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A critical appraisal of methods used in early clinical development of novel drugs for the treatment of asthma

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Introduction

Asthma is a chronic disorder of the airways characterized by at least partly reversible airways obstruction, airway inflammation, hyperresponsiveness and remodeling. Furthermore, there is a systemic link to atopy, predisposing for IgE-related comorbidities, such as atopic dermatitis, allergic rhinitis/rhinosinusitis and blood eosinophilia (1;2). Presently, asthma and related disorders are considered complex and heterogeneous diseases. This has resulted in a paradigm-shift from a general to an individualized, and from a local to a systemic therapeutic approach for this 'systemic airways disease'.

Measurement of airway inflammation being the hallmark of asthma is crucial to assess the disease's activity and severity. Although bronchial mucosal biopsies are still the gold standard (3), there are many disadvantages to this invasive and costly procedure. Therefore, an increasing number of non-invasive sampling methods have been developed closely approaching or complementary to the gold standard (Figure 1) (4). Some of these methods have been validated and even included into clinical evaluation and therapy monitoring, whereas others are still in a more explorative phase.

Airway hyperresponsiveness (AHR) has been shown to correlate with the degree of airway inflammation and remodeling and can be quantified by a direct bronchoprovocation test (5, 6). Such tests have been standardized and validated for the diagnosis and evaluation of asthma (7). In addition, some indirect bronchoprovocation tests (BPTs), mimicking specific features of asthma, serve as disease models in early drug development trials.

With this review we aim to evaluate standardized and upcoming non-invasive and semi-invasive sampling methods and (in)direct BPTs providing practical recommendations on their applicability for clinical monitoring or early clinical trials. This review provides an extension on the recommendations for clinical intervention trials in asthma (8).

| eNO |
| EBC |
| Sputum |
| BAL |
| Biopsy |
| AMP BPT |

Airway inflammation ⟷ Airway remodeling

| Transbronchial biopsy |
| Imaging |
| Methacholine BPT? |

Airway hyperresponsiveness

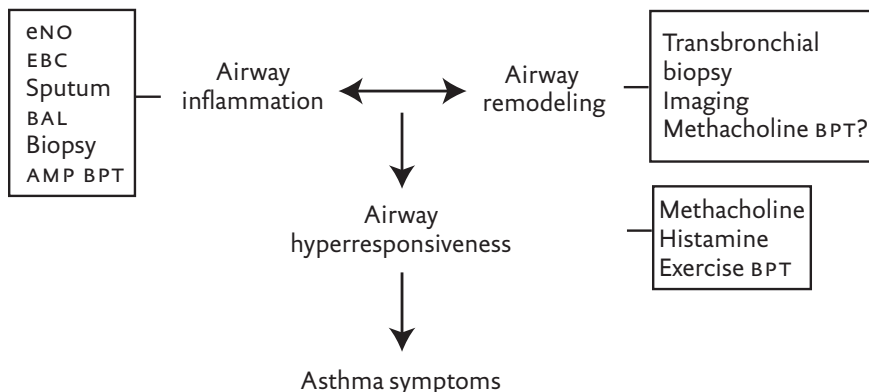| Methacholine |
| Histamine |
| Exercise BPT |

Asthma symptoms

FIGURE 1   Key features of asthma and possible sampling methods. eNO = exhaled NO, EBC = exhaled breath condensate, BAL = bronchoalveolar lavage, AMP = adenosine 5' monophosphate, BPT = bronchoprovocation test.

Sputum

Sputum induction and processing technique

Sputum induction has been explored since the 1990s and in 2002 the European Respiratory Society (ERS) issued guidelines to harmonize the different techniques used worldwide (9). The recommended induction technique can be summarized as follows: prior to sputum induction, 200-400 µg salbutamol is administered as a safety measure, followed by spirometry to assess baseline Forced Expiratory Volume in one second (FEV_1). Subsequently, the entire procedure is carefully explained to the subject prior to commencing. The procedure has to be performed in a quiet, secluded environment to obtain the subject's full cooperation. The hypertonic saline (NaCl 4.5%) aerosols are generated by an ultrasonic nebuliser with an average output of 1 ml/min hence producing a dose of about 5-7 ml per inhalation. During the procedure, subjects perform three to four inhalations by tidal breathing through a mouthpiece for 5-7 minutes each. According to guidelines, the same inhalation time should be maintained throughout the procedure with a total duration of 15-20 minutes. After each induction, subjects are instructed to blow their nose, rinse their mouth and take a sip of water to minimize nasal and saliva contamination before expectoration of sputum.

To further minimize saliva contamination, subjects are either requested to spit saliva into another cup or sputum and saliva can be separated directly after collection but before processing. Both methods yield satisfactory results but are not interchangeable (10;11). During the procedure, all expectorations can be pooled in a pre-weighed plastic cup. After every inhalation, spirometry should be performed as a safety procedure. If the FEV$_1$ decreases by more than 20% from baseline, further induction should be discontinued and salbutamol administered. After the entire collection procedure, the obtained secretions should be processed within 2 hours according to standardized guidelines by a laboratory technician (11;12).

Biomarkers in sputum

CELLULAR PHASE

When performed according to ERS guidelines, sputum cell counts can be performed in a reproducible and validated manner (13, 14). This applies especially for eosinophil and neutrophil counts (15). Eosinophils (and neutrophils in severe persistent asthma) are considered as key effector cells of the asthmatic airway inflammation (16, 17). Increased eosinophil counts have been demonstrated in sputum samples both in validated models of asthma, such as allergen-induced late response, and in the actual disease (18). As compared with non-asthmatic volunteers, Louis *et al* showed increased sputum eosinophils and neutrophils in asthmatics (19). Moreover, the percentage inflammatory cells appeared to be related to disease severity, with further increase during exacerbations (20, 21). Conversely, anti-inflammatory interventions reduced sputum eosinophils both following allergen challenge and in 'wild type' asthma (22-27). In most studies, the reduction in sputum eosinophils was accompanied by an improvement in symptoms scores and lung function parameters. Green *et al* achieved superior asthma control applying a treatment regimen aimed at reducing sputum eosinophils rather than aiming at improving symptom scores and lung function parameters (28). In general, sputum eosinophil count is a validated biomarker to sample airway inflammation that can be employed both in clinical setting and in early drug development.

FLUID PHASE

Presently, numerous inflammatory mediators can be measured in the fluid phase of sputum ('supernatant'), however the validity and reproducibility of several techniques has not yet been determined. Apart from the induction

technique, there are at least two other reasons that can account for this. First, processing of sputum may affect mediator measurements. Dithiothreitol (DTT) is added to the sputum sample, in most processing protocols, to free mediators by dispersing the mucus layer through cleavage of the disulphide bonds (11). However, DTT may also affect the disulphide bonds in the mediators (29). Second, varying dilutions may account for inaccurate measurements among the samples and presently there is not yet a validated dilution factor to correct for this (12). ERS guidelines recommend immunoassays as the method of choice to quantify mediators in sputum due to their reproducibility, specificity and improving sensitivity (12).

GRANULOCYTE PROTEINS

Eosinophil cationic protein (ECP) and myeloperoxidase (MPO) are granulocyte proteins that can serve as activation markers of eosinophils and neutrophils, respectively. In sputum of asthmatics, (increased levels of) ECP have been found to be well-correlated with the eosinophil cell counts (30). In addition, anti-inflammatory treatment decreases both the eosinophils and ECP within the airways (25, 31). While measurements of ECP in sputum have been shown to be reproducible, MPO concentrations appear to be affected by sputum induction and/or processing technique and therefore immunoassays are not always reproducible (29, 32, 33).

LEAKAGE MARKERS

Microvascular leakage is another aspect of airway inflammation that can be assessed by measuring the concentrations of leakage markers in the sputum of asthmatic patients. Based on several studies applying different induction and processing techniques, albumin and fibrinogen have been shown to be reproducible leakage markers correlating with the degree of airway inflammation. Pizzichini *et al* demonstrated increased levels of albumin and fibrinogen in sputum of asthmatics as compared with healthy controls (14). Two other studies found that increases in albumin and fibrinogen corresponded with asthma severity (20, 33). Apart from albumin and fibrinogen, another potential leakage marker has been studied in asthmatic subjects is α2-macroglobulin. Applying BPTS with pro-inflammatory tachykinins in patients with asthma, van Rensen *et al* showed in patients with asthma that α2-macroglobulin (and albumin) appeared the best leakage markers (34).

CYTOKINES AND CHEMOKINES

These biomarkers are degraded by DTT. An extensive overview on cytokine and chemokine recovery from sputum is reported in the ERS guidelines (13).

SECTION 1 – A CRITICAL APPRAISAL OF METHODS USED IN EARLY CLINICAL DEVELOPMENT OF NOVEL DRUGS FOR THE TREATMENT OF ASTHMAS

Several research groups have investigated modified sputum processing techniques to optimize recovery of these biomarkers with overall good results (35-37). However, these processing techniques are not fully validated and most of them prevented recovery of other mediators from the samples. As an exception, IL-8, potent neutrophil chemoattractant, seems less affected by DTT and can be quantified by a validated immunoassay (29, 38). In several studies, increased reproducible levels of IL-8 have been demonstrated during asthma exacerbations and in patients with severe persistent asthma (38, 39). Hence, IL-8 is a validated marker for the assessment of drug efficacy in more severe asthma and for monitoring of asthma exacerbations.

EICOSANOIDS

Leukotrienes (LTs) and isoprostanes are derivates of arachidonic acid. Both groups of inflammatory mediators are involved in the pathophysiology of asthma (40, 41). Cysteinyl leukotrienes (Cys-LTs) are mainly released from activated mast cells and eosinophils. As compared to healthy controls, increased levels of these bronchoactive mediators have been measured in several body fluids of asthmatic subjects, including sputum (42, 43). Moreover, the concentration of Cys-LTs was found to correlate with disease-severity and was not affected by corticosteroids (57).

F_2-isoprostanes are considered specific markers of oxidative stress (44). These mediators are involved in the pathophysiology of inflammatory diseases, including asthma and COPD. 8-Isoprostane is the most extensively studied isoprostane, and reproducible levels have been measured in sputum and exhaled breath condensate (EBC) of both healthy and asthmatic subjects, with higher levels in those with more severe disease (45). In agreement with these data, increased levels of this eicosanoid have been reported during asthma exacerbations (45). Similar to Cys-LTs, 8-isoprostane is relatively inert to treatment with corticosteroids (46). Hence, both Cys-LTs and 8-isoprostane are useful markers of airway inflammation and oxidative stress in disorders including asthma.

PROTEASES

Matrix metalloproteinases (MMPs) are members of a large family consisting of calcium and zinc dependent enzymes. Several MMPs are involved in the process of extracellular matrix degradation and are considered key players in airway remodeling occurring in e.g. asthma, COPD and lung fibrosis (47). In the process of remodeling, there is a critical balance between MMP-9 and its counterpart, tissue inhibitor of metalloproteinases (TIMP). In asthma, increased levels of MMP-9 have been found in sputum, BAL and bronchial

biopsies (48-52). In addition, several investigators reported an imbalance between MMP-9 and TIMP, resulting in a disease-severity dependent increase of the MMP-9/TIMP ratio (48, 49, 53). In agreement with these data, allergen challenge has been shown to induce further increase in MMP-9, without increasing TIMP levels both in sputum and bronchoalveolar lavage (BAL) of asthmatic subjects (48, 49, 52). This MMP-9/TIMP imbalance may at least partly account for the allergen-induced airway remodeling in asthma. In conclusion, MMP-9/TIMP ratio in sputum is a potential marker for monitoring effects of interventions directed against airway remodeling.

Limitations of induced sputum

Only trained personnel should perform sputum inductions and careful patient instruction is needed prior to the procedure to ensure safety of the subject. Processing and analysis of the sputum samples are time-consuming and expensive procedures which require a well-equipped laboratory including a technician and an experienced cytopathologist. Furthermore, the results are not immediately available.

Not all patients are able to expectorate sputum and not all sputum samples are suitable for analysis. On average, sputum induction is successful in only 80-90% of adult patients and approximately 30% of asthmatic patients have normal sputum eosinophil counts (19, 28, 33, 54-56). In children (6 years and older) the percentage of successful inductions is significantly lower, around 80% (57, 58). In addition, the procedure should not be performed in patients with unstable or severe persistent asthma and those with moderate to severe disease should be carefully monitored during the induction process for the occurrence of sudden severe bronchoconstriction (59, 60).

Since sputum induction may affect the composition of the inflammatory cells and mediators within the airways, serial sputum inductions cannot be readily performed within a short time-interval, which may be a disadvantage in a clinical trial setting (61, 62). According to recommendations, a wash-out interval of at least 2 days should be allowed between two serial inductions to prevent potential carry-over effects (9, 63). Another disadvantage of the procedure is that most patients find the procedure strenuous and sometimes even embarrassing.

Summary and recommendations - sputum

Sputum is defined as secretion originating from the lower airways. Sputum induction by inhalations of hypertonic saline promoting expectoration is a validated method both for research and diagnosis. The obtained sputum

SECTION 1 – A CRITICAL APPRAISAL OF METHODS USED IN EARLY CLINICAL DEVELOPMENT OF NOVEL DRUGS FOR THE TREATMENT OF ASTHMAS

samples can be divided into a 'solid' phase consisting of cells, and a fluid phase containing soluble mediators. Both components can be quantified to assess the presence and activity of inflammatory markers. Sputum induction can be regarded as a semi-invasive procedure and is safer, cheaper and generally easier to perform than bronchial biopsy or BAL but more troublesome than exhaled nitric oxide (ENO) or EBC. Over the last fifteen years, a large amount of research has contributed to validation and standardization of the technique. A recent ERS Task Force document has been issued relating on recommendation and guidelines for standardized induction, collection, processing and analysis of sputum (13). Although airway sputum, BAL and bronchial mucosal biopsy provide samples from different lower airway compartments (64), a reasonable relationship has been found between these techniques providing similar information on the inflammatory airway components (54, 65). In addition, the recommended sputum induction protocol can be modified to enable differentiation between inflammatory cells from central and distal airways (66). In conclusion, induced sputum is a validated tool suitable for monitoring lower airway inflammation both in patient care and in early drug development. The pros and cons of sputum induction are summarized in Table 1.

Nitric Oxide (NO)

NO measurement technique

In 2005, the American Thoracic Society (ATS) issued updated recommendations for the measurements of NO from the upper and lower respiratory tract (67). Although various methods have been reported, the online measurement during a single-breath exhalation against a fixed resistance is currently the recommended sampling technique. This highly reproducible method has been standardized and is now widely used (68, 69). This technique can be summarized as follows: subjects are seated in front of a PC-screen while wearing a nose-clip, and instructed to blow into the NO-analyser with a constant flow rate (50 mL/s) for approximately 20 seconds. Blowing against a resistance ensures soft palate closure and prevents contamination with NO from the upper respiratory tract. A constant flow rate is important for a representative measurements, since NO is markedly flow dependent (70). After several seconds of expiration, an NO plateau is reached and the NO level is measured online by a chemoluminescence analyser. NO is expressed in parts per billion (ppb) and measurements are repeated until three reproducible values are obtained within 10%. Repeated measures do not affect the results.

	SPUTUM INDUCTION	EXHALED NO	EXHALED BREATH CONDENSATE	BRONCHOPROVO-CATION TESTS
Pro's	-Multiple biomarkers -Reproducible cell differentials on cyto-spins -Valid tool for assessment of anti-inflammatory therapy	-Non-invasive -Reproducible -Inexpensive measurements -Direct results -Allows serial measurements -Tool for assessment of anti-inflammatory therapy	-Non-invasive -Multiple biomarkers -Allows serial measurements -Potential tool for assessment of anti-inflammatory therapy	-Reproducible -Direct results -Valid tools/models for assessment of anti-inflammatory therapy
Contra's	-Semi-invasive -Expensive & time-consuming procedure & analysis -Representative samples available in approx. 80-90% of subjects -Soluble markers subject to dilution -Non-repeatable over short time-period (<48 h) -Experienced personnel needed (MD/cytopatho-logist/lab) -Rescue medication needed -Contraindicated in severe persistent asthma/COPD/active cardiovascular disorders	-Expensive equipment -Many perturbing factors -Longitudinal samplings within 1 patient are more informative than single measurements	-Assays not fully reproducible -Expensive & time-consuming proce-dure/assays -Soluble markers subject to dilution -Specialized lab needed	-Semi-invasive -Experienced personnel (MD/technician) needed -Non-repeatable over short time-period (>6h-3wk) -Rescue medication needed -Contraindicated in severe persis-tent asthma/COPD/active cardiovascular disorders
Overall assessment	-Validated tool for monitoring of anti-inflammatory drug-effects - Lengthy, expensive procedure - Not suitable for patients with severe bronchoconstric-tion/comorbidities	-Validated tool for monitoring of anti-inflammatory drug-effects - Patient & researcher-friendly method	- Procedure awaits further validation - Patient & researcher-friendly method	-Validated tools for monitoring of AHR and anti-inflammatory drug-effects -Some BPTs are standardized models of asthma - Not suitable for patients with severe bronchoconstric-tion/comorbidities
Refs*	(9, 11, 12, 14, 60, 208)	(4, 67, 108, 209)	(110, 112, 113, 148, 210)	(7, 153, 154)

* Position papers and reviews/AHR: airway hyperresponsiveness/BPTS: bronchoprovocation tests

Although ambient NO appears to have no effect on eNO, most analyzers are equipped with a scrubber to ensure subjects inhale NO-free air prior to the exhalation manoeuvre. The results can be viewed online, which allows incorrect manoeuvres and values to be discarded. Correct operation of the equipment is relatively easy and does not require extensive training. In addition, performing (serial) measurements does not impose a great burden on patients and can be easily explained, which is ideal for young children (applicable from 4-5 years).

NO as a biomarker of airway inflammation

Exhaled NO is a sensitive marker of acute airway inflammation. In asthma, acute airway inflammation implies either loss of control or exacerbation. In clinical trials, this can be induced either by allergen challenge or by tapering off anti-inflammatory therapy (mainly corticosteroids). Allergen challenge, especially the late asthmatic response (LAR), is a well-known inducer of airway inflammation (71). Kharitonov *et al* reported a clear correlation between the size of the LAR and allergen-induced increase in eNO 10 hours post-allergen (72). Similarly, several tapering studies have shown that loss of asthma control is associated with an increase in eNO (21, 58, 73). In addition, these studies demonstrated that the change in eNO is a better predictor for loss of asthma control than baseline eNO per se. However, Leuppi *et al* found no increase in eNO during asthma exacerbations as a result of tapering off inhaled corticosteroids (ICS) (74). This aberrant observation may be due to measuring eNO offline in contrast with online measurements used in other studies.

Exhaled NO is very responsive to anti-inflammatory therapy. ICS have been shown to produce a dose-dependent reduction in eNO, preceding the decline of other disease-related parameters (75, 76). Other anti-inflammatory therapies for asthma, including leukotriene receptor antagonists (LTRA) and anti-IgE, have also been shown to reduce eNO both in children and adults (77, 78). Several studies report a correlation between eNO and other markers of airway inflammation and responsiveness in asthma which adds to its applicability as a valid biomarker for clinical monitoring and early drug development. Jatakanon *et al* showed significant correlations between eNO, sputum eosinophils and PC_{20} methacholine in steroid naïve patients with mild persistent asthma (79). These data have been confirmed and extended by Dupont *et al* who found a correlation between eNO and PC_{20} histamine in patients with similar asthma characteristics (80). However in asthmatics using ICS, the correlation between the different markers of airway inflammation and responsiveness is lost (81, 82). This is due to a fast decrease of eNO attaining

a maximal response even on low dose ICS therapy, resulting in almost normal ENO levels, while airway inflammation and hyperresponsiveness are still present. Therefore, ENO should probably not be used as the sole marker of airway inflammation in asthmatics using corticosteroids.

TABLE 2   REFERENCE ENO VALUES MEASURED IN CLINICAL STUDIES

SUBJECTS	N* (NUMBER OF SUBJECTS)	ENO MEAN** (PPB)	INTERQUAR- TILE RANGE (PPB)	REFERENCE
Atopic asthmatic adults***	44	58.5	30.4 – 85.5	(68, 211)
Non atopic asthmatic adults	30	18.9	14.6 - 33.4	(211, 212)
Atopic asthmatic children***	118	25.7	11.4 – 56.2	(68, 105, 106)
Atopic non asthmatic adults	67	27.6	11.3 – 49.3	(211, 212)
Non atopic non asthmatic adults	158	15.9	11.5-21.7	(68, 211, 212)
Non atopic non asthmatic children	332	8.8	N.A.	(213)
Cystic fibrosis	83	5.3	3.3 – 8.5	(214, 215)

Effect of corticosteroid intervention on ENO levels in patients with asthma

SUBJECTS		ENO BEFORE	ENO AFTER	% CHANGE	REFERENCE
Corticosteroid treatment > stable asthma	52	75.2	34.0	-54.8%	(75, 216, 217)
Tapering off corticosteroids > unstable asthma	67	9.8	21.8	+122.4%	(21, 73)

All measurements were performed with an online chemoluminescence analyzer.
N.A. = not applicable, ENO = exhaled NO. *Total number of subjects from all studies combined, **Median was used as mean when necessary, ***Patients with mild to moderate persistent asthma without maintenance therapy.

Limitations of ENO

The equipment used for online measurement of ENO is bulky and expensive. Recently, a handheld and less costly NO meter, NIOX MINO®, has been introduced, yielding reproducible NO measurements similar to the stationary chemoluminescence NO-analyzer (83). The availability of smaller and cheaper devices will enable future NO measurements to be applied at larger scale, including non-academic hospitals and extramural care.
Exhaled NO appears to be dependent on airway calibre. Recent studies have shown decreased ENO on bronchoconstriction, e.g. following allergen

challenge, with an increase after bronchodilatation (84-87). As asthmatic patients often have unperceived bronchoconstriction, eNO values may be underestimated. Applying maximal bronchodilation with short-acting β2-agonists before eNO measurements, may be a good option to correct for this perturbing factor (84).

Unlike EBC and sputum, eNO depicts only one single component of the inflammatory response within the airways, while its origin is difficult to assess. Recent studies have proposed a two-compartment model to discriminate between alveolar (representing the small airways) and conducting airways contribution (88, 89). Using this model, Lehtimaki et al demonstrated that increased eNO is mainly derived from the larger airways, as in both steroid-naïve patients with mild-to-moderate persistent asthma and nonasthmatic controls comparable alveolar NO levels were measured. Moreover, treating these patients with ICS established a decrease in airway NO levels but failed to reduce alveolar NO (90, 91). Similar findings have been reported in patients with mild-to-moderate persistent asthma and nonasthmatic controls, whereas in those with severe disease, alveolar NO was increased despite ICS and reduced by oral prednisolon (92). In this study alveolar NO appeared to be well correlated with eosinophils in BAL. Hence, these findings suggest that in severe persistent asthma alveolar NO is a potential marker for distal airway inflammation that cannot be easily reached by ICS.

Many factors appear to affect eNO levels including atopy status, airway caliber, medication, nitrate rich food, smoking and airway infections (67, 93-96). Both in clinical practice and in clinical trials, these perturbing factors should be anticipated or corrected for to enable comparison of representative eNO values within one subject, among subjects and across studies.

Summary and recommendations - NO

Nasal NO is produced in the upper respiratory compartment, while eNO mainly comes from the lower airways following synthesis from L-arginine by constitutive (cNOS) and inducible NO (iNOS) synthases (97). cNOS is expressed in neuronal, endothelial and epithelial cells under baseline conditions, whereas iNOS is mainly expressed in inflammatory, epithelial and airway smooth muscle cells and can be further upregulated by inflammatory cytokines (98). Exhaled NO can be measured in exhaled breath of several species, including humans (99, 100). Increased iNOS expression has been demonstrated in bronchial biopsies of asthmatic patients as compared with healthy controls (101). In agreement with these data, increased NO levels occur in exhaled air from patients with asthma with further increase during

exacerbations (72,102). In addition, iNOS inhibitors, such as alcohol and corticosteroids, have been shown to reduce the expression of iNOS and consequently reduce eNO levels in asthmatic subjects, but not in (non) atopic, non-asthmatic controls (103,104). Furthermore, eNO concentrations appeared to correlate with IgE levels and the number of positive skin prick test wheals (105). Similarly, atopic asthmatics have been shown to produce higher levels of eNO than non-atopic asthmatics and higher eNO levels have been reported in atopic subjects as compared with non-atopic controls (Table 2) (93,106,107).

Taken together, upregulated iNOS expression accounts for high eNO concentrations in asthma and eNO levels have been shown to relate to atopy status and disease severity. Although increased eNO is not a prerequisite for asthma only, measuring eNO is an established tool for the assessment of airway inflammation and a validated marker in interventional trials with anti-inflammatory asthma therapies. The pros and cons of eNO measurements are summarized in Table 1.

Exhaled Breath Condensate

Collection of EBC

Several collectors and condensers are currently available (109-111). All devices are easy to use and both young and old subjects can easily perform the procedure (Figure 2). In most protocols, subjects have their nose clipped while breathing through a mouthpiece into a non-rebreathing valve connected to a tube of variable length for approximately 15-30 minutes (112). During the procedure, the exhaled breath travels through the tube that serves as a cooling chamber and the thus formed condensate is collected (usually around 2 mL/sample) in a cooled collection chamber. Cooling of the samples is advised to preserve "thermo-labile" markers (112). Subsequently, samples can be directly analysed or frozen pending analysis. The non-rebreathing valve ensures separation of in- and expiratory air and prevents rebreathing of exhaled samples. Exhalation flow affects the composition of EBC. Therefore, flow variations should be limited during each procedure. The long duration of sample collection makes fixed exhalation flow rate - commonly applied in eNO measurements - impossible. Hence, following subject's acclimatization, tidal breathing over a predefined time-interval is probably the best way to minimize influence of exhalation flow rate on the EBC composition. Using a fixed exhaled volume may be a good alternative.
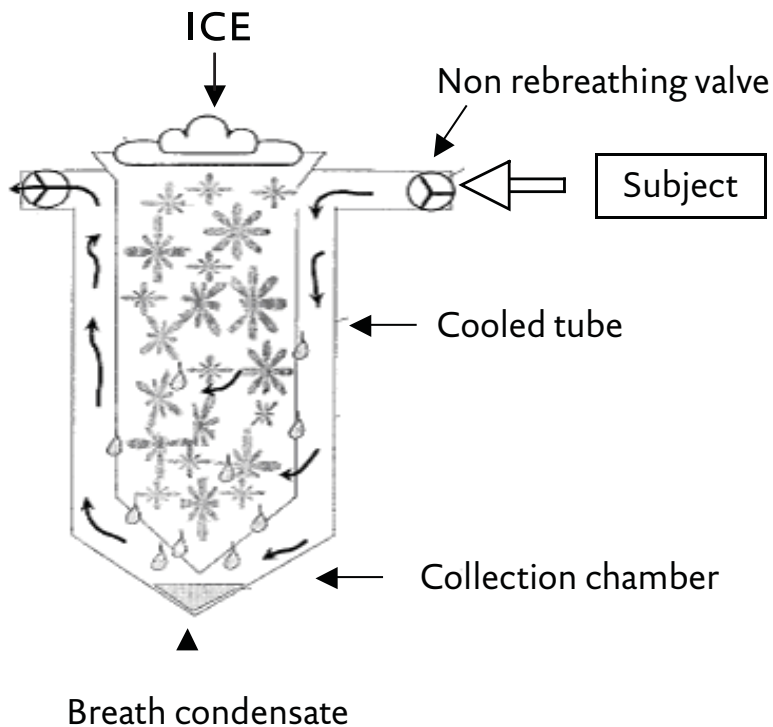
ICE

Non rebreathing valve

Subject

Cooled tube

Collection chamber

Breath condensate

FIGURE 2 Schematic diagram of an exhaled breath condensate collecting device (reproduced and modified with permission from (207)

Biomarkers in EBC

HYDROGEN PEROXIDE

Hydrogen peroxide (H_2O_2) is formed when O_2- released by activated inflammatory cells such as neutrophils, macrophages and eosinophils, undergoes spontaneous or enzymatic dismutation. Hence, H_2O_2 is a potential marker of oxidative stress common in many inflammatory conditions (113). Indeed, reproducible increased concentrations of H_2O_2 have been measured in active smokers and patients with more severe asthma and an inverse correlation between FEV_1 and H_2O_2 has been found (114-117). In these patients anti-inflammatory drugs, such as ICS, have been shown to reduce exhaled H_2O_2 accompanied by improvements in FEV_1 (114, 118).

The stability after collection is an important advantage of measuring H_2O_2 (119, 120). However, in several healthy subjects and in some asthmatics with

mild disease H_2O_2 concentrations remained below the detection limit (116, 120). Therefore, more sensitive assays are required to study airway inflammation in these patients.

EICOSANOIDS

Leukotrienes and isoprostanes are involved in the pathophysiology of inflammatory disorders including asthma and COPD. These eicosanoids can be measured in EBC by an enzyme immunoassay (EIA) or by gas chromatography/mass spectrometry (121). As compared with non-asthmatic controls, increased levels of cys-LTs have been detected in asthmatic subjects. Similar with measurements in sputum, cys-LTs levels in EBC appeared to be correlated with disease severity and could be reduced by anti-inflammatory drugs (122, 123). Leukotriene B4 (LTB$_4$) is released by activated neutrophils; increased levels have been found in patients with severe persistent asthma and COPD (122,124, 125). In these patients, sputum LTB$_4$ levels have been shown to correlate with those in EBC.

Conform with sputum, in EBC 8-isoprostane is the most extensively studied prostanoid for its stability and good detectability in both healthy state and disease (126). In asthma, 8-isoprostane EBC concentrations have been found to be correlated with levels of eNO (127). However, 8-isoprostane levels appeared to correlate with asthma severity while eNO does not. Unlike eNO, 8-isoprostane is not completely suppressed by corticosteroid treatment. Hence, 8-isoprostane is a potential indicator for ongoing airway inflammation despite anti-inflammatory treatment and hence may be a useful marker for asthma control (123, 127, 128). Moreover, recent data suggest a link between 8-isoprostane concentration in EBC and small airways inflammation (129).

NO DERIVED PRODUCTS

NO within the airways can react with O_2 yielding NO-derived products, NO_2^- and NO_3^-. Unsurprisingly, NO_2^- and NO_3^- levels are increased in EBC of asthmatics and corticosteroids have been shown to reduce these inflammatory markers (130, 131).

Nitrotyrosine is another stable end-product of NO and O_2^-, detectable in EBC of healthy subjects and three-fold increased in steroid-naïve patients with mild persistent asthma (124). In correspondence with other NO-derivates, inhaled and oral corticosteroids reduced nitrotyrosine levels in asthma below those of healthy controls (124). Unsurprisingly, nitrotyrosine and NO appeared to be correlated (124).

Nitrosothiol a metabolite of NO and gluthatione is yet another NO-derivate. Elevated concentrations of this biomarker have been found in EBC of patients with severe persistent asthma, as compared with mild asthmatics and

healthy, non-asthmatic controls (132). Similar to other NO-derived products, nitrosothiol is a potential marker of asthma severity and control.

PH

Measurement of pH in EBC is relatively simple and inexpensive as compared with other biomarkers. Furthermore, several studies have reported that pH measurements are reproducible and not affected by temperature or duration of collection, airway obstruction, oral ammonia or storage (133, 134). Using different collection devices, a number of research groups have measured pH in EBC of healthy subjects yielding an average pH of around 7.8, whereas in asthma the average pH was found below 7.5 (133-137). Asthma exacerbations result in further decline of pH with reversal following corticosteroid treatment (137). This corresponds with other studies showing a higher pH in steroid-treated patients as compared with steroid-naive asthma controls. The low costs, good reproducibility in combination with the availability of reference values are major advantages of pH measurements over other inflammatory markers in EBC.

CYTOKINES AND CHEMOKINES

Several cytokines and chemokines including IL-4, TNF-α, macrophage-derived chemokine, and eotaxin have been detected in EBC of asthmatics (138, 139). The most striking EBC data so far have been reported in steroid-naïve asthmatic children by Shahid *et al* showing an increase in IL-4 combined with a decrease of IFN-Y (140). Following treatment with ICS, the IL-4 and IFN-Y levels returned within the ranges of healthy controls. These data clearly demonstrate the Th2-driven cytokine profile in human asthma and the modulating effects of anti-inflammatory therapy. However, in all these studies some cytokine and chemokine measurements could not be reproduced in all subjects and other investigators fail altogether in measuring cytokines in EBC (141). Hence, the methodology awaits validation and more sensitive assays are needed.

Limitations of EBC and potential remedies

EBC has several limitations. Until recently there has been no consensus on the collection and storage techniques which complicates correct comparison and interpretation of data across different studies. Commercially available condensers in combination with a recent Task Force publication providing guidelines for standardization of collection and storage procedures are expected to solve most of these issues in the future (112, 142-144). The ERS/ATS task force document provides a review of several protocols using differ-

ent condensers yielding reproducible data of specific measurements in EBC. Using the RTube® (Respiratory Research, Inc., Charlottesville, VA, USA), Vaughan *et al* showed reproducible pH measurements in EBC of asthmatics (134). The Cryocond® (Boehringer Ingelheim, Burlington, Canada) has been used in several studies yielding reproducible H_2O_2 measurements in EBC of subjects with asthma and allergic rhinitis (77, 145). Ecoscreen® (Jaeger GmbH, VIASYS Healthcare, Hoechberg, Germany) is still another commercially available condenser providing reproducible levels of sodium and chloride in EBC of healthy volunteers, children with asthma and cystic fibrosis (146).

An even greater concern is the poor availability of sensitive assays yielding reproducible measurements (116, 146). Ideally, biomarkers should present well within the concentration range of sensitive and reproducible assays. Moreover, there should be a well-defined concentration range between healthy state and disease. However, partly due to dilution, the concentration of the majority of the inflammatory markers in EBC is generally in the range of the lower detection limit of most assays. Tufvesson *et al* report two potential processing strategies to improve measurements of several inflammatory markers in EBC, i.e. sample concentration and coating of collectors to minimize absorption to plastic material (147). Both methods await standardization.

Another obstacle in interpreting EBC data is the dilution factor. The airway lining fluid captured in EBC is variably diluted during the collection process (148). Extrapolation to the original concentrations within the airways is only possible applying a dilution factor (149, 150). Presently there is no validated dilution factor.

Yet another inconsistency is the composition of an EBC sample including various components of different origin, coming both from different parts of the respiratory and from the digestive tract. Unfortunately, it is not possible to designate the relative contribution of these sites to the exhaled mediators. However, there are some devices and methods being applied to prevent excessive saliva contamination, such as a saliva trap or allowing the subject to rinse the oro-pharynx prior to collection and swallow accumulated saliva during collection. Furthermore, applying different flow-rates may enable discrimination between central and peripheral airways (151).

Summary and recommendations – EBC

EBC is a new sampling technique for various markers of inflammation in inflammatory airway diseases including asthma. EBC is a potential tool for monitoring anti-inflammatory effects of novel drugs. Being a simple, non-invasive

procedure requiring uncomplicated and inexpensive equipment are its main advantages over bronchial biopsies, BAL and induced sputum (134, 148). Exhaled breath consists of two phases: the gaseous phase, containing volatile substances such as NO and carbon dioxide (CO_2), and a liquid phase containing non-volatile components including water-soluble inflammatory markers (113). The non-volatile ions and proteins originate from the airway lining fluid. These entities are aerosolized due to local turbulence to become liquid constituents of EBC (143).

So far, there has been no standardization of EBC sample collection or analysis and this needs to be solved before results can be interpreted, compared and its clinical applicability can be assessed. These issues have recently been largely addressed by an ATS/ERS task force resulting in novel EBC guidelines (112). Overall, EBC seems a promising tool for both research and clinical monitoring. So far, several markers of airway inflammation have been detected in the EBC of asthmatics, including hydrogen peroxide, leukotrienes, isoprostanes, NO-derived products, pH, chemokines, cytokines and adenosine (113, 148,152). In addition, measurement of drug concentrations in EBC may allow studying the link between pharmacokinetic properties and pharmacodynamic effects in the future. The pros and cons of EBC measurements are summarized in Table 1.

Bronchoprovocation tests

Methodology of BPTs

The ATS and ERS have issued several guidelines for standardized BPTs or challenges with both direct and indirect challenges. These guidelines address methodology, recommended equipment and preparation of pharmacologic agents (7, 153, 154).

Methacholine is the most common direct bronchoconstrictor stimulus applied for both diagnostic and research purposes (Figure 3 and Table 3). Two challenge methods are being recommended in the guidelines: the 2-minutes tidal breathing method with 2-fold concentration increases and the 5-breath dosimeter method with 4-fold increases (7). In the 2-min tidal breathing method, up to 10 consecutive doubling concentrations of methacholine are placed in a jet-nebulizer and aerosolized (0.13 mL/min) with a constant-output compressor. Patients are instructed to perform tidal breathing for 2 min through the mouth with the nose clipped. Airway response is measured after each concentration step over the subsequent 3-min period by FEV_1 and expressed as % fall from baseline FEV_1. In the 5-breath dosimeter method,

patients slowly inhale 5 breaths from functional residual capacity to total lung capacity (i.e. a slow deep breath) through a dosimeter-driven nebulizer containing up to 5 fourfold concentration increases of methacholine. After each manoeuvre, patients are instructed to hold the breath for 3-5 seconds. Airway response is measured by FEV_1 before and 3 min after each concentration step. In both protocols, challenges are repeated at 5 min intervals until the FEV_1 falls by at least 20% from baseline FEV_1. The provocative concentration causing a 20% fall in FEV_1 (PC_{20}) is calculated by extrapolation and is indicative of the degree of AHR (range: no AHR, mild, moderate, severe AHR, respectively). After challenge, a short-acting β2-agonist is administered for immediate bronchodilation and FEV_1 is measured after 10 minutes to assure lung function returns within baseline values. BPTs usually last up to 60-70 minutes, depending on the degree of AHR and the protocol used.

Although both methods are reported to be reproducible in clinically stable subjects, there are important differences that may affect the outcome in some asthma patients (7, 153). Cockcroft and co-workers found that specifically in subjects in whom mild AHR was found using the 2-min tidal breathing method, no measurable AHR could be detected using the 5-breaths dosimeter method (155). This difference can be explained by the bronchodilator effect of the dosimeter method, which reduces the sensitivity of the test specifically in patients with mild AHR.
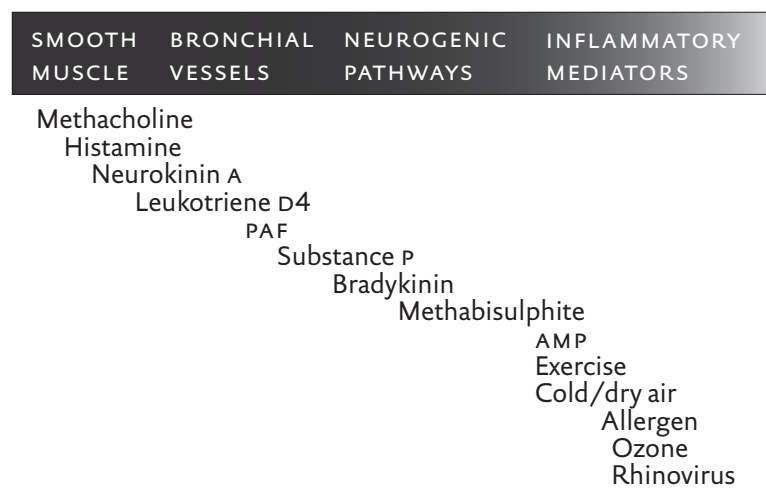
SMOOTH MUSCLE	BRONCHIAL VESSELS	NEUROGENIC PATHWAYS	INFLAMMATORY MEDIATORS

Methacholine
Histamine
Neurokinin A
Leukotriene D4
PAF
Substance P
Bradykinin
Methabisulphite
AMP
Exercise
Cold/dry air
Allergen
Ozone
Rhinovirus

FIGURE 3   Overview of direct and indirect stimuli and their mechanism of action. PAF = platelet activating factor, AMP = adenosine 5' monophosphate. direct challenges

Indirect challenges comprise a heterogeneous group of pro-inflammatory stimuli. The methodologies slightly differ across the spectrum of the challenges. The majority of indirect challenges has been standardized and basically follows the same principle as a direct challenge. During these tests subjects are exposed to serial increasing concentrations of a pharmacological agent or increasing exposure to exercise resulting in a fall in FEV_1 as compared with baseline. Airway response measurements and safety precautions are similar to those in methacholine challenge and are described in the ERS guidelines and a recent ERS task force report (7, 154). The most commonly used indirect challenges have been standardized and validated (Table 3).

Bronchoprovocation tests and their clinical relevance

PC_{20} METHACHOLINE: MARKER OF AHR AND AIRWAY REMODELING VERSUS AIRWAY INFLAMMATION

Methacholine BPT is the preferential test to assess and quantify AHR by PC_{20}. According to previous evidence, the wash-out period between two consecutive methacholine challenges varies from zero up to 24 hours in asthma and up to 6 hours in nonasthmatic subjects (156, 157). Two landmark studies investigated the relationship between methacholine-induced AHR and airway inflammation and/or remodeling (5, 6). In patients with mild-to-moderate persistent asthma, Sont *et al* compared a treatment strategy aimed at improving AHR with the existing strategy aimed at improving symptoms and lung function (5). After 24 months, patients treated according to the AHR-strategy had a lower exacerbation rate corresponding with a reduced number of eosinophils in bronchial biopsies as compared with the reference strategy. Furthermore, subepithelial reticular basement membrane (rbm) thickness, a feature of airway remodeling, was significantly reduced in the AHR-group as compared with the reference group. Comparable observations were reported in patients with similar asthma characteristics by Ward *et al* after 12 months of treatment with high doses of ICS (6). Following longterm anti-inflammatory therapy, the changes in PC_{20} methacholine and rbm-thickness, being one of the characteristics of airway remodeling, appear to be interrelated (6).

In contrast with these data, the relationship between PC_{20} methacholine and markers of airway inflammation has not been unanimously demonstrated. Although several studies reported a good correlation between methacholine sensitivity and markers of airway inflammation (5, 28, 158, 159), other

studies did not find such relationship (160). These contradictive data may be the result of different patient characteristics, methodologies and/or treatment regimes and remain to be clarified.

INDIRECT CHALLENGES: INDUCERS OF AIRWAY INFLAMMATION AND MODELS OF ASTHMA

In contrast with the direct stimuli, there is a variety of indirect airway challenges interfering with different pathophysiological mechanisms within the airways. These indirect stimuli possess varying mechanisms of action with a gliding scale between direct to indirect as schematically depicted in figure 3. Depending on their (specific) mode of action, several indirect challenges can be applied for various purposes, including monitoring or research of pathophysiological mechanisms and proof of principle/concept studies in asthma. In the following paragraphs, the most important and validated indirect challenges will be addressed (7, 153, 154).

AMP CHALLENGE

Adenosine 5' monophosphate (AMP) releases inflammatory mediators from activated mast cells. Recent studies in asthma have shown that airway responsiveness to AMP - expressed as PC_{20} AMP - is a valid marker of airway inflammation (154). Furthermore, PC_{20} AMP has been shown to be more sensitive to ICS and high altitude than PC_{20} of other direct or indirect agents including methacholine or bradykinin (6, 154, 161, 162). In several intervention studies, there was a fast-onset, ICS-dose-dependent improvement in PC_{20} AMP which correlated with a significant reduction in inflammatory markers and improvement in asthma symptom scores (163-165). Thus, AMP is a sensitive marker of fluctuations in airway inflammation during a relatively short time interval. Whether this is relevant for monitoring chronic inflammation remains to be established.

EXERCISE CHALLENGE AND COLD, DRY AIR CHALLENGE

Exercise and the related cold, dry air challenge have been shown to induce bronchoconstriction in 70-80% of asthmatics (166). Although the underlying mechanisms are still incompletely understood, there is evidence of mast-cell triggered mediator release and neural reflexes contributing to the pathophysiology of these airway responses (153, 167). Indeed, intervention with antileukotrienes, antihistamines and neurokinin receptor antagonists have been shown to protect against exercise/cold dry air-induced bronchoconstriction in patients with asthma (168-171). Presently, these indirect challenges are applied as standardized models of mast-cell mediated mechanisms in asthma in proof of concept studies with new drugs or for diagnostic purposes (7, 154).

ALLERGEN CHALLENGE

Inhalation of serial increasing concentrations of a standardized allergen extract, the so-called allergen BPTs or allergen challenge, is the most useful preclinical model of asthma (7). This indirect challenge can be conducted in two ways: inhalation of aerosolized allergen or local instillation of allergen into a pulmonary segment via bronchoscope. The first method allows investigation of the relation between the allergen-induced airway and inflammatory responses, whereas the latter only allows to study the allergen-induced inflammatory response (7, 18, 172). The characteristics of the inhalational allergen challenge include early asthmatic response (EAR) occurring within 10 minutes after inhalation of an effective dose of a relevant allergen, and a LAR which is associated by allergen-induced AHR, occurring in approx. 50% of the subjects. The EAR is a mast-cell-triggered event, resulting in acute, transient airway smooth muscle (ASM) contraction (0-3 h), whereas the LAR is characterized by a chronic inflammatory response (over 24 h), in which eosinophils and their mediators prevail and induce the associated AHR (up to 3 weeks). Recent evidence has been provided that allergen challenge may induce features of airway remodeling in both animals and humans (173, 174). These characteristics render this model a suitable tool to study both the acute and chronic sequelae of asthma.

STEROID TAPERING

Steroid tapering is an exacerbation model in patients with moderate-to-severe persistent asthma in which gradual reduction of corticosteroid therapy results in a gradual deterioration of FEV_1 and PC_{20} accompanied by an increase in symptom scores and inflammatory markers (21, 175, 176). In clinical trials, steroid tapering is a validated tool to test the effect of potential anti-inflammatory therapy on asthma control. Recent intervention studies applying tapering off corticosteroids have been performed with leukotriene receptor antagonists and anti-IgE (177-180).

RHINOVIRUS AND OZONE CHALLENGES

Rhinovirus and ozone challenge tests are still other, more experimental, exacerbation models of asthma. Similar to allergens, virus infections are important causative agents of asthma exacerbations. Rhinoviruses have been detected in 10-44% cases of adults and 23-86% cases of children with asthma exacerbations (181, 182). Grünberg *et al* showed that experimental rhinovirus infections induce airway inflammation and associated AHR in asthmatics, closely resembling spontaneous asthma exacerbations (183, 184). The inflammatory influx caused by a rhinovirus mainly consists of eosinophils,

neutrophils and their respective mediators (183-186). In analogy with the airway inflammation in severe persistent asthma, the neutrophil component may – at least partly – explain why corticosteroids do not effectively protect against rhinovirus-induced airway inflammation (187,188,189). Experimental viral infections are exacerbation models of asthma suitable to study pathophysiological mechanisms and for early drug development (190). Ozone is another indirect, bronchoactive stimulus inducing acute neutrophil inflammation and oxidative stress into the airways of (non) asthmatic subjects (191-193). Consequently, ozone may be a useful tool to investigate new drugs with anti-oxidant properties (194,195).

Limitations of bronchoprovocation tests and models

Overall, airway challenges with direct stimuli are valuable tools to assess AHR, while indirect challenges are useful to study the relationship between a specific stimulus and the airway inflammatory response. Due to different modes of action, care should be taken when selecting a challenge for a specific protocol.

If performed according to guidelines by adequately trained technicians, BPTs are safe and generally well-tolerated (7). The most common adverse reactions are transient, usually mild respiratory problems (dyspnea, chest tightness, cough) and sometimes headache. The most important health-related contraindications for challenges are active cardiovascular diseases and uncontrolled or severe persistent asthma with an FEV_1 less than 1.2 L (7).

In clinical trials, it should be taken into account that indirect airway challenges may affect markers of inflammation, which also applies for histamine that has been shown to induce an airway exudative response (196). When performed serially, an adequate washout period should be allowed to correct for these "carry-over effects". The length of a washout period is dependent on the pro-inflammatory response of the indirect stimulus and varies between at least 6 hours for histamine and as long as 6 weeks for a rhinovirus challenge (197,198).

Summary and recommendations - Bronchoprovocation tests

AHR and chronic airway inflammation are the hallmarks of asthma (199). Several interventional trials have demonstrated that targeting either component resulted in better asthma control (5, 28). AHR is defined as an exaggerated bronchoconstrictor response to (non)specific stimuli such as cigarette smoke, exercise, viruses or allergens.

TABLE 3   OVERVIEW OF DIRECT AND INDIRECT CHALLENGES/ASTHMA MODELS

STIMULUS	MODE OF ACTION	CHARACTERISTICS	SPECIFIC DRAWBACKS	REFS
Metha-choline	Directly on ASM	Gold standard to quantify AHR by PC_{20}methacholine Potential marker of airway remodeling	Less related to inflammation Not specific for asthma	(7, 153, 155)
AMP	Release of mast cell-derived mediators	Sensitive marker of airway inflammation	No sharp cut-off between atopic asthmatics and atopic controls	(7, 154, 200)
Allergen	Acute release of mast cell-derived mediators (EAR), in ±50% of cases followed by a more chronic inflammatory response primarily mediated by activated eosinophils and their products (LAR)	Most suitable model with both acute and chronic features of asthma (EAR, LAR and AHR)	Strenuous and long-lasting procedure Extra safety needed Long washout period Contraindicated in severe persistent asthma/COPD/ active cardiovascular disorders	(7, 18, 172)
Exercise/ cold dry air	Release of mast cell-derived mediators	Diagnostic tool Suitable model to test efficacy of therapy directed against mast cell-derived mechanisms	Strenuous test, not suitable for elderly or handicapped	(7, 153, 154)
Virus/ Ozone	Activated eosinophils and neutrophils and their mediators	Experimental models to test therapy interfering with virus-induced mechanisms of asthma, including airway eosinophils/ neutrophils; unresponsive to ICS	Extra safety and laboratory procedures needed due to viral inoculation	(183, 194, 218)
Steroid tapering	Induction of exacerbation by withdrawal of anti-inflammatory treatment and subsequent flare up of airway inflammation	Validated model to test efficacy of potential anti-inflammatory therapy on asthma control	Stepwise dose reduction necessitates lengthy trials	(175, 176)

ASM = Airway Smooth Muscle, AHR = airway hyperresponsiveness, AMP = Adenosine 5' mono-phosphate, EAR = early asthmatic response, LAR = late asthmatic response, ICS = inhaled cortico-steroids

Bronchoactive stimuli can either directly or indirectly act on ASM cells and consequently induce bronchoconstriction. Methacholine and histamine are direct stimuli, inducing bronchoconstriction through direct interaction with specific receptors on ASM cells (7, 200). AMP, non-isotonic aerosols, exercise and allergen challenges are examples of indirect stimuli causing ASM contraction through the release of bronchoactive mediators from inflammatory cells and neurons (Figure 3). Challenges with methacholine, histamine and exercise can test the (severity of) AHR, while challenges with most indirect stimuli may serve as functional estimate of airway inflammation in a clinical or research setting. Furthermore, some of these tests have been standardized and have become diagnostic tools or validated models of asthma. For example, AMP challenge appeared a sensitive test to assess (the severity) of airway inflammation (162, 201), while challenges with exercise, allergen, ozone and rhinovirus can induce specific features of asthma, including a transient worsening of airway inflammation and AHR. These challenges are used as validated models for early drug development to test specific pharmacological activity. The pros and cons of BPTs have been summarized in Table 1 and 3.

Recommendations

Chronic airway inflammation is the hallmark of asthma and related disorders, resulting in various features including airway hyperresponsiveness and airway remodeling. Several studies have shown that symptoms and lung function parameters do not reflect the activity of the airway inflammation in asthma, and hence do not provide adequate information on asthma control (5, 28, 203, 204). Alternatively, inflammatory indices, such as sputum eosinophils and eNO, appeared to be good indicators of asthma severity and control (205). Therefore, sampling of airway inflammation is becoming an integral part of the diagnosis, and monitoring of asthma and a valuable tool to test the pharmacological efficacy of anti-inflammatory therapy in early clinical trials. Presently, there is an increasing array of non-invasive methods aimed at sampling the airway inflammation. Standardized and validated markers of airway inflammation include eNO and sputum eosinophils, while exhaled breath condensate is a promising method that still awaits refinement of technique and more sensitive assays. In addition, there are more or less standardized and validated bronchial provocation tests for quantification and or assessment of airway hyperresponsiveness (methacholine, histamine, exercise challenge), airway inflammation (adenosine monophosphate), or to mimic

specific pathways during a flare up of asthma – mostly applied in early clinical trials (exercise, allergen, rhinovirus, ozone). Although the methacholine BPTs has been shown to be correlated with some features of airway remodeling, there is still a paucity of non-invasive markers for this complicated asthma feature, except imaging (Figure1).

The most promising future biomarker for asthma remains to be identified and should be disease-specific, non-invasive, reproducible, inexpensive, fast and simple and well- responding to the pharmacological activity of anti-asthma therapy. The most recent developments point towards the potential application of so-called 'omics' techniques to blood, sputum and recently even to exhaled breath (206, 207). Such systems may drastically change the screening and monitoring of airway disease.

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SECTION 1 – A CRITICAL APPRAISAL OF METHODS USED IN EARLY CLINICAL DEVELOPMENT OF NOVEL DRUGS FOR THE TREATMENT OF ASTHMAS

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