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ERS TASK FORCE

Indirect airway challenges

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ABSTRACT: Indirect challenges act by causing the release of endogenous mediators that cause the airway smooth muscle to contract. This is in contrast to the direct challenges where agonists such as methacholine or histamine cause airflow limitation predominantly *via* a direct effect on airway smooth muscle.

Direct airway challenges have been used widely and are well standardised. They are highly sensitive, but not specific to asthma and can be used to exclude current asthma in a clinic population. Indirect bronchial stimuli, in particular exercise, hyperventilation, hypertonic aerosols, as well as adenosine, may reflect more directly the ongoing airway inflammation and are therefore more specific to identify active asthma. They are increasingly used to evaluate the prevalence of bronchial hyperresponsiveness and to assess specific problems in patients with known asthma, *e.g.* exercise-induced bronchoconstriction, evaluation before scuba diving.

Direct bronchial responsiveness is only slowly and to a modest extent, influenced by repeated administration of inhaled steroids. Indirect challenges may reflect more closely acute changes in airway inflammation and a change in responsiveness to an indirect stimulus may be a clinically relevant marker to assess the clinical course of asthma. Moreover, some of the indirect challenges, *e.g.* hypertonic saline and mannitol, can be combined with the assessment of inflammatory cells by induction of sputum.
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Bronchial hyperresponsiveness is an abnormal increase in airflow limitation following exposure to a nonallergic stimulus [1, 2]. Bronchial hyperresponsiveness is a characteristic feature of both asthma and chronic obstructive pulmonary disease (COPD). Thus, bronchial hyperresponsiveness is frequently used to aid in diagnosis and characterisation of individuals with airway disease. Although bronchial hyperresponsiveness is not specific for asthma, nearly all patients with asthma exhibit increased responsiveness, which is more marked during symptomatic episodes. Bronchial hyperresponsiveness to methacholine is also present in a majority of patients with mild to moderate COPD [3]. Moreover, the severity of bronchial hyperresponsiveness predicts the response to inhaled corticosteroids in patients with asthma [4] and the progression of airflow limitation in patients with COPD [5].

Most investigators assess bronchial responsiveness using methacholine or histamine as a provocative stimulus. Methacholine and histamine cause airflow limitation predominantly *via* a direct effect on airway smooth muscle. By contrast, indirect challenges induce airflow limitation by acting on cells other than smooth muscle cells *e.g.* inflammatory cells, epithelial cells and nerves, which upon stimulation release mediators or neurotransmitters that provoke smooth muscle contraction. Nearly all the published studies on asthma and COPD have utilised histamine and methacholine provocation tests for clinical characterisation of patients. Furthermore, hyperresponsiveness testing is widely used in clinical research settings to evaluate potential new therapies. For example, direct challenges with histamine or methacholine are used to establish a dose response and time course of the acute bronchoprotective effects of β-agonists. These challenges have also been used to assess the potential anti-inflammatory effects of prolonged treatment with new agents. There are limitations to this model. Inhaled corticosteroids, the current gold standard anti-inflammatory treatment for asthma, reduces bronchial responsiveness to histamine or methacholine only to a small degree, an effect that is both dose and time dependent. In recent years an increasing number of studies have investigated the relative usefulness of indirect airway challenges in monitoring anti-inflammatory treatment in asthma, but almost none in COPD.

In 1998 the European Respiratory Society (ERS) approved a Task Force on Indirect Airway Challenges. The objectives of this Task Force were to develop recommendations concerning the role of indirect airway challenges in the assessment and monitoring of airway diseases. The recommendations in this report are based on a review of the published literature and were developed during workshops held at the American Thoracic Society in San Diego (April 1999), ERS Congress in Madrid (October 1999), ERS Meeting in Ghent (June 2000) and the ERS Congress in Florence (August 2000). The following topics were included. 1) Mechanisms and receptors involved in the airway narrowing caused by indirect airway challenges. 2) Diagnostic value of indirect challenges. 3) Value of indirect challenges in the monitoring of asthma, including the use of these challenges as an outcome measure in clinical trials. 4) Value of indirect challenges in epidemiological studies. 5) The importance of standardisation of challenge methods. 6) Areas for further research.

Definition and main properties of an indirect challenge

The concept of indirect challenges was developed at the end of the eighties [6]. Several publications had confirmed that many different nonspecific stimuli induced airway narrowing in patients with asthma. Thus a distinction had to be made between direct and indirect stimuli. Methacholine and histamine are direct stimuli because they cause airflow limitation by acting on effector cells, predominantly on airway smooth muscle but also on mucus glands and on airway microvasculature without involving intermediate pathways. By contrast indirect stimuli, *i.e.* physical stimuli such as exercise, osmotic challenge or pharmacological stimuli such as adenosine, cause airflow limitation by acting on cells, most notably inflammatory cells and neuronal cells which release mediators or cytokines to cause secondary bronchoconstriction.

The fact that the pattern of airway narrowing induced by indirect stimuli differs from that provoked by direct stimuli is shown by the following clear evidence. 1) Bronchial responsiveness to direct and indirect challenges are rather poorly correlated with each other [6]. 2) A wide array of mediators including histamine, leukotrienes, prostaglandins, acetylcholine, neuropeptides are involved in the airway narrowing induced by the indirect stimuli [7]. 3) The airway narrowing caused by an indirect, but not a direct challenge, can be prevented by acute pretreatment with a cromone (cromoglycate, nedocromil), inhaled frusemide and/or heparin [7]. 4) After the administration of an indirect challenge tachyphylaxis to a second stimulus, with the same or another indirect acting agent (cross refractoriness) is frequently observed [7]. The tachyphylaxis observed with the indirect challenges, is far more pronounced than the small changes seen when histamine or methacholine is repeatedly inhaled [8–10]. 4) In patients with asthma, bronchial responsiveness to an indirect airway challenge is more closely associated with airway inflammation than bronchial responsiveness to a direct stimulus [11]. Bronchial responsiveness to an indirect stimulus may also better reflect acute changes in airway inflammation induced by allergen avoidance [12] or by treatment with inhaled steroids [13, 14].

The authors propose the following practical, working definition of an indirect challenge: "Indirect challenges act by causing the release of endogenous mediators that cause the airway smooth muscle to contract, with or without effect in inducing microvascular leakage. Because the responses to these challenges are modified or even completely inhibited by inhaled steroids, the airway response to these challenges may be a closer reflection of active airway inflammation".

Table 1. – Overview of direct and indirect stimuli

Indirect stimuli	Direct stimuli
Physical stimuli	Cholinergic agonists (acetylcholine, methacholine, carbachol)
Exercise	
Nonisotonic aerosols (hyper-, hypotonic, distilled water aerosols, mannitol)	Histamine
Eucapnic voluntary hyperpnoea of dry air	Prostaglandin D_2
	Leukotriene $C_4/D_4/E_4$
Pharmacological stimuli	
Adenosine	
Tachykinins	
Bradykinin	
Metabisulphite/SO_2	
Propranolol	
Endotoxin (LPS)	
Platelet activating factor	
Ozone	
Selective agents	
Aspirin and NSAID	
Allergen	

LPS: lipopolysacharides; NSAID: nonsteroidal anti-inflammatory drugs; SO_2: sulphur dioxide.

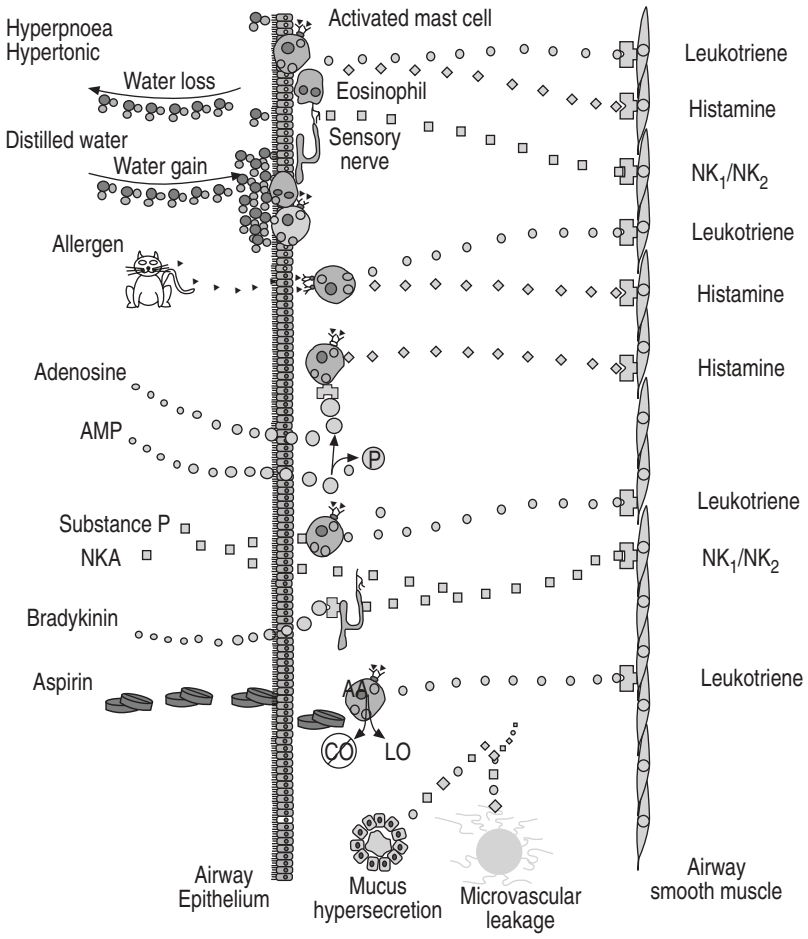

Fig. 1. – The contribution of different intermediate pathways in airway-narrowing induced by various indirect stimuli. NK: neurokinin receptor; AMP: adenosine 5′-monophosphate; P: phosphate group; AA: arachidonic acid; CO: cyclooxygenase; LO: 5-lipoxygenase. For details on the different pathways see the Mechanisms and receptors involved in indirect challenges section of this report and the report by VAN SCHOOR *et al* [7].

Mechanisms and receptors involved in indirect challenges

An overview of the different indirect and direct airway stimuli is given in table 1. In figure 1 the contribution of the different intermediate pathways involved in indirect bronchoconstriction are outlined.

Mechanisms involved in the airway narrowing to physical stimuli: evidence from studies on exercise-induced bronchoconstriction

Exercise causes airway narrowing by the loss of water *via* evaporation from the airway surface. The mechanism, whereby the loss of water causes the airways to narrow, is thought to relate to the thermal (cooling and rewarming) [15] and osmotic (increase in airway osmolarity) effects of dehydration [16]. The dehydration results in cell shrinkage and leads to a complex sequence of biochemical events, as part of the homeostatic response, producing a restorative increase in the cell volume. For cells such as the epithelial cell, the mast cell and the sensory nerve cell these biochemical events are likely to stimulate the release of mediators [16]. *In-vitro studies* of human lung mast cells show that increasing the osmolarity of the solution bathing the cells is a potent stimulus to release of histamine [17]. The major clinical evidence to support a role for histamine release is the finding that some histamine H1 receptor antagonists have an inhibitory effect on exercise-induced bronchoconstriction (EIB) [18–20]. Because the inhibitory effect is incomplete, histamine cannot be the only mediator involved in EIB.

There are other mast cell mediators that are likely to be involved in EIB, most notably prostaglandin D2 (PGD_2) and the cysteinyl leukotrienes. Recent studies have demonstrated that the PGD_2 metabolite 9-α 11-β prostaglandin F2 is significantly increased in the urine 30, 60 and 90 min postexercise [21, 22]. This finding is also supported by the observation that flurbiprofen, a cyclooxygenase inhibitor, also has a partial inhibitory effect on EIB [19].

Leukotrienes are involved in the genesis of EIB and in sustaining the bronchoconstriction following exercise. Repeated studies have reported increases in urinary leukotriene E_4 following EIB [23, 24]. Some investigators have also reported a significant increase in leukotrienes in bronchoalveolar lavage following dry air hyperpnoea [25]. Also, there are now many studies demonstrating that both 5-lipoxygenase inhibitors [26–28] and leukotriene receptor antagonists [24, 29] inhibit EIB and enhance recovery of lung function to pre-exercise values. The inhibition is incomplete confirming that more than one mediator is involved.

The epithelial cell is a rich source of mediators. One such mediator is prostaglandin E2 (PGE_2) which may act to protect the airways from narrowing [30]. The release of PGE_2 may in part be dependent on stimulation by leukotrienes [8]. Thus, PGE_2 may play an important role in the refractoriness that follows exercise. In a recently reported study [31], human epithelial cells in culture, when stimulated with hypertonic solutions rapidly produced interleukin (IL)-8. IL-8 promotes

Table 2. – Mediators and neurotransmitters involved in indirect bronchial responsiveness

	Mediator Release	Neuronal Stimulation	References
Adenosine	+(Hi, LT, PG)	+(ACh, TK?)	[27, 39–49]
Tachykinins	+(Hi, LT, PG)	+(ACh)	[50–56]
Bradykinin	+(Hi, PG, NO)	+(ACh, TK?)	[57–63]
Propranolol	±(Hi)	+(ACh)	[64–68]
Metabisulphite/SO_2	+(Hi, LT, PG)	+(ACh, TK?)	[47, 69–75]
Exercise	+(Hi, LT, PG)	+(ACh,TK)	[19, 21, 22, 24, 27–30, 34, 76, 77–82]
Nonisotonic aerosols	+(Hi, LT, PG)	+(ACh, TK?)	[17, 83–88]
EVH of dry air	+(Hi, LT)	+(ACh, TK?)	[26, 30, 89–94]
PAF	+(LT?)	±	[95]
Aspirin	+(PG, LT)	?	[96]
Allergen	+(Hi, PG, LT, TK?)	±	[97–103]

Hi: histamine; LT: leukotriene C_4, D_4, E_4; PG: prostaglandins; ACh: acetylcholine; TK: tachykinins; NO: nitric oxide; EVH: eucapnic voluntary hyperpnoea; PAF: platelet activating factor; ?: not known for human airways. This table was modified from [7].

neutrophil chemotactic activity, which has been reported to be increased during EIB [32].

Airway sensory nerves may also be affected by alterations in osmolarity and cell volume. There is abundant evidence from animal studies that an increase in osmolarity stimulates sensory nerves. In addition, exercise-induced respiratory water loss can cause coughing in humans, an effect that is blocked by inspiring humid air [33]. There is some evidence to support the role of tachykinins in EIB; the selective tachykinin neurokinin-receptor type-1 (NK_1) antagonist FK888 hastened the recovery in lung function to baseline after exercise [34].

EIB is significantly inhibited or even completely blocked by single doses of nedocromil sodium, sodium cromoglycate [35], frusemide [36] and by repeated dosing with inhaled steroids [37]. These drugs have no direct effect on airway smooth muscle but reduce the functional activity of mast cells, epithelial cells and sensory nerves, implying a significant role for these cells in EIB.

The other physical stimuli, nonisotonic aerosols and eucapnic voluntary hyperpnoea of dry air, work through similar mechanisms (table 2).

Mechanisms involved in the airway narrowing caused by pharmacological stimuli: evidence on adenosine-, tachykinin-, and bradykinin-induced bronchoconstriction

Several cells and mediators are involved in the airway narrowing due to indirect stimuli, these include epithelial cells, inflammatory cells (incorporating mast cells), nerve cells and blood vessels. A summary is given in figure 1 and table 2 and more details can be found in a recent review on this subject by VAN SCHOOR *et al.* [7]. The effect exerted by an indirect acting pharmacological agent on the airways differs from stimulus to stimulus, depending on the targets and receptors involved and by the presence of degrading enzymes [7].

Adenosine. Adenosine exerts its effects on human cells through interaction with specific adenosine (P1) receptors, of which four subtypes (A_1, A_{2A}, A_{2B}, and A_3) have been described [104]. The A_1, A_{2B}, and A_3 receptors have been shown to be involved in various animal and human models of inflammation. In particular, the potential role of A_{2B} receptors is being increasingly recognised [105]. The future development of specific and potent adenosine-receptor agonists and antagonists for use *in vivo* in asthma will clarify the relative importance of these receptors [106].

Tachykinins. The airway effects of the tachykinins are mediated *via* tachykinin NK_1 and NK_2 receptors; there is no evidence for the presence of tachykinin NK_3 receptors in human airways. Substance P has the greatest affinity for the NK_1 receptor, whereas neurokinin A has the greatest affinity for the NK_2 receptor, although there is cross-reactivity [107]. *In vitro*, tachykinins constrict the smooth muscle of human airways, mainly through tachykinin NK_2 receptors [108–110]; in small and medium sized bronchi, tachykinin NK_1 receptors are also involved [50, 111]. *In vivo*, inhaled neurokinin A causes bronchoconstriction mainly by indirect mechanisms [112]. Both tachykinin NK_1 and NK_2 receptors are involved in the bronchoconstrictor effect of neurokinin A [113, 114]. Following their release from sensory cells and immune cells, the tachykinins are degraded by at least two enzymes: these are neutral endopeptidase (NEP; EC 3.4.24.11) [115, 116] and angiotensin converting enzyme (ACE; EC 3.4.15.1). NEP is widely distributed on a variety of airway cells, and in the airway epithelium. NEP appears to be the most important enzyme for the breakdown of tachykinins in tissues. ACE, on the other hand, is localised predominantly around the vascular endothelium and therefore degrades intravascular peptides [117].

Bradykinin. Bradykinin causes contraction of the airways by stimulation of B2 receptors [57, 58, 118]. Bradykinin is metabolised by several peptidases, the most important of which are carboxypeptidase N (kininase I), ACE and NEP [119]. Pretreatment with inhaled N^G-monomethyl-L-arginine, a nitric oxide (NO) synthase inhibitor, significantly potentiated airflow limitation in response to inhaled bradykinin in asthmatics; this suggests that bradykinin activates the NO synthase pathway, leading to the release of NO, which in turn counteracts the bronchoconstrictor response to bradykinin [120]. The involvement of histamine and prostaglandins in bradykinin-induced airflow limitation appears to be limited [59, 60]. The bronchoconstrictor effect of bradykinin is, at least in part, mediated *via* cholinergic vagal nerves, since pretreatment with ipratropium bromide significantly reduced airflow limitation in asthmatics [60]. Although bradykinin has been shown to release tachykinins in guinea-pig airways [121–123], conclusive evidence for an involvement of tachykinins in bradykinin-induced bronchoconstriction in man is lacking [51, 52, 61].

Diagnostic value of the indirect challenges: a comparison with direct challenges

Diagnostic value of the direct challenges, histamine and methacholine

Physicians need objective measurements such as a bronchial provocation test, to diagnose asthma [124]. For historical

reasons, bronchial responsiveness has been most commonly assessed using the direct stimuli histamine and methacholine [125]. Widely used methods include the 2-min tidal breathing method [126, 127], the counted-breath dosimeter method [128] which produce comparable results with appropriate calibration [129] and the portable counted breath technique [130]. The results are usually expressed as the provocation concentration (or dose) producing a 20% fall in forced expiratory volume in one second (PC20; PD20; FEV1). Histamine and methacholine are approximately equivalent on a μg [131], or μmol [132] basis. Bronchial responsiveness to histamine and methacholine (PC20, PD20) is unimodally log-normally distributed within the population; this continuous distribution plus the 95% confidence interval (CI) of repeatability in the range of ±1–1.6 doubling concentrations [133] leads to a significant grey area when trying to define a "normal" response.

Inhalation tests have been arbitrarily defined so that the majority of current asthmatics are identified generally by a cut-off point that is at the higher end of the borderline range. Bronchial hyperresponsiveness is considered to be present when the histamine or methacholine PC20 is <8–16 $mg \cdot mL^{-1}$ [127] or the PD20 is <3.9–7.8 μmol [130]. These arbitrary definitions make the test highly sensitive for the detection of hyperresponsiveness in a pulmonary function laboratory or hospital clinic population. This has been confirmed by a number of studies documenting sensitivity and closely related negative predictive values of histamine and methacholine challenges, approaching 100% for clinically current asthma (symptoms within previous few days) as opposed to epidemiologically current asthma (symptoms within the past year) [134–137]. By contrast, the specificity and positive predictive value of these challenges for asthma symptoms perform less well in the field. For example, the positive predictive value of histamine PC20 <8 $mg \cdot mL^{-1}$ for current symptoms of asthma in a random sample from the general population was shown to be well below 50% [137]. When the cut-off point is reduced, the specificity and positive predictive value can approach 100% (for example, PC20 <1 $mg \cdot mL^{-1}$ [127]) but the sensitivity and negative predictive value perform poorly [137]. Thus, methacholine and histamine at a cut-off point of PC20 of 8–16 $mg \cdot mL^{-1}$ are highly sensitive tests and are best used to exclude current active disease as opposed to the application of the highly specific cut-off point of PC20 of 1 $mg \cdot mL^{-1}$, which permits these tests to confirm disease.

Patients with nonasthmatic fixed airflow limitation (chronic airflow limitation, COPD) also demonstrate bronchial hyperresponsiveness to histamine and methacholine [138–141]. The characteristics are somewhat different in that there is a strong linear relationship between bronchial hyperresponsiveness and the obstructive reduction in FEV1 in subjects with chronic airflow limitation. Subjects with COPD also are less hyperresponsive than asthmatics. However, bronchoprovocation with direct stimuli lack specificity to be able to detect asthma in the presence of resting airflow obstruction. Thus, bronchoprovocation with the directly acting stimuli, histamine and methacholine is extremely sensitive for current asthma symptoms, but lack specificity both in differentiating asthma from normal and asthma from chronic airflow limitation.

Diagnostic value of indirect challenges

Physical stimuli. Exercise challenge. Many comparisons of exercise challenges (EIB) with histamine and methacholine challenges have produced somewhat variable results. There is a weak, if statistically significant, correlation between EIB and log histamine, or methacholine PC20 [142, 143]. Exercise challenge, to a preset threshold, is consistently less sensitive but more specific than the direct challenges in differentiating asthma from normal [142–149]. There are many asthmatics with mild bronchial hyperresponsiveness to direct stimuli who have negative exercise challenges but there are individuals who have positive exercise challenges and negative histamine or methacholine challenges [150]. The imperfect relationship between EIB and PC20 and the existence of a number of EIB-positive, methacholine-negative individuals are indicative of the difference in mechanisms involved.

The fewer studies in nonasthmatic lung disease are due in part to the difficulty such individuals have in performing exercise challenges. In children, an exercise challenge is better than methacholine at distinguishing asthma from chronic airway disorders such as cystic fibrosis, bronchiolitis obliterans, pulmonary ciliary dyskinesia and bronchiectasis [151, 152]. Additional studies showing that allergen avoidance resulted in a greater improvement in EIB than in methacholine PC20 [153] and that EIB correlates better with markers of inflammation than methacholine PC20 [154], would support the possibility that EIB may be more clinically relevant than methacholine PC20.

The investigations described in the previous paragraphs confirm that a positive exercise challenge is highly specific to identify clinical asthma, but generally is somewhat insensitive to the presence of clinically relevant mild bronchial hyperresponsiveness. In this regard, the sensitivity-specificity profile of exercise challenge, resembles that of a histamine or methacholine PC20 of 1 or 2 $mg \cdot mL^{-1}$ [137]. There are two possible explanations. First, as the physical stimulus affects many cells that are abnormal in asthma, it may more readily identify patients with this disease than with other airway inflammatory diseases and therefore has a high specificity. Secondly, there is a limit to the extent of stimulus that can be achieved, due to the technical and safety constraints of exercise, this prevents maximal airway provocation, resulting in low sensitivity.

The indications for exercise testing have been summarised in statements from the ERS [1] and the American Thoracic Society (ATS) [155]. Exercise may be used in the following ways. 1) In making a diagnosis of EIB in asthmatic patients with a history of breathlessness during or after exertion. 2) To evaluate the ability of performing demanding or lifesaving work (*e.g.* military, police, or firefighting work) in persons with a history suggesting asthma. 3) To determine the effectiveness and optimal dosing of medications prescribed to prevent EIB. 4) To evaluate the effects of anti-inflammatory therapy given acutely (*e.g.* cromones) or chronically (*e.g.* steroids and leukotriene antagonists).

The recommendations for conducting an exercise test to identify those with exercise-induced bronchoconstriction have been described in detail in both the ERS [1] and the ATS [155] guidelines. The recommendations are similar in both documents. In brief the subjects should exercise for 6 (children, 12 yrs) to 8 (adults) min breathing dry air (<25 °C and <50% relative humidity or <10 mg $H_2O \cdot L^{-1}$) at an intensity to raise the minute ventilation 14 times above the FEV1 and preferably to 21 times the FEV1 (60% maximum voluntary ventilation) for the last 4 min of exercise. In the absence of a measure of ventilation the heart rate should achieve 90% predicted maximum in the last 4 min of exercise. Values for FEV1 are measured before and after exercise. Providing the air is dry and the intensity of exercise appropriate it is only necessary to increase the time of exercise, to increase the severity of the airway response. A reduction in FEV1 of 10% of the pre-exercise value is widely accepted as outside the response observed in healthy individuals without asthma.

Eucapnic voluntary hyperpnoea with dry air. Although there are fewer studies available, the results are consistent

with the findings for exercise challenge. Eucapnic hyperpnoea with dry air is more specific and less sensitive than histamine or methacholine challenges [147, 156–158]. Dry air challenge is clearly more able to separate asthmatics from subjects with chronic airflow limitation than is histamine challenge [139, 140]. Eucapnic voluntary hyperpnoea (EVH) of dry air containing 5% carbon dioxide (CO_2) for 6 min at a ventilation equivalent to 30 times the FEV1 mimics the effects of exercise as described above and has the same clinical significance [156]. As with exercise a 10% reduction in FEV1 is outside the range for healthy subjects without asthma [156]. EVH was recommended to assess winter athletes competing in the Olympic Games in Salt Lake City, as higher levels of ventilation could be more easily achieved during EVH compared with exercise ergometers. Further with EVH it is possible to simulate the conditions of exercise (ventilation, duration, inspired air temperature *etc.*) in a laboratory setting [156]. In contrast to exercise, dose/response curves can be constructed.

Hypertonic aerosols. Bronchial responsiveness to hypertonic saline challenge correlates better with serum markers of inflammation than bronchial responsiveness to methacholine [159]. It improves more than bronchial responsiveness to histamine after a course of inhaled corticosteroids. A challenge with hypertonic saline is easy to perform and allows construction of a dose/response curve [160].

A recently developed highly portable test using mannitol capsules and a dry-powder inhaler has shown promise as an indirect challenge with good correlation with the other indirect physical challenges, exercise, hypertonic saline and hyperventilation [161, 162]. In one study, there was a reasonable correlation between mannitol PD15 and methacholine PC20 [161]. There appears to be no published data on comparative sensitivity and specificity. However, since some subjects with positive mannitol tests had mild bronchial responsiveness to methacholine, the mannitol inhalation test may be more sensitive than other indirect challenges for detecting mild bronchial responsiveness. In a study by BRANNAN *et al.* [162], 22 of the 23 subjects with exercise asthma were identified with mannitol and the only subject who did not respond had a 10% fall in FEV1 to exercise.

The major indications for using hypertonic aerosols are to identify bronchial hyperresponsiveness consistent with active asthma or exercise-induced asthma and to evaluate bronchial responsiveness that will respond to treatment with anti-inflammatory drugs. In a study by RIEDLER *et al.* [163], children with a history of current wheeze were seven times more likely to have a positive response to hypertonic saline than asymptomatic children. In an occupational study in people responding positively to the question "have you ever had an attack of asthma" the mean percentage fall in FEV1 was 17.6% compared with 5.8% for those who responded negatively [164]. From the evidence to date, it would appear that bronchial responsiveness to a hypertonic aerosol is consistent with an asthma diagnosis.

A test using a hypertonic aerosol is an alternative to exercise or hyperventilation to identify patients with EIB [76, 162, 165, 166]. Although some patients can have EIB and be negative to hypertonic saline or mannitol, this is unusual and has only been found in persons with very mild EIB [162, 163].

A challenge with a hypertonic aerosol can be used in the assessment of a patient with a past history of asthma that wishes to scubadive. In a study using 4.5% saline to assess potential divers with a past history of asthma (usually >5 yrs), 17% were found to have an abnormal response, consistent with a diagnosis of current asthma [167].

Another indication for the use of hypertonic aerosols may be in the identification of persons with other airway diseases, *e.g.* chronic airflow limitation or cystic fibrosis, who have an asthmatic component to their disease. Many patients with cystic fibrosis are considered to have asthma. As some of the inhaled medication used in the treatment of cystic fibrosis is hyperosmolar, it would also seem important to identify those in whom airway narrowing may occur in response to treatment of their primary disease [168]. Both hypertonic saline and mannitol increase mucociliary clearance in subjects with asthma, bronchiectasis and cystic fibrosis [169–171]. When given daily, hypertonic saline has been shown to improve lung function in patients with cystic fibrosis [172]. Thus a recommendation for use of a hypertonic aerosol as a therapeutic agent may need to be preceded by an inhalational challenge, with the same hypertonic aerosol [173].

A challenge with a hypertonic aerosol may also be indicated in persons with cough-variant asthma. Hypertonic aerosols can provoke cough [174, 175], so documenting excessive cough in the absence of airway narrowing may indicate that the cough is not due to asthma. Further, the cough normally provoked by inhaling hypertonic saline stops very quickly within 1–2 min suggesting a form of refractoriness to cough in healthy subjects.

Finally, a challenge with a hypertonic aerosol may be indicated in pregnancy when a patient chooses not to be challenged with a pharmacological agent.

The inhalation of hypertonic saline has been widely used to induce sputum and to collect inflammatory cells and cytokines in asthmatics [176–179]. What is unique to hypertonic challenge is that it can be used to document bronchial responsiveness at the same time as collecting sputum [179, 180]. This makes hypertonic challenge attractive for assessing both acute and chronic treatment with corticosteroids.

Distilled water. ALLEGRA and BIANCO [181] performed the first inhalation challenge with ultrasonically nebulised distilled water (UNDW) in asthmatic patients. The technique was later modified and standardised by other investigators [182, 183]. Inhalation of UNDW evokes only a cough in some normal subjects and a cough and bronchoconstriction in asthmatic patients [184]. Bronchial response to UNDW is normally distributed. Most asthmatic patients develop bronchoconstriction after inhaling <2 mL of UNDW [185]. A positive response to UNDW is more likely when PD20 methacholine is <2 μmol [185, 186]. Bronchial response to UNDW correlates poorly with methacholine responsiveness [187]. The degree of bronchial responsiveness to UNDW is in good concordance with the response to exercise and to eucapnic hyperpnoea [165]. A refractory period is evident after UNDW in ~50% of patients [185, 188]. Refractoriness of bronchial airways to UNDW is decreased by histamine-induced bronchoconstriction [189], the UNDW-induced refractoriness cross reacts with exercise-induced refractoriness [190].

Pharmacological stimuli. Adenosine. CUSHLEY *et al.* [191] reported the first observation that inhaled adenosine, but not related nucleotides, caused bronchoconstriction in patients with asthma. Subsequently, PHILLIPS *et al.* [38] have shown that atopic subjects, when compared to non-atopic controls, are relatively more responsive to inhaled adenosine and adenosine 5′-monophosphate (AMP), than they are to methacholine. The airway response to these purines may be an index of mast-cell priming, probably through A_{2B} receptor stimulation, linked to mobilisation of intracellular calcium stores. Indeed, nasal challenge with AMP elicits rhinitic symptoms and an immediate rise in histamine levels in the lavage fluid with the greatest increase occurring in atopic compared to nonatopic volunteers [192].

This indicates that atopy and other conditions, where mast cells are primed for mediator release, are important determinants of enhanced adenosine-induced histamine release and that this response may be used as an index of mast cell priming *in vivo*. The capacity of adenosine to augment mediator release from mast cells *in vivo* indicates that adenosine-induced bronchoconstriction in asthmatics may depend on the state of airway mast-cell priming and might be useful as an *in-vivo* test for this.

There are limited data available for comparison of sensitivity and specificity of AMP challenge with the direct-acting stimuli. It requires ~30 times as much AMP as methacholine to induce bronchoconstriction. AMP and exercise challenges are better than methacholine challenges for separating paediatric asthma from paediatric "chronic obstructive lung disease" *i.e.* AMP and exercise challenges tended to be negative in the children with cystic fibrosis, bronchiolitis obliterans, ciliary dyskinesia and bronchiectasis [151, 152]. Nonsmoking adults with COPD are significantly less responsive to inhaled adenosine than nonsmoking asthmatics, whereas the sensitivity to methacholine is similar in both groups [193]. Taken together, these findings indicate that adenosine challenge may be a useful tool in the differential diagnosis of asthma and COPD in patients of all ages in whom the diagnosis is clinically uncertain. This is especially the case in nonsmokers, since smokers with COPD may show AMP responsiveness as well [193]. In addition, the specificity of adenosine bronchoprovocation for asthma together with the high repeatability of this test could be useful for epidemiological studies.

Propranolol. On a molar basis, the dose of propranolol required to induce bronchoconstriction in patients with asthma is ~10–15 times larger than methacholine or histamine [194]. The limited data supports higher specificity and lower sensitivity for propranolol compared to histamine or methacholine. Propranolol inhalation tests were negative in the majority of subjects with chronic airflow limitation, supporting better specificity of propranolol challenge for asthma [195]. Bronchoconstriction induced by propranol is usually less well tolerated by patients compared to that caused by histamine, methacholine or adenosine. Nevertheless no serious events have ever been reported following propranolol-induced bronchoconstriction either in asthmatics or in patients with COPD. In addition, propranolol-induced bronchoconstriction can be weakly reversed by inhaled adrenergic and anticholinergic drugs.

Metabisulphite, sulphur dioxide. In epidemiological studies, airway responsiveness to the indirect stimulus sulphur dioxide (SO_2) and the direct stimulus methacholine were compared in a sample of 790 adults aged 20–44 yrs. In this cohort the prevalence of hyperresponsiveness to SO_2 was 3.4%. Among the subjects who had hyperresponsiveness to methacholine, 22.4% had hyperresponsiveness to SO_2. There was no significant correlation between the degrees of hyperresponsiveness to methacholine and SO_2 [196].

Aspirin. While there is no *in-vitro* test available for the detection of intolerance to aspirin and cross-reacting non-steroidal anti-inflammatory drugs (NSAIDs) in patients with asthma, oral provocations with incremental doses of aspirin have been used to diagnose this syndrome [197]. However, the challenge procedure is fairly time consuming, potentially dangerous and should only be performed in a laboratory with considerable experience of aspirin elicited reactions. More recently, the lysine-aspirin inhalation challenge introduced by BIANCO *et al.* [198] has proven very useful in identifying aspirin-intolerant asthmatic subjects [199–201]. In a prospective comparative study, the lysine-aspirin challenge was found to be as sensitive as oral provocation, with respect to production of airway obstruction. In a study on 22 consecutive patients with a history and/or clinical findings suggestive of aspirin-intolerance (asthma, rhinorrhea, nasal polyposis) challenges by both routes were performed at least two weeks apart. A total of 10 subjects developed significant bronchoconstriction ($\geqslant$20% drop in FEV1) during either challenge, with the same absolute sensitivity for both tests (9/10). Inhalation challenge provoked responses that developed more promptly (within 20–30 min), were limited to the airways, caused a lesser degree of airway obstruction (mean maximal fall in FEV1 29$\pm$6% *versus* 38$\pm$16% for oral challenge) and were more easily reversed [200]. In 19 aspirin-tolerant control subjects with the same baseline pulmonary function, inhalation of lysine-aspirin caused no significant changes in FEV1, supporting the specificity of the test.

Although oral administration is necessary for the detection and investigation of extrapulmonary reactions, inhalation challenge has the benefit of safety for use in clinical practice. For research purposes, the safety and good repeatability of inhalation challenge provide a considerable advantage over oral challenge, particularly since a significant proportion of aspirin-intolerant asthmatics suffer from moderate-to-severe asthma.

Reports on the repeatability of lysine-aspirin challenge [96, 199] have shown that it is repeatable approximately within a single doubling concentration or dose difference. With the methodology described below, the 95% CI for the difference in results between two challenges separated by 10–75 days was 0.6–1.8-fold. A positive provocation response to inhaled (or oral) aspirin results in a state of refractoriness to further doses of aspirin or other NSAIDs [198]. The refractory period lasts between 2–5 days and desensitisation, as well as cross-desensitisation, may be retained provided aspirin is ingested within a maximum interval of 48 h. Complete sensitivity to aspirin and other NSAIDs, reappears ~7 days after the last exposure to these drugs [202]. Therefore, repeated challenges for diagnosis or research purpose should be separated by at least 1 week. Another pitfall that may produce false-negative aspirin provocation is indicated by observations that high doses of glucocorticosteroids may mask aspirin intolerance [203]. Moreover, it has been documented that treatment with antileukotrienes [96] and salmeterol [204] blunt the lysine-aspirin induced airway response.

The major indication for using lysine-aspirin inhalation challenge is to identify aspirin-sensitive asthmatic patients and to study mechanisms involved in bronchoconstriction elicited by aspirin and other NSAIDs.

Direct *versus* indirect airway challenges to monitor asthma

The monitoring of symptoms, airflow obstruction and exacerbations is essential to asthma management. Regular monitoring by physicians improves health outcomes, provided it includes monitoring of control of asthma, medication and skills at regular intervals [205]. Bronchial responsiveness can be assessed at regular clinic visits and is related to asthma severity and airway inflammation [205]. It has been demonstrated repeatedly that, despite significantly improving symptoms and decreasing airway inflammation, inhaled corticosteroids produce, at best, a modest decrease in bronchial hyperresponsiveness as measured by histamine or methacholine challenges. This observation has been made in adults [206] as well as children with asthma [207]. Despite these limitations, direct airway challenges may be useful in the titration of anti-inflammatory therapy [208]. Indeed SONT *et al.* [208] have reported that a treatment protocol aimed at

improving bronchial hyperresponsiveness to methacholine, as well as symptoms and lung function, led to better asthma control, fewer exacerbations and reduced chronic airway inflammation.

In view of the clinical and physiological relevance of indirect challenges, it is desirable to design studies that compare the improvement in symptoms and markers of airway inflammation induced by anti-asthmatic therapy with their effects on direct and indirect airway challenges. The view that bronchial responsiveness to adenosine is a more robust marker of disease activity, in relation to allergic airway inflammation than other nonspecific stimuli, such as histamine or methacholine, is supported by a number of clinical studies. In subjects with active allergic rhinitis, bronchial responsiveness to AMP, but not methacholine, is strongly correlated to sputum eosinophilia [209]. In a large group of patients with asthma, PC20 AMP was more closely associated with eosinophilic airway inflammation than PC20 methacholine [11]. A series of clinical studies have confirmed the potential utility of AMP in detecting inflammatory changes in adult and paediatric asthma. Regular treatment with inhaled corticosteroids results in a significantly greater reduction in AMP responsiveness compared to that of direct (methacholine and histamine) and neurally acting stimuli (sodium metabisulphite and bradykinin) [210, 211]. In keeping with this, several studies have shown that β-agonists cause greater bronchoprotection against AMP than against histamine or methacholine challenge in patients with asthma [212, 213]. VAN VELZEN *et al.* [12] have shown that improvements in clinical asthma occurred in a group of 16 allergic asthmatic children admitted to a high-altitude clinic. This was believed to be due to the lower allergen levels encountered and was accompanied by a significant reduction in bronchial responsiveness to AMP but, interestingly, not to methacholine. On the basis of these observations, the authors believe that adenosine bronchoprovocation may provide an index that could be used to survey disease progression, monitor therapy and assess prognosis.

Osmotic stimuli, such as hypertonic (4.5%) saline and mannitol, hold promise for monitoring asthma. A challenge with hypertonic saline or mannitol can be used to assess the severity of asthma, the effect of treatment and the compliance with treatment. In a recent study in well-controlled asthmatics, LEUPPI *et al.* [214] demonstrated that failure of successful reduction in steroids could be predicted by responsiveness to mannitol. The use of 4.5% saline, as an indication of severity of asthma and need for steroids, is supported by the findings of RODWELL *et al.* [215]. In their study patients with a PD20 to 4.5% saline of $\geqslant$3.0 mL, *i.e.* those with moderate-to-mild asthma, were most likely to become negative to hypertonic saline during treatment with steroids and to plateau in response to acute administration of nedocromil sodium. BRANNAN *et al.* [216] reported similar findings for mannitol and nedocromil sodium. In the study of ANDERSON *et al.* [217] the increase in PD20 to hypertonic saline in response to 8 weeks of treatment with budesonide was predicted by the increase in PD20, following a single dose of sodium cromoglycate given 10 min before challenge [217]. A negative response to challenge with 4.5% saline suggests that the person either does not have asthma, or that their asthma is currently under control with treatment. For example, a patient taking budesonide daily for 4–8 weeks has a 50% likelihood of becoming negative to challenge with hypertonic saline [160, 215] and to mannitol [218]. These findings are in keeping with 50% of the subjects no longer having EIB after treatment with budesonide [37]. By contrast, it is highly likely that the same people would remain responsive to inhaled histamine or methacholine [160, 206, 219].

A bronchial challenge with hypertonic saline can be combined with an induction of sputum to assess airway inflammation [176, 220]. IN'T VEEN *et al.* [179] compared provocation with methacholine (PC20), hypertonic saline and sputum induction, as outcome parameters in patients with severe asthma during steroid withdrawal [179]. During both induced and spontaneously occurring exacerbations, increased bronchial responsiveness for methacholine was noted. However, only the induced exacerbations were associated with increased bronchial responsiveness to hypertonic saline and increased percentage of sputum eosinophils.

Response to indirect challenges can be an interesting outcome parameter in the evaluation of anti-inflammatory treatment by inhaled steroids or leukotriene receptor antagonists. In a comparative study on the effects of 4-week treatment periods with three different doses of budesonide (100, 200 and 400 $\mu g \cdot day^{-1}$), PEDERSEN and HANSEN [221] found a dose/response effect on lung function and EIB, but not on symptoms or peak expiratory flow rate in the evening. Approximately 53% of the maximum effect against EIB was achieved by the lowest budesonide dose and ~83% by the highest dose. In a study on the effects of two doses of fluticasone propionate (100 and 250 μg *b.i.d.* compared to placebo), the severity of EIB decreased significantly as compared to placebo within 3 weeks [13]. These reductions in EIB did not differ between the two doses and were sustained during the study period of 6 months. In contrast, responsiveness to methacholine improved during the first 6 weeks of the treatment with fluticasone, and steadily increased with time: after 24 weeks of treatment, the difference in improvement of PD20 methacholine was 1.6 dose steps for 100 μg fluticasone *b.i.d.* and 3.3 dose steps for 250 μg *b.i.d.* The new inhaled steroid ciclesonide (50, 200 and 800 $\mu g \cdot day^{-1}$) reduced responsiveness to AMP and eosinophils in induced sputum. In contrast to sputum eosinophilia, the reduction in responsiveness to AMP was dependent on the dose of inhaled steroid [14].

The studies that have compared direct and indirect challenges to monitor asthma during anti-inflammatory therapy with inhaled corticosteroids and leukotriene-receptor antagonists are summarised in table 3. Inhaled corticosteroids led to an attenuation of bronchial responsiveness to the majority of different stimuli, although to different extents, thereby underlining the antiasthmatic efficiency of inhaled corticosteroids. All authors found a significant, although small reduction in histamine or methacholine responsiveness. Results were less consistent for bradykinin responsiveness and inhalation challenges using hyperventilation of air which contained SO_2 [211, 222]. It has even been argued that AMP responsiveness, at least in children, is a more sensitive predictor of the effect of anti-inflammatory therapy than bronchial responsiveness to methacholine or bradykinin [211]. In a study on the effect of a 2-week treatment with oral or inhaled steroids in adult, asthmatic patients, PC20 AMP was found to be more sensitive to changes in acute airway inflammation compared to PC20 methacholine [228]. This would underline the assertion that indirect challenges may be better suited to assess therapeutic efficacy than direct challenges. Following the same line of reasoning, LEFF *et al.* [29] demonstrated that EIB was significantly attenuated by long-term treatment with a leukotriene receptor antagonist, whereas methacholine responsiveness was not significantly reduced. It should be noted however that the relatively modest benefit of inhaled steroids on direct challenges should not *per se* be considered as a disadvantage. This modest benefit may in fact be highly relevant, as parts of bronchial responsiveness to histamine or methacholine may not be sensitive to steroids, or may require very prolonged therapy. The slow response to steroids may actually be more informative on *e.g.* remodelling aspects, which may be more important for the long-term management and prognosis of the disease [208].

Table 3. – Direct and indirect challenge tests to monitor asthma during anti-inflammatory therapy

First author [ref. no.]	Year	Compound	Duration of treatment weeks	Dose	Challenge			
					Direct		Indirect	
					Type	Reactivity	Type	Reactivity
WIEBICKE *et al.* [222]	1990	Salbutamol+ BDP	3	0.2/0.5 mg *q.i.d*	Histamine Methacholine	↓ ↓	SO_2 Hypervent.	Ø Ø
VATHENEN *et al.* [223]	1991	Budesonide	6	800 μg *b.i.d.*	Histamine	↓	Exercise Cold air hypervent.	↓
FULLER *et al.*[224]	1991	Budesonide	3	1200 $\mu g \cdot day^{-1}$	Histamine	↓	Bradykinin	↓
GROOT *et al.* [225]	1992	BDP	8	200 μg *q.i.d*	Histamine	↓	Dist. water	↓
O'CONNOR *et al.* [210]	1992	Budesonide	2	0.8 mg *b.i.d.*	Methacholine	↓	Metabisulphite AMP	↓ ↓ ↓
BOOTSMA *et al.* [226]	1995	Fluticasone BDP	6	750 $\mu g \cdot day^{-1}$ 1500 $\mu g \cdot day^{-1}$	Histamine Histamine	↓ ↓	Dist. water Dist. water	↓ ↓
DOULL *et al.* [211]	1997	BDP	12	400 $\mu g \cdot day^{-1}$	Methacholine	Ø	Bradykinin	Ø
DU TOIT *et al.* [160]	1997	Budesonide	8	1000 $\mu g \cdot day^{-1}$	Histamine	↓	Hypertonic saline	↓ ↓
WEERSINCK *et al.* [227]	1997	Salmeterol Fluticasone Salmeterol+ Fluticasone	6	50 μg *b.i.d.* 250 μg *b.i.d.* 50+250 μg *b.i.d.*	Methacholine	↓ ↓ ↓	Adenosine	↓ ↓ ↓ ↓ ↓
LEFF *et al.* [29]	1998	Montelukast	12	10 $mg \cdot day^{-1}$	Methacholine	Ø	Exercise	↓ ↓

BDP: beclomethasone dipropionate; Dist. water: distilled water; ↓: modest reduction; ↓ ↓: more pronounced reduction; Ø: no change.

Use of indirect airway challenges in epidemiological studies

Questionnaires are most frequently used to diagnose asthma or other respiratory disorders in epidemiological studies. They may, however, be subjective and the level of awareness of the condition in the community may influence the pattern of response. Similar problems may occur with a doctor's diagnosis of asthma. These differences in defining respiratory diseases often cause problems with comparisons of epidemiological studies between different populations and over time. Thus, an objective marker closely associated with diseases like asthma is desirable.

In the past, direct-airway challenges using histamine and methacholine have been considered to be more sensitive for a diagnosis of asthma or asthma symptoms, when compared with indirect tests. However, recent laboratory and epidemiological studies have shown that this concept might be in question. In a laboratory based study of elite summer athletes HOLZER *et al.* [150] found that methacholine PD20 had a sensitivity of only 36% to identify the athletes with positive response to EVH, a surrogate challenge used to identify exercise-induced bronchoconstriction. For those 16 subjects positive to EVH and negative to methacholine the mean±SD percentage fall in FEV1 was 17.8±19.5% after EVH and the top dose of methacholine the fall in FEV1 was 7.6±4.9%. In a field study by HABY *et al.* [229], in which children were studied with histamine and exercise, 45% of those positive to a standardised exercise challenge were negative to inhaled histamine with reduction in FEV1 to the highest cumulative dose of histamine being <10%. A histamine challenge in 2,363 Australian schoolchildren aged 8–11 yrs, yielded a sensitivity of 53% and a specificity of 90% to detect subjects with a diagnosis of asthma [230]. Sensitivity and specificity of the histamine challenge were similar to sensitivity and specificity of a hypertonic saline challenge and an exercise challenge in another epidemiological study in children from the same country [163].

For many participants in field studies, particularly children, indirect challenges, involving more natural stimuli, are more appealing. Parents will often not allow their child to inhale a pharmacological agent in epidemiological surveys. Consequently, there has been increasing interest in the use of indirect airway challenges for epidemiological studies. These tests mainly comprise of the inhalation of nonisotonic solutions such as hypertonic saline or distilled water, hyperventilation of dry air and various sorts of exercise tests. Hypertonic saline challenge is a relatively inexpensive test that is safe, well tolerated and reproducible. It can be performed readily in the field. It produces few complaints of dryness or irritation of the throat. In a study on 500 children, only 1.5 % of participating children felt that they could not continue the challenge because of irritation to the throat or cough. Similarly, 1.6 % of the same subjects were unwilling to complete a free-running exercise test because of fatigue [163].

The hypertonic saline challenge appears to have some practical advantages compared to exercise challenge in a field study. A challenge with hypertonic saline is not dependent on weather conditions (temperature, humidity), nor is it influenced by the level of the child's fitness and it allows for dose increments and measurement of dose response curves, making the challenge safer. The EVH challenge is well standardised [156, 157] but needs a special gas mixture source which makes it less suitable for field studies.

Safety aspects of indirect airway challenges

The safety of standardised histamine and methacholine challenge tests is recognised all over the world. Previous guidelines on provocation challenges have stressed the precautions that need to be taken as well as the relative and absolute contraindications for challenge testing [1]. These precautions apply also to indirect airway challenges, and include: laboratory materials, personnel training and written safety protocols. With regard to physical challenges there is general consensus that standardised exercise tests are safe [155]. In the literature there is one documented case of a fatal

asthma attack during inhalation challenge with distilled water [231]. Recent studies have reported inhalation of hypertonic saline, eventually in conjunction with sputum induction, to be safe [232, 233]. In the appendices (1–4) safety and performance issues concerning physical challenges with exercise or hypertonic saline and pharmacological challenges with adenosine or lysine-aspirin are described in detail.

Conclusions

The direct airway challenges, methacholine and histamine, cause airflow limitation predominantly *via* a direct effect on airway smooth muscle. Indirect airway challenges induce airflow limitation by an action on cells other than smooth muscle cells which, upon stimulation, release mediators that provoke smooth muscle contraction.

A challenge with methacholine or histamine is a highly sensitive measure for the detection of hyperresponsiveness in patients suspected of having asthma when referred to a pulmonary function laboratory or clinic. They are useful to exclude current asthma in these populations. However direct challenges are not specific to asthma, do not exclude exercise-induced bronchoconstriction and perform less well in the epidemiological setting. Indirect bronchial stimuli, in particular exercise, hyperventilation, nonisotonic aerosols, as well as adenosine, may reflect more directly the ongoing airway inflammation and are more specific, but less sensitive, to asthma. They are increasingly used to evaluate the prevalence of bronchial hyperresponsiveness and to assess specific problems in patients with known asthma (*e.g.* exercise-induced bronchoconstriction, evaluation before scuba diving).

Bronchial responsiveness can be assessed at regular intervals and is related to asthma severity and airway inflammation. It is well known that anti-inflammatory therapy with inhaled corticosteroids results in an improvement of symptoms and a decrease in airway inflammation. Direct bronchial responsiveness is only slowly and to a modest extent, influenced by the repeated administration of inhaled steroids. Indirect challenges may reflect more closely acute changes in airway inflammation and be clinically relevant markers to assess the clinical course of asthma. Moreover, some of the indirect challenges, *e.g.* hypertonic saline and mannitol, can be combined with the assessment of inflammatory cells by induction of sputum. In view of the clinical and physiological relevance of indirect challenges, it is desirable to design studies that compare the improvement in symptoms and markers of airway inflammation induced by antiasthmatic therapy with their effects on direct and indirect airway challenges.

Areas for future research

Mechanisms and receptors

The following are areas that require further research to improve the understanding within this field. 1) Further characterisation of receptor(s) involved in bronchoconstrictor effects of adenosine, *e.g.* by use of specific antagonists. 2) The identification of adenosine targets on cells other than mast cells (*e.g.* epithelial cells). 3) The relation between mediator release and the response in individual patients; combination of indirect challenges with measurements in breath condensate and exhaled air. 4) The use of transgenic technology, *i.e.* knock-outs and knock-ins, to define in more detail the molecular targets for some of the indirect stimuli.

Diagnosis

Further questions that need to be addressed to improve the diagnostic development in this field include. 1) How do indirect challenges relate to mucosal inflammation and to noninvasive measures of airway inflammation such as induced sputum and exhaled air? 2) What is the relationship between bronchial responsiveness of different indirect challenges and airway remodelling? 3) Can an indirect challenge be used as an index of asthma severity? 4) To assess risk for an exacerbation? 5) Are indirect challenges useful in assessing risks, *e.g.* occupational exposure? 6) How can indirect challenges be incorporated in genetic/phenotyping studies?

Monitoring

To improve knowledge on the value of indirect airway challenges for monitoring of asthma the following questions need to be investigated further. 1) How can indirect challenges be applied in the short-term and long-term monitoring of an asthma patient? 2) How do they compare to the direct stimuli histamine and methacholine? 3) Can indirect challenges be used to evaluate the efficacy of allergen avoidance measures? 4) Can indirect challenges be used to assess the minimum effective dose of an inhaled steroid and to monitor compliance to treatment with inhaled steroids? 5) Do indirect challenges have a prognostic value in allergic rhinitis?

Epidemiology

A question concerning epidemiology that needs to be addressed is "what is the epidemiology of responsiveness to lysine-aspirin or to adenosine?" Further research into the phenotype-genotype correlation and the standardisation of protocols for indirect challenges in infants and toddlers is also needed.

Safety, performance

There is a need for better standardisation and also to know more about reproducibility, in order to improve both safety and performance of indirect airway challenges.

Appendix 1: Safety issues for exercise challenges in the lung function laboratory and in field studies

A distinction has to be made between challenges applied in the laboratory and in a field study. General issues, including safety issues, have been discussed in recent documents, European Respiratory Society (ERS) 1993 [1] and American Thoracic Society (ATS) 2000 [155].

Safety issues for exercise challenge in the laboratory

Safety issues for the exercise challenge in the laboratory include the following. 1) Two experienced people in attendance; if patient at high risk one should be a physician. 2) Adult of >60 yr to have normal electrocardiogram. 3) Ventilation to be measured as this is the stimulus. 4) Heart rate measured. 5) Bronchodilator plus oxygen (O_2) at hand. 6) Medical help/resuscitation available within 2 min. 7) Forced expiratory volume in one second (FEV1) pre-exercise >70% predicted. 8) No long-acting β_2 agonist for 48 h. 9) Cessation of exercise test if: patient is distressed; the arterial oxygen saturation (Sa,O_2) is falling during exercise; ventilation is

reduced; or the breathing is laboured. 10) The FEV1 in distressed patients must be measured.

Safety issues for exercise challenge in the field

The safety issues for exercise challenge in the field are as follows. 1) Baseline FEV1 >70% predicted. 2) Actual value for FEV1 is to be considered. 3) Known asthmatics should be identified. 4) Subject exercising observed by one person at all times during and especially after exercise. 5) Bronchodilator and O_2 are to be at hand. 6) Large volume-spacer and pressurised metered-dose inhalers available. 7) Medical or nursing help at hand. 8) Transport available to nearest accident and emergency facility. 9) Oximeter for monitoring O_2 saturation and heart rate. 10) Bronchodilator given when fall in FEV1 >10%.

Appendix 2: Safety issues for airway challenges with hypertonic saline in the lung function laboratory and in field studies

Safety issues for hypertonic saline challenge in the laboratory

The safety issues for hypertonic saline challenge in the laboratory are as follows. 1) Baseline FEV1 >75% pred or 65% for some laboratories. 2) First exposure 30 s only. 3) Patient must be attended at all times. 4) Patient must be free to come off mouthpiece. 5) Bronchodilator and oxygen to be in immediate vicinity. 6) Medical help/resuscitation available within 2 min. 7) Oximeter available for monitoring oxygen saturation. 8) No long-acting bronchodilator for 48 h. 9) Equipment must be properly cleaned.

Safety issues for hypertonic saline challenge in the field

Safety issues for hypertonic saline challenge in the field are as follows. 1) Baseline FEV1 >65–75% of pred, or >1.2 L. 2) First exposure 30 s only. 3) Stop at 15 or 20% fall in FEV1; give bronchodilator. 4) Subject must be attended at all times. 5) Subject must be free to come off mouthpiece. 6) Bronchodilator and O_2 at hand. 7) Medical or nursing help at hand. 8) Transport available to nearest accident and emergency facility. 9) Oximeter for monitoring O_2 saturation and heart rate. 10) Subjects should understand test. 11) Bronchodilator given if fall in FEV1 >10%.

Appendix 3: Performance standards, safety issues and protocol recommendations for airway challenges with adenosine

Contraindications and safety

As for more traditional means of bronchial challenge, contraindications to adenosine challenge testing are conditions that may compromise the quality of the test (*e.g.* inability to perform acceptable spirometric manoeuvres, significant airway obstruction) or that may subject the patient to increased risk or discomfort (*e.g.* low baseline–lung function, recent heart attack or stroke, and pregnancy). Moreover to ensure good-quality results and patient safety the technician/physician who performs the test should be proficient in bronchial challenge testing. However, hundreds of adenosine challenge tests have been performed by laboratories with no serious side-effects.

Patient preparation

Patients undergoing adenosine 5′-monophosphate (AMP) challenge testing should be given a list of items/medications to avoid before the test. Medications such as inhaled bronchodilators (β_2-agonists, anticholinergics) [39], theophylline [234], antihistamines [40, 234], cromones (sodium cromoglycate, nedocromil) [235], nonsteroidal anti-inflammatory drugs (NSAIDs) [41, 42], and oral antileukotrienes [27] can reduce bronchial responsiveness to adenosine, potentially causing a false-negative response. Moreover, as adenosine responses are extremely sensitive in detecting changes after inhaled steroids, much attention should be dedicated to this confounder especially when monitoring bronchial responsiveness in the long term. Factors such as allergen exposure, recent respiratory infection, and cigarette smoking may temporarily increase bronchial responsiveness to AMP and generate false-positive results.

Making of adenosine 5′-monophosphate solution

The sodium salt of AMP (Sigma-Aldrich, product no. A1752), available as a dry crystalline powder, is the agent of choice for challenge testing. This is preferred to adenosine because it is more soluble in sterile normal saline. Bulk powder should be stored with a desiccator in a freezer. Sterile normal saline (0.9% sodium chloride) may be used as the diluent. AMP solutions should be properly mixed, labelled, and stored (~4 °C). AMP solutions $\geqslant 3.125$ mg·m^{-1} remain stable for up to 25 weeks at 4 °C.

Dosing protocols

Doubling concentrations are widely recommended and are mathematically attractive. Many authors favour the five-breath method (either using a dosimeter at the beginning of a deep inhalation or by continous nebulisation dosing during a deep inhalation) over the others. The 2-min tidal breathing method is slightly more time consuming, but has also been used successfully in both adults and children [152].

For the five-breath dosimeter technique the authors recommend a dosing schedule using AMP concentrations of 3.125, 6.25, 12.5, 25, 50, 100, 200 and 400 mg·mL^{-1}. The five-breath dosimeter protocol was first standardised by the National Institutes of Health (NIH) Institute of Allergic and Infectious Diseases in 1975 [128] and is presented as an alternative method by the ERS [1]. Dosimeters may improve the accuracy and repeatability of the dose delivered to the airways but adds additional expense. They are widely used in both clinical and research settings. The protocol is as follows. 1) Set up and check the dosimeter. 2) Prepare AMP solutions (3.125–400 mg·mL^{-1}) in sterile vials; place them in a holder; and store them in a refrigerator. 3) Remove the vials from the refrigerator 30 min before testing, so that the contents warm to room temperature before use. 4) Most current protocols start with a diluent step with normal saline. 5) The patient is seated throughout the test. 6) Perform baseline spirometry. 7) Ask the patient to hold the nebuliser upright with the mouthpiece in their mouth. Watch the patient during the breathing manoeuvres to ensure that the inhalation and breathhold are correct. 8) Instruct the patient to inhale slowly and deeply from the nebuliser. Trigger the dosimeter soon after the inhalation begins; dosimeters may do this automatically. 9) Repeat step eight for a total of five inspiratory capacity inhalations. Take no more than a total of 2 min to perform these five inhalations. 10) Measure the FEV1 at ~60 and 180 s after the fifth inhalation from the nebuliser. Obtain

a good-quality FEV1 at each time point. This may require repeated attempts. 11) Report the highest FEV1 from acceptable manoeuvres. The postsaline FEV1 is the reference point for comparison, and it should not exceed a 10% fall in FEV1 from baseline. 12) Pour the first concentration of AMP solution into the nebuliser, using a sterile syringe and repeat steps 7–9. 13) Measure the FEV1 at 60 and 180 s after the fifth inhalation from the nebuliser. The timing of FEV1 measurements at 60 and 180 s after the inhalation is based on the results of time course studies with AMP. 14) At each dose, report the highest FEV1 from acceptable manoeuvres. 15) If the FEV1 falls <20%, empty the nebuliser, shake it dry, and add 2.0 mL of the next higher concentration, and repeat steps 12–14. 16) If the FEV1 falls >20% from baseline (or the highest concentration has been given), give no further AMP, administer inhaled salbutamol, wait 10 min and repeat spirometry.

Recommendation on nebulisers and dosimeters

The nebuliser must deliver an aerosol with a particle mass median diameter (MMD) between 1.0–3.6 μm. Avoid the use of nebulisers with MMD <1.0 μm. Nebulisers for the five-breath method should deliver 9 μL±10% of solution per 0.6-s actuation during inhalation [129]. A single nebuliser may be used for all concentrations, provided it is emptied and the nozzle dried between doses. Alternatively, six or seven separate calibrated nebulisers may be filled before the test. If separate nebulisers are used, they must be carefully labelled to avoid dosing errors. Inexpensive plastic nebulisers are generally not manufactured with tight output tolerances and their volume output should be checked before use. At least 1 mL of solution should remain at the end of nebulisation, because output decreases below this level.

Common end-point measures

Change in FEV1 is the primary outcome measure for adenosine challenge testing. Special care should be taken to obtain good, quality baseline FEV1 measurements because unacceptable manoeuvres may result in false-positive or false-negative results. The quality of the flow/volume curves should be examined after each manoeuvre.

Measures of airway resistance (*R*aw), usually expressed as specific conductance (*s*Gaw), are alternative end-points for adenosine challenge testing, but both Raw and sGaw are more variable and less reproducible than FEV1. Changes in airway resistance may be more sensitive than changes in FEV1 for detecting bronchoconstriction, but FEV1 is superior to other parameters for discriminating relatively healthy persons from those with asthma. Changes in peak expiratory flow often parallel changes in FEV1 during bronchoconstriction but have the disadvantages of being more effort dependent and less reproducible [236].

Data presentation

The percentage fall in FEV1 from baseline is plotted on the ordinate against the log concentration of AMP on the abscissa and the provocation concentration required to produce a 20% decrease in FEV1 (PC20) from the postsaline treatment baseline value is determined by linear interpolation. The PC20 value may be used to summarise the results. If the FEV1 does not fall by at least 20% after the highest concentration then the PC20 should be reported as "> 400 $mg \cdot mL^{-1}$". The PC20 is recommended as the outcome variable because it is simple to calculate and avoids the complicated and controversial aspects of estimating a provocative dose (PD20).

Table 4. – Concentrations and doses of Aspirin used in the dosimeter-controlled jet-nebuliser protocol

Aspirin M	No. of breaths	Dose μmol	Cumulated dose μmol	log10	log10 units increase
0.1	1	1	1	0	0
0.1	2	2	3	0.5	0.5
0.1	7	7	10	1.0	0.5
1.0	2	20	30	1.5	0.5
1.0	7	70	100	2.0	0.5
1.0	8	80	180	2.26	0.26
1.0	12	120	300	2.48	0.23
1.0	30	300	600	2.77	0.29

Appendix 4: Performance standards, safety issues and protocol recommendations for airway challenges with lysine-aspirin

Challenges must be performed in the hospital under close supervision of the patients and with emergency resuscitative equipment readily available. Moreover, the responsible physician and the technician performing the test should be experienced with bronchial challenge testing. The protocol described below has been used repeatedly at the Dept of Respiratory Medecine at Karolinska Hospital (Stockholm, Sweden), in cohorts of NSAID-intolerant asthmatic subjects and in patients with NSAID-tolerant asthma on >250 occasions and with no serious adverse events.

Lysine-aspirin is administered by a dosimeter-controlled jet-nebuliser (Spira Elektro 2, Respiratory Care Center, Hameenlinna, Finland). As indicated in table 4, by the use of two or sometimes three different solutions of lysine-aspirin and by variations in the number of tidal breaths, step-wise increments in the dose of inhaled aspirin produce the desired protocol for cumulative challenge.

Nebuliser settings

The settings for the nebuliser are shown in table 5. These settings provide an aerosol with 80% of the particles being <5.8 μm and an MMD of 4.1 μm [237].

Lysine-aspirin solutions

Crystalline lysine-aspirin (Aspisol™, Horby Bayer AG, Germany, Flectadol™, Maggioni-Winthrop, Italy) is provided in vials containing 1 g (Aspisol™) or 2 g (Flectadol™) of lysine-aspirin. This corresponds to 500 and 1000 mg of acetylsalicylic acid respectively.

The lysine-aspirin solutions are prepared fresh just before

Table 5. – Nebuliser settings

Procedure	Parameter
Inspiratory flow rate $L \cdot s^{-1}$	0.5
Starting volume mL	50
Tidal volume L	0.5–0.6
Duration of nebulisation s	0.8
Output $\mu L \cdot breath^{-1}$	10.3

the start of the challenge by dissolving the crystalline lysine-aspirin in saline (0.9% sodium chloride). Crystalline lysine-aspirin is stable and may be kept at room temperature for prolonged periods, whereas solutions of lysine-aspirin are only stable for 2 h in the refrigerator.

For most challenges in sensitive subjects, it is sufficient to make up two concentrations of lysine-aspirin (0.1 and 1 M). Using Aspisol^{TM} the 1 M stock solution (360 mg·mL^{-1} lysine-aspirin, 180 mg·mL^{-1} aspirin) is made by dissolving one vial of crystalline lysine-aspirin (1 g lysine-aspirin contains 0.5 g aspirin) in 2.8 mL of saline. The 0.1 M solution (36 mg·mL^{-1} lysine-aspirin, 18 mg·mL^{-1} aspirin) is produced by adding 4.5 mL of saline to 0.5 mL of the 1 M stock solution. Please make sure that the 1.0 M stock solution is dissolved before performing the dilution.

In subjects who are less sensitive to aspirin, it may be required to prepare also a 2.0 M solution by dissolving 1 g of lysine-aspirin in 1.4 mL of saline (720 mg·mL^{-1} lysine-aspirin, 360 mg·mL^{-1} of aspirin). This more concentrated solution, reduces the number of breaths required to produce the highest dose of lysine-aspirin in the protocol or may be used, if necessary, to increase the dose even further in doubtful cases (table 4). However, it should be borne in mind that aspirin is absorbed through the airways and gives rise to measurable plasma levels.

The solutions are kept in the refrigerator during the provocation but must be brought to room temperature before each administration. A minimum of 1 mL of solution is required in this particular nebuliser.

Challenge protocol

Pulmonary function is measured as FEV1 and the baseline defined as the best of three efforts. If baseline FEV1 is >70% of pred, the test is started by administration of the diluent (seven breaths of saline). Provided FEV1 at 10 and 20 min after inhalation of the diluent does not change by >10%, the aspirin challenge is started and the postdiluent FEV1 value used as baseline.

The lysine-aspirin solution is inhaled every 30 min and ~0.5–0.25 log-dose increments are administered according to the table. FEV1 is obtained at 10, 20 and 30 min after each dose. The provocation is stopped when FEV1 has fallen ⩾20% from the postdiluent baseline, or the maximum dose of aspirin has been reached (600 μmol cumulative dose).

If the decrease in FEV1 at 30 min after an inhaled dose is between 15–20%, indicating the development of a positive reaction, it is advised to wait another 15 min before a further dose increment. If the drop in FEV1 remains between 15–20%, the responsible physician must make a decision as to whether or not the next dose in the protocol should be given. In subjects with a steep dose-response relation for lysine-aspirin and/or moderate to severe asthma, it is recommended for safety purposes to repeat the previous dose, rather than giving the next dose in the protocol.

After a positive reaction FEV1 is followed every 15 min until it returns to within 10% of the post diluent baseline. The patient should always be observed for at least 1 h after the termination of provocation. Although typical late reactions have not been documented following aspirin challenge, the challenged subject should be advised to record peak expiratory flow rate (PEFR) in the case of airway symptoms. Before leaving the clinic, PEFR should be recorded and a predefined level marked out on the PEFR-chart to alert for rescue medication and/or contact with the hospital.

Dose-response relations for aspirin are constructed and used for calculation of the PD20. The lowest FEV1 measurement at 10, 20 or 30 min after each dose is plotted against the log cumulated dose of aspirin and the PD20 value is derived from linear interpolation between the two last doses.

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