induced asthma by salmeterol. Lancet. 1990;336(8727):1338–42.
45. Bleecker ER, Mason PL, Moore WC. Clinical effects of nedocromil sodium on allergen-related mechanisms. J Allergy Clin Immunol. 1996;98(5 PART 2):S118–23.
46. Singh D, Cadden P, Hunter M, Collins LP, Perkins M. Inhibition of the asthmatic allergen challenge response by the CRTH2 antagonist OC000459. Eur Respir J. 2013;41(1):46–52.
47. Singh D, Richards D, Knowles RG, Schwartz S, Woodcock A, Langley S, O'Connor BJ. Selective inducible nitric oxide synthase inhibition has no effect on allergen challenge in asthma. Am J Respir Crit Care Med. 2007; 176:988–93.
48. Gauvreau GM, O'Byrne PM, Boulet L-P, Wang Y, Cockcroft D, Bigler J, et al. Effects of an anti-TSLP antibody on allergen-induced asthmatic responses. N Engl J Med. 2014;370(22):2102–10.
49. Gauvreau GM, Boulet LP, Leigh R, Cockcroft DW, Killian KJ, Davis BE, et al. A nonsteroidal glucocorticoid receptor agonist inhibits allergen-induced late asthmatic responses. Am J Respir Crit Care Med. 2015;191(2):161–7.
50. Thorn J. The inflammatory response in humans after inhalation of bacterial endotoxin: A review. Inflamm Res. 2001;50(5):254–61.
51. Rylander R, Bake B, Fischer JJ, Helander IM. Pulmonary function and symptoms after inhalation of endotoxin. Am Rev Respir Dis. 1989;140(4):981–6.

52. Sandstrom T, Bjermer L, Rylander R. Lipopolysaccharide (LPS) inhalation in healthy subjects increases neutrophils, lymphocytes and fibronectin levels in bronchoalveolar lavage fluid. Eur Respir J. 1992;5(8):992–6.

53. Michel O, Nagy AM, Schroeven M, Duchateau J, Neve J, Fondu P, et al. Dose-response relationship to inhaled endotoxin in normal subjects. Am J Respir Crit Care Med. 1997;156(4 I):1157–64.

54. Michel O, Duchateau J, Plat G, Cantinieaux B, Hotimsky A, Gerain J, et al. Blood inflammatory response to inhaled endotoxin in normal subjects. Clin Exp Allergy. 1995;36(4):425–31.

55. Michel O, Ginanni R, Le Bon B, Content J, Duchateau J, Sergysels R. Inflammatory response to acute inhalation of endotoxin in asthmatic patients. Am Rev Respir Dis. 1992;146(2):352–7.

56. Kitz R, Rose MA, Borgmann A, Schubert R, Zielen S. Systemic and bronchial inflammation following LPS inhalation in asthmatic and healthy subjects. J Endotoxin Res. 2006;12(6):367–74.

57. Calzetta L, Luongo L, Cazzola M, Page C, Rogliani P, Facciolo F, Maione S, Capuano A, Rinaldi B, Matera MG. Contribution of sensory nerves to LPS-induced hyperresponsiveness of human isolated bronchi. Life Sci. 2015; 131:44–50.

58. Franciosi LG, Diamant Z, Banner K, Zuiker R, Morelli N, Kamerling IM, de Kam ML, Burggraaf J, Cohen AF, Cazzola M, Calzetta L, Singh D, Spina D, Walker MJA, Page CP. Efficacy and safety of RPL554, a dual PDE3 and PDE4 inhibitor, in healthy volunteers and in patients with asthma or chronic obstructive pulmonary disease: findings from four clinical trials. Lancet Respir Med. 2013; 1(9):714–27.

59. Maris NA, De Vos AF, Dessing MC, Spek CA, Lutter R, Jansen HM, et al. Antiinflammatory effects of salmeterol after inhalation of lipopolysaccharide by healthy volunteers. Am J Respir Crit Care Med. 2005;172(7):878–84.
60. Loh LC, Vyas B, Kanabar V, Kemeny DM, O'Connor BJ. Inhaled endotoxin in healthy human subjects: A dose-related study on systemic effects and peripheral CD4+ and CD8+ T cells. Respir Med. 2006;100(3):519–28.
61. Janssen O, Schaumann F, Holz O, Lavae-Mokhtari B, Welker L, Winkler C, Biller H, Krug N, Hohlfeld JM. Low-dose endotoxin inhalation in healthy volunteers--a challenge model for early clinical drug development. BMC Pulm Med. 2013; 13(1):19.
62. Singh D, Siew L, Christensen J, Plumb J, Clarke GW, Greenaway S, Perros-Huguet C, Clarke N, Kilty I, Tan L. Oral and inhaled p38 MAPK inhibitors: effects on inhaled LPS challenge in healthy subjects. Eur J Clin Pharmacol. 2015; 71:1175–84.
63. Cockcroft DW, Killian DN, Mellon JJ, Hargreave FE. Bronchial reactivity to inhaled histamine: a method and clinical survey. Clin Allergy. 1977;7:235–43.
64. Ryan G, Dolovich MB, Obminski G, Cockcroft DW, Juniper E, Hargreave FE, Newhouse MT. Standardization of inhalation provocation tests: influence of nebulizer output, particle size, and method of inhalation. J Allergy Clin Immunol. 1981; 67(2):156–61.
65. Cockcroft DW. Provocation Methods: Direct Challenges. Clin Rev Allergy Immunol. 2003;24:19–26.
66. Polosa R, Lau LCK, Holgate ST. Inhibition of adenosine 5'-monophosphate- and methacholine-induced bronchoconstriction in asthma by inhaled frusemide. Eur Respir J. 1990;3:665–72.

67. Brusasco V, Crimi E, Barisione G, Spanevello A, Rodarte JR, Pellegrino R. Airway responsiveness to methacholine: effects of deep inhalations and airway inflammation. J Appl Physiol. 1999;87(2):567–73.
68. Kapsali T, Permutt S, Laube B, Scichilone N, Togias A. Potent bronchoprotective effect of deep inspiration and its absence in asthma. J Appl Physiol. 2000;89:711–20.
69. Scichilone N, Kapsali T, Permutt S, Togias A. Deep inspiration-induced bronchoprotection is stronger than bronchodilation. Am J Respir Crit Care Med. 2000;162:910–6.
70. Lexmond AJ, Hagedoorn P, Frijlink HW, de Boer AH. Challenging the two-minute tidal breathing challenge test. J Aerosol Med Pulm Drug Deliv. 2013;26(6):380–6.
71. Mercer TT. Production and characterization of aerosols. Arch Intern Med. 1973;131(1):39–50.
72. Cockcroft DW, Hurst TS, Gore BP. Importance of evaporative water losses during standardized nebulized inhalation provocation tests. Chest. 1989;96(3):505.
73. De Boer AH, Gjaltema D, Hagedoorn P, Frijlink HW. Can "extrafine" dry powder aerosols improve lung deposition? Eur J Pharm Biopharm. 2015;96:143–51.
74. Naji N, Keung E, Beaudin S, Kane J, Killian KJ, Gauvreau GM. The effects of particle size on measurement of airway hyperresponsiveness to methacholine. Ann Allergy, Asthma Immunol. 2013;110(5):359–63.
75. Cohen J, Postma DS, Douma WR, Vonk JM, De Boer AH, Ten Hacken NHT. Particle size matters: diagnostics and treatment of small airways involvement in asthma. Eur Respir J. 2011;37(3):532–40.
76. Lieutier-Colas F, Purohit A, Meyer P, Fabries JF, Kopferschmitt MC, Dessanges JF, Pauli G, de Blay F. Bronchial challenge tests in patients with asthma sensitized to cats:

The importance of large particles in the immediate response. Am J Respir Crit Care Med. 2003;167(8):1077–82.

77. Casset A, Purohit A, Birba E, Chenard M-P, Uring Lambert B, Bahram S, Meyer P, Pauli G, de Blay F. Bronchial challenge test in asthmatics sensitized to mites: role of particle size in bronchial response. J Aerosol Med. 2007;20(4):509–18.

78. Doyen V, Pilcer G, Dinh PHD, Corazza F, Bernard A, Bergmann P, Lefevre N, Amighi K, Michel O. Inflammation induced by inhaled lipopolysaccharide depends on particle size in healthy volunteers. Br J Clin Pharmacol. 2016;82:1371–81.

79. Laube BL, Janssens HM, de Jongh FHC, Devadason SG, Dhand R, Diot P, Everard ML, Horvath I, Navalesi P, Voshaar T, Chrystyn H. What the pulmonary specialist should know about the new inhalation therapies. Eur Respir J. 2011;37(6):1308–31.

80. Usmani OS, Biddiscombe MF, Barnes PJ. Regional lung deposition and bronchodilator response as a function of beta2-agonist particle size. Am J Respir Crit Care Med. 2005;172(12):1497–504.

81. Brand P, Friemel I, Meyer T, Schulz H, Heyder J, Haussinger K. Total deposition of therapeutic particles during spontaneous and controlled inhalations. J Pharm Sci. 2000;89(6):724–31.

82. Köhler E, Sollich V, Schuster-Wonka R, Jorch G. Lung deposition after electronically breath-controlled inhalation and manually triggered conventional inhalation in cystic fibrosis patients. J Aerosol Med. 2005;18(4):386–95.

83. Fischer A, Stegemann J, Scheuch G, Siekmeier R. Novel devices for individualized controlled inhalation can optimize aerosol therapy in efficacy, patient care and power of clinical trials. Eur J Med Res. 2009;14 Suppl 4:71–7.

84. Wubbel C, Asmus MJ, Stevens G, Chesrown SE, Hendeles L. Methacholine challenge testing* Comparison of the two American Thoracic Society-recommended methods. Chest. 2004;125(2):453–8.
85. Cockcroft DW, Davis BE, Todd DC, Smycniuk AJ. Methacholine challenge: comparison of two methods. Chest. 2005;127(3):839–44.
86. Allen ND, Davis BE, Hurst TS, Cockcroft DW, Donald W. Difference between dosimeter and tidal breathing methacholine challenge - Contributions of dose and deep inspiration bronchoprotection. Chest. 2005;128(6):4018–23.
87. Prieto L, Ferrer A, Domenech J, Pérez-Francés C. Effect of challenge method on sensitivity, reactivity, and maximal response to methacholine. Ann Allergy, Asthma Immunol. 2006;97(2):175–81.
88. Prieto L, Lopez V, Llusar R, Rojas R, Marin J. Differences in the response to methacholine between the tidal breathing and dosimeter methods: influence of the dose of bronchoconstrictor agent delivered to the mouth. Chest. 2008;134(4):699–703.
89. Coates AL, Leung K, Dell SD. Developing alternative delivery systems for methacholine challenge tests. J Aerosol Med Pulm Drug Deliv. 2014;27(1):66–70.
90. Schulze J, Rosewich M, Riemer C, Dressler M, Rose MA, Zielen S. Methacholine challenge - Comparison of an ATS protocol to a new rapid single concentration technique. Respir Med. 2009;103(12):1898–903.
91. Dell SD, Bola SS, Foty RG, Marshall LC, Nelligan KA, Coates AL. Provocative dose of methacholine causing a 20% drop in FEV1 should be used to interpret methacholine challenge tests with modern nebulizers. Ann Am Thorac Soc. 2015;12(3):357–63.
92. Coates AL, Dell SD, Cockcroft DW, Gauvreau GM. The PD 20 but not the PC 20 in a methacholine challenge test is device independent. Ann Allergy, Asthma Immunol. 2017;10–2.

93. El-Gammal AI, Killian KJ, Scime TX, Beaudin S, Schlatman A, Cockcroft DW, Gauvreau GM. Comparison of the provocative concentration of methacholine causing a 20% fall in FEV1 between the AeroEclipse II breath-actuated nebulizer and the wright nebulizer in adult subjects with asthma. Ann Am Thorac Soc. 2015;12(7):1039–43.
94. Anderson SD, Brannan J, Spring J, Spalding N, Rodwell LT, Chan KIM, Gonda I, Walsh A, Clark AR. A new method for bronchial-provocation testing in asthmatic subjects using a dry powder of mannitol. Am J Respir Crit Care Med. 1997; 156:758–65.
95. Brannan JD, Anderson SD, Perry CP, Freed-Martens R, Lassig AR, Charlton B. The safety and efficacy of inhaled dry powder mannitol as a bronchial provocation test for airway hyperresponsiveness: a phase 3 comparison study with hypertonic (4.5%) saline. Respir Res. 2005; 6:144.
96. Brannan JD, Gulliksson M, Anderson SD, Chew N, Kumlin M. Evidence of mast cell activation and leukotriene release after mannitol inhalation. Eur Respir J. 2003;22(3):491–6.
97. Brannan JD, Koskela HO, Anderson SD, Chan HK. Budesonide reduces sensitivity and reactivity to inhaled mannitol in asthmatic subjects. Respirology. 2002;7(1):37–44.
98. Koskela HO, Hyvärinen L, Brannan JD, Chan H-K, Anderson SD. Responsiveness to three bronchial provocation tests in patients with asthma. Chest. 2003;124(6):2171–7.
99. Brannan JD, Koskela H, Anderson SD, Chew N. Responsiveness to mannitol in asthmatic subjects with exercise- and hyperventilation-induced asthma. Am J Respir Crit Care Med. 1998;158(4):1120–6.
100. Currie GP, Haggart K, Brannan JD, Lee DKC, Anderson SD, Lipworth BJ. Relationship between airway hyperresponsiveness to mannitol and adenosine monophosphate. Allergy. 2003;58(8):762–6.

101. Holzer K, Anderson SD, Chan H-K, Douglass J. Mannitol as a challenge test to identify exercise-induced bronchoconstriction in elite athletes. Am J Respir Crit Care Med. 2003;167(4):534–7.
102. Canning BJ, Chang AB, Bolser DC, Smith JA, Mazzone SB, McGarvey L. Anatomy and neurophysiology of cough. CHEST guideline and expert panel report. Chest. 2014;146(6):1633–48.
103. Koskela HO, Hyvärinen L, Brannan JD, Chan HK, Anderson SD. Coughing during mannitol challenge is associated with asthma. Chest. 2004;125(6):1985–92.
104. Spicuzza L, Di Maria G, Polosa R. Adenosine in the airways: implications and applications. Eur J Pharmacol. 2006;533(1–3):77–88.
105. Van den Berge M, Kerstjens HAM, De Reus DM, Koëter GH, Kauffman HF, Postma DS. Provocation with adenosine 5'-monophosphate, but not methacholine, induces sputum eosinophilia. Clin Exp Allergy. 2004;34(1):71–6.
106. Van den Berge M, Polosa R, Kerstjens HAM, Postma DS. The role of endogenous and exogenous AMP in asthma and chronic obstructive pulmonary disease. J Allergy Clin Immunol. 2004;114(4):737–46.
107. Brown RA, Clarke GW, Ledbetter CL, Hurle MJ, Denyer JC, Simcock DE, Coote JE, Savage TJ, Murdoch RD, Page CP, Spina D, O'Connor BJ. Elevated expression of adenosine A1 receptor in bronchial biopsy specimens from asthmatic subjects. Eur Respir J. 2008; 31(2):311–9.
108. Lexmond AJ, Hagedoorn P, Van der Wiel E, Ten Hacken NHT, Frijlink HW, De Boer AH. Adenosine dry powder inhalation for bronchial challenge testing, part 1: Inhaler and formulation development and in vitro performance testing. Eur J Pharm Biopharm. 2014;86(1):105–14.

109. Lexmond AJ, Van der Wiel E, Hagedoorn P, Bult W, Frijlink HW, Ten Hacken NHT, De Boer AH. Adenosine dry powder inhalation for bronchial challenge testing, part 2: Proof of concept in asthmatic subjects. Eur J Pharm Biopharm. 2014; 88:148–52.
110. Lexmond AJ, Boudewijn IM, Hagedoorn P, Schokker S, Cox CA, Vonk JM, Ten Hacken NHT, Frijlink HW, Vroegop S, Van den Berge M. Bronchial provocation testing can be improved by using dry powder adenosine instead of nebulized AMP. Am J Respir Crit Care Med. 2017;https://doi.org/10.1164/rccm.201704-0715LE.
111. Münster AB, Bendstrup E, Jensen JI, Gram J. Jet and ultrasonic nebulization of single chain urokinase plasminogen activator (scu-PA). J Aerosol Med. 2000;13(4):325–33.
112. Khatri L, Taylor KM, Craig DQ, Palin K. An assessment of jet and ultrasonic nebulisers for the delivery of lactate dehydrogenase solutions. Int J Pharm. 2001;227(1–2):121–31.
113. Michel O, Dentener M, Cataldo D, Cantinieaux B, Vertongen F, Delvaux C, Murdoch RD. Evaluation of oral corticosteroids and phosphodiesterase-4 inhibitor on the acute inflammation induced by inhaled lipopolysaccharide in human. Pulm Pharmacol Ther. 2007;20(6):676–83.
114. Michel O, Duchateau J, Sergysels R. Effect of inhaled endotoxin on bronchial reactivity in asthmatic and normal subjects. J Appl Physiol. 1989;66(3):1059–64.
115. Nightingale JA, Rogers DF, Hart LA, Kharitonov SA, Chung KF, Barnes PJ. Effect of inhaled endotoxin on induced sputum in normal, atopic, and atopic asthmatic subjects. Thorax. 1998;53(7):563–71.
116. Aul R, Armstrong J, Duvoix A, Lomas D, Hayes B, Miller BE, Jagger C, Singh D. Inhaled LPS challenges in smokers: A study of pulmonary and systemic effects. Br J Clin Pharmacol. 2012;74(6):1023–32.

117. De Pee S, Timmers MC, Hermans J, Duiverman EJ, Sterk PJ. Comparison of maximal airway narrowing to methacholine between children and adults. Eur Respir J. 1991: 4(4):421–8.