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The added value of targeting airway hyperresponsiveness by blocking thymic stromal lymphopoietin (TSLP) in the management of severe asthma

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Key words

Severe asthma; airway hyperresponsiveness; airway epithelium; TSLP; Tezepelumab.

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Impact statement

Correct use of diagnostics and novel drugs targeting AHR will improve the treatment of severe asthma.

Summary

Airways hyperresponsiveness (AHR) is a pathognomonic event of asthma in which the airways are reactive to various bronchoconstrictor stimuli at 'doses' that normally have no bronchoconstrictor effect in non-asthmatics. AHR is an objective measure of clinical efficacy, and the introduction of biologics revived interest as a marker of disease and its pathophysiologic mechanism.

This article aims to discuss the mechanisms of AHR, focusing on the role of epithelial damage and thymic stromal lymphopoietin (TSLP) production, and promote its correct assessment for the evaluation of patients with severe asthma, to predict the risk of exacerbations and outcomes, and the eligibility for treatment with an anti-TSLP agent.

AHR is a complex trait of asthma, induced by the concurrence of many pathophysiological factors and related to different clinical manifestations. Recent evidence demonstrates the important role of airway epithelial damage and TSLP production in many of these events.

A therapeutic response based on AHR control could be considered as a condition of disease remission and seems a promising new goal for the management of patients with severe asthma.

Introduction

Airways hyperresponsiveness (AHR) is a pathognomonic event of asthma in which the airways are reactive to various bronchoconstrictor stimuli at 'doses' that normally have no bronchoconstrictor effect in non-asthmatics (1). Measurement of AHR is an objective methodology for asthma diagnosis (2) and for the assessment of response to asthma treatments. The European Medicines Agency (EMA) guideline on the clinical investigation of medicinal products for the treatment of asthma states that broncho protection (*i.e.*, the ability of a drug to provide protection against bronchial challenge) is an acceptable objective measure of clini-

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cal efficacy (3). Positive response to AHR tests is usually present during asthma attacks and is a parameter related to variable expiratory flow rates, clinical symptoms of asthma, risk of exacerbations and functional respiratory decline in patients with asthma (4-7). AHR has also been proposed as a prognostic tool for the assessment of exacerbation risk (8). Moreover, AHR induced by allergens could reproduce allergic-specific asthma reactions and detect the impact of the epithelial barrier in asthma pathogenesis (9-11).

While the role of AHR testing in the diagnosis of mild to moderate asthma is widely shared, its role in severe asthma is less well understood. In-depth advancements in the therapeutics of asthma, with the introduction of biologics, revived interest in AHR as a marker of disease and its pathophysiologic mechanism. Additionally, the role of AHR in the algorithm for the definition of severe asthma clinical remission is being investigated, aiming at assessing its role as a prognostic biomarker of response to biologic treatments (12, 13), as symptoms could be a consequence of hyperreactivity (14).

Therefore, a reappraisal of evidence on AHR is necessary to understand the use and new perspectives of AHR tests in clinical practice. This article aims to discuss the mechanisms of AHR, focusing on the role of epithelial damage and TSLP production, and promote its correct assessment for the evaluation of patients with severe asthma, to predict the risk of exacerbations and outcomes, and the eligibility to treatment with an anti-TSLP agent.

Methods

This article presents the established experience of the authors in the assessment of AHR for the management of asthma, focusing on severe patients. They propose a narrative review of the literature, providing a historical and up-to-date overview of mechanisms of AHR and technical methods for assessment and supporting their interpretation of current evidence on the role of bronchial epithelium and TSLP in this context, as well as tezepelumab as a new antibody for addressing AHR in severe asthma. PubMed has been searched by cross-matching relevant keywords: “asthma”, “airways hyperresponsiveness”, “direct test”, “indirect test”, “methacholine”, “histamine”, “allergy”, “inflammation”, “remodeling”, “airway epithelium”, “TSLP”, “tezepelumab”, “diagnosis”, “therapy”, “prognosis”, “symptom”. Articles in English or with English abstracts have been considered, evaluated, and included based on the expertise of the authors and the relevance to the subject.

The pathogenesis of AHR

AHR is a common pathophysiologic event in asthma, and many mechanisms, including inflammation, airway remodeling, and hyperreactivity of bronchial smooth muscle cells, contribute differently to its development in individuals. Heterogeneity of mechanisms, variable impact of environmental factors, aging, therapy, genetics, and epigenetic factors result in a great variability of AHR (4, 6, 15-17).

Contraction of bronchial musculature is the effector step of AHR, and the anomalous contractility of airway smooth muscle cells (ASMC) is an important component in the increased bronchoconstrictor response to stimuli in asthma. The abnormal response of ASMC may be linked to intrinsic or microenvironment changes. Pathologic changes that result in epithelial damage, bronchoconstriction, mucus secretion, bronchial wall edema, muscle hypertrophy and reversible airway obstruction (18, 19) are all strictly related to the physiopathology of AHR. Each patient could express a special phenotype of such a network, and this could be relevant to therapy (15, 20). Changes in cells playing a role in these interconnected mechanisms are also interrelated, and the network change should be understood better than single-cell type changes.

Inflammation and AHR

Epithelial-induced inflammation is one of the major contributors to the physiopathology of AHR, with subjective and environmental factors impacting its relevance (**figure 1**). The presence of AHR in subjects with asthma has commonly been correlated with the number of inflammatory cells in sputum and airway tissue (21-25), but recent evidence showed that specific epithelial-derived cytokines (*i.e.*, TSLP) are the ultimate master drivers of inflammation and AHR in asthma, as also confirmed by genomic studies (26, 27).

The presence of inflammation in airways was associated with the severity of AHR (4, 17, 28-31).

Inflammatory phenotypes: role of eosinophils

The intensity of bronchial eosinophilic inflammation was related to the response to indirect AHR tests (15, 32-39), but data are inconsistent (35, 40-45). Indeed, the main mechanism of AHR may be allergic or eosinophilic inflammation in some subjects, airway remodeling, non-T2 inflammation, or neuronal dysfunction in others. AHR is independent of the inflammatory phenotype (46) and may be considered a marker of mast cell activation through the epithelium (47).

Indeed, the severity of exercise-induced bronchoconstriction is not correlated with the concentration of eosinophils in induced sputum, and direct AHR persists after depletion of sputum eosinophilia obtained through IL-5 blockage (48-54). Contractility is increased by neurokinins released from nerve terminals in patients with exercise-induced bronchoconstriction (44, 50). Additionally, Al-Shaikhly *et al.* (41) could not find a significant correlation between sub-epithelial or epithelial eosinophils and direct AHR, while intraepithelial eosinophil density correlated with severity of exercise-induced bronchoconstriction. These data showed that intraepithelial eosinophils are only a specific feature of asthma

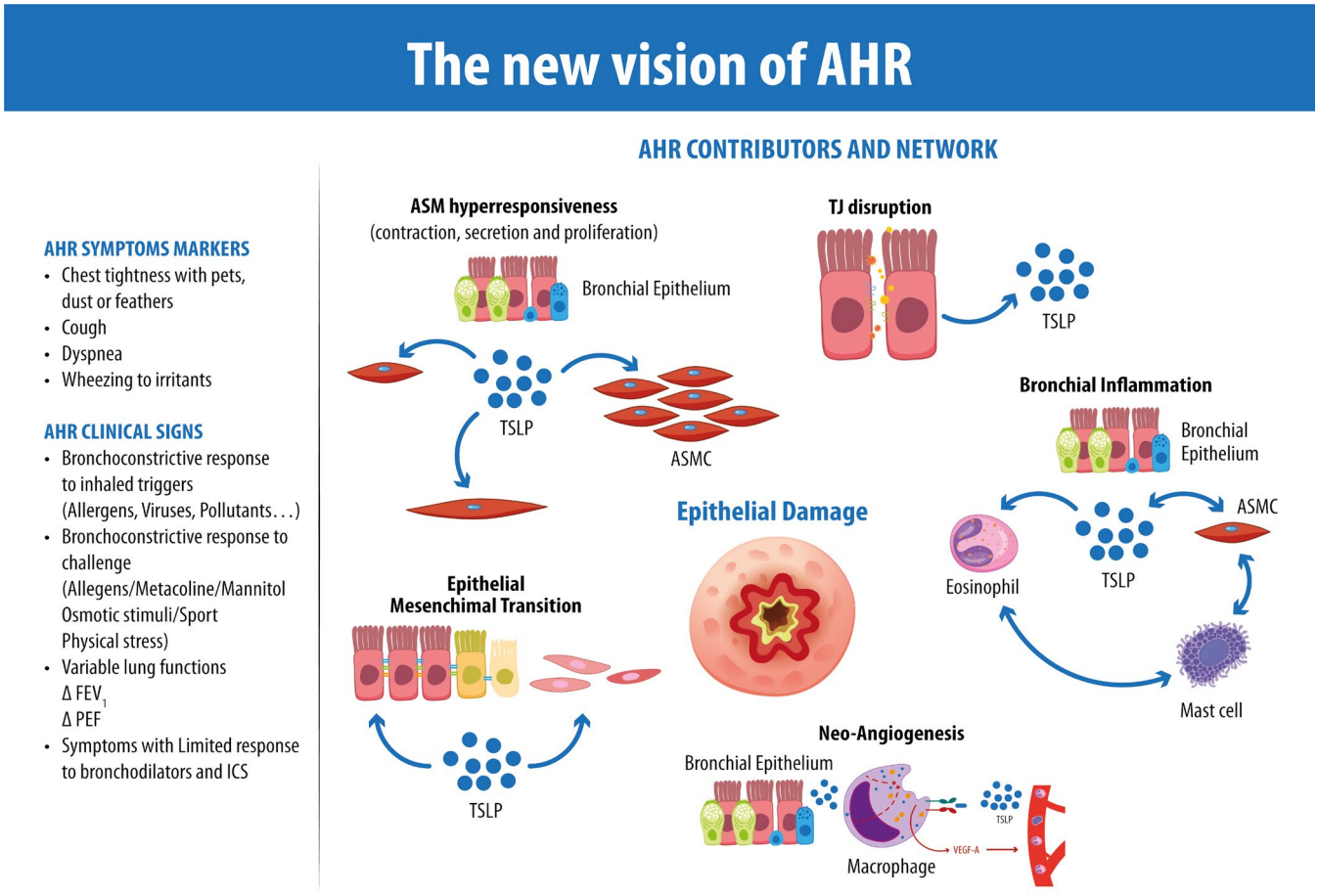
and are related to the severity of indirect AHR and T2 inflammation, in contrast with previous studies showing a correlation of indirect AHR with eosinophils under the mucosa (55). Intraepithelial eosinophils may be easily stimulated by external factors, explaining the reactivity of patients with asthma (56-58). Differently expressed genes in the epithelium of patients with only direct AHR and in patients with indirect AHR were found, correlating with the density of mast cells and eosinophils in the epithelium (26).

Mast cells

In the airways, mast cells with high expression of chymase and tryptase (MC-tc) are prevalent in the submucosa (prevalent in healthy subjects), and those with high expression of tryptase (MC-t) mainly infiltrate the mucosa (prevalent in asthma) (40). Indeed, the number of intraepithelial MCs is correlated with the presence of indirect AHR and the presence of type 2 inflammation (40, 59, 60). MCs in patients with asthma have signs of degranulation and activation, suggesting an increased turn-over. Additionally, MC-derived mediators are increased in bronchoalveolar lavage (BAL) from patients with asthma (61-64). The relation of the epithelium with the MCs is a pivotal factor for the development of indirect AHR, as found in exercise-induced asthma (65-68).

The epithelial-MC cross talk is involved in AHR both with type 2 and non-type 2 inflammation, suggesting that this could be a therapeutic target across all asthma phenotypes (42). Moreover, MCs and eosinophils ("allergic effector unit") activate each other, contributing to the development and persistence of inflammation and AHR (69-72). Nevertheless, active MCs may contribute to AHR also independently of eosinophils (73, 74). MCs infiltrate within the airway musculature was observed in patients with asthma, indirect AHR and a non-type 2 inflammatory phenotype (42).

Figure 1 *- The pivotal role of epithelial damage in mechanisms contributing to the development of airway hyperresponsiveness.*



MCs may be activated by neurotransmitters and neuropeptides and have a role in neurogenic inflammation, and indirect AHR (75-77). Also, neuropeptides from MCs promote the innervation of the muscular layer, increasing contractility and responsivity (78, 79).

Arachidonic acid derivatives

The epithelium exerts its regulating activity of the airway tonus also by modulating cytokines and arachidonic acid derivatives production. It produces arachidonic acid metabolites with bronchoprotective and broncho-dilating activity; the alarmins TSLP and IL-33 may increase the production of the bronchoconstrictors PGD2 and cysLT by inflammatory cells (59). The airway epithelium modulates the production of inflammatory eicosanoids by MCs and eosinophils (60, 50, 80, 81) highlighting its considerable potential as a target through its cytokines to modulate AHR. In conclusion, current evidence shows that TSLP is involved in many inflammatory mechanisms with pathophysiological relevance in asthma and may be an important therapeutic target in intercepting and modulating such mechanisms.

Epithelial damage, remodeling, and AHR

Structural changes in large and small airways are typical of asthma and include disruption of the epithelial layer, increased osmolarity of periciliary fluid, hyperplasia, metaplasia of goblet cells and submucous glands, thickening of the basement membrane (subepithelial fibrosis), increased bronchial smooth muscle cell number, angiogenesis, and lost relationship between small airways and lung parenchyma. All these events are strictly related to AHR and need to be considered when evaluating the response to bronchoconstriction direct and indirect stimuli (**figure 1**).

Epithelium

Aeroallergens or microbial pathogens induce epithelial cells to secrete interleukins (IL), alarmins (IL-25, IL33, and TSLP), and chemotactic factors (CXCL8, CCL5, CCL17, and CCL20), cooperating in the initiation of innate or acquired immune responses (11, 20, 82-86) and inflammation (20, 87-89). These reactions contribute to the disruption of the epithelial barrier and promote further factor release while inhibiting the production of antimicrobial peptides (20, 90-94). The increased permeability of the damaged epithelium allows changes in osmolarity and the entrance of pathogens and irritants that may reach nerve terminals and inflammatory cells. Concurrently, epithelial-derived cytokines directly activate inflammatory cells of the innate and acquired immune system and ASMC. Once activated, immune cells synthesize secondary mediators like IL-5, IL-13, and IL-4, resulting in inflammation amplification (20, 59, 84).

Osmolarity

The airway epithelium regulates the electrolytic balance, volume and osmolarity of the periciliary fluid. High changes in osmolarity induce cell damage and may be a bronchoconstrictor trigger in patients with asthma (95). The epithelial regulation of osmolarity is the main target of indirect stimuli of AHR, such as exercise, hypertonic and hypotonic solutions (4, 95-99). Indeed, the response to stimuli that directly increase the periciliary fluid osmolarity, such as hyperosmotic solutions, and those that act indirectly, such as hyperventilation, are strictly related to AHR (100-102). Epithelial cells under osmotic stress produce alarmins (59), suggesting that mechanical and osmotic stress of the airway epithelium is related to the development of AHR. This mechanism may explain the asthma and exercise-induced bronchoconstriction in athletes practicing winter sports who have extreme hyperventilation in cold and dry air (103). So, the epithelium results easily damaged, with desquamation and layer breaks, inducing cytokine releasing (29, 48, 50, 103-105). Chronic epithelial damage with increased permeability and reduced bronchoprotective molecules may promote exercise-induced asthma in athletes and subjects with asthma (29, 106-108). Asthma of elite athletes is a clinical model showing that epithelial injury, production of inflammatory mediators, and epithelial cytokines are important factors in the development of AHR in subjects with asthma.

Mucus

The main changes in the epithelium of patients with asthma (thickened basement membrane, loss of cilia and junctions, anomalous mucus with overexpression of MUC5AC and MUC2) (20, 82-85, 90, 109) have been associated to AHR and tissue remodeling (90, 110-117).

Neo-angiogenesis

Neo-angiogenesis is a fundamental player of airway remodeling and is correlated to limited airflow, AHR, and asthma severity (118-122). Human endothelial cells express TSLP receptors and TSLP induces their proliferation and vascular endothelial growth factor A (VEGF-A) release from human lung macrophages (123).

Smooth muscle cells

Additionally, the abnormal bronchoconstriction response to stimuli could be due to increased velocity of ASM shortening in asthmatic AHR and increased constriction (124, 125). Epithelial injury is associated with increased ASMC proliferation mediated by IL-6, IL-8, MCP-1, and MMP-9 (126) and by growth factors (TGF-β, PDGF, FGFs, and VEGF) (127). Additionally, damaged epithelial cells release soluble mediators and Ca+ ions activating ASMC (128), induce muscular hypertrophy and increase ASMC migration (129-131). These data suggest that asthma may develop independently of inflammation through the reinforcing effects of bronchoconstriction and epithelial injury on each other (52).

Epithelial-mesenchymal transition

Stressing stimuli induce epithelial-mesenchymal transition in primary airway epithelial cells from patients with asthma by pro-

duction of transforming growth factor-beta1 (132). This factor promotes the production of extracellular matrix by fibroblasts, expressing the receptor for TSLP, and the change in the extracellular matrix induces ASMC proliferation (133). Besides this indirect stimulation, also ASMC express the receptor for TSLP (TSLPR) (134, 135) and are directly stimulated by TSLP through transcription factors (MAP kinases ERK1/2, p38 and JNK), increasing their contractility (134, 136).

Mast cells

MCs are activated by TSLP, and produce CysLTs and PGD2 inducing ASMC migration and non-type 2 cytokines, including TNF-α and IL-1β that may activate ASMCs, and thus induce AHR by a non-type 2 mechanism (137, 138).

Indirect AHR is correlated to MC density in the airway epithelium in subjects with type 2 inflammation, while it is correlated with MC infiltration of bronchial muscularis in subjects with non-type 2 inflammation. Reduction of indirect AHR following therapy was associated with reduction of the MC infiltrate in the epithelium (subjects with type 2 inflammation) and the muscularis (subjects with non-type 2 inflammation) (42).

Clinical impact of airway changes

Clinical signs of asthma are the result of epithelial dysfunction, inflammation, large and small airway remodeling, and increased contractile response of ASMCs (139, 140). The reversible component of AHR is conventionally attributed to inflammatory mechanisms, and the non-reversible one to the remodeling of airways (17, 35, 141). Airways remodeling, assessed as the thickness of the bronchus wall, is associated with progressively impaired FEV_1 (142), asthma severity (143), irreversible obstruction and air trapping (142, 144, 145). An increased response to direct AHR stimuli in patients with non-reversible asthma may be due to an altered shape of airways, resulting in a reduced FEV_1 independent of the smooth muscle reactivity level (145-147). Boulet *et al*. also demonstrated that remodeling, assessed as intermediate bronchus wall thickness, is correlated with direct AHR in patients with asthma and irreversible obstruction (148).

Different pathways of remodeling may thus act differently on AHR, with effects varying from increased intrinsic responsiveness of ASMC to a geometric structural change of airways. Current evidence suggests that a great amount of remodeling is correlated with a greater ventilatory disparity, air trapping, airway closure and small airway dysfunction, which may together contribute to the development of AHR (141, 144, 145, 149-151).

Ventilation heterogeneity is correlated with AHR independently of airway inflammation and with clinical features of asthma (35, 152). ASMC remodeling as well as bronchial obstruction, are critical only in some areas of airways (149, 153, 154). With uniform, smooth muscle contraction, minimal heterogeneity of airway caliber may lead to clusters of poorly ventilated lung units and, at critical muscle contraction, induce sudden airway obstruction (155). Heterogeneous remodeling and areas of poor ventilation are stable, and the thickening of muscularis is a patchy defect, not involving the whole airways (156, 157).

Although the mean difference in bronchial wall thickness is not different in asthmatic and healthy subjects, patients with asthma have thickened airways in some areas only, especially in near-fatal asthma (154). Thus, measuring mean thickness or a single section thickness of airways may not be a reliable assessment of airway remodeling. The hypertrophy of some areas may induce dramatic obstruction, although other areas have only a low level of remodeling (155, 158, 159). The heterogeneity of ventilation is mainly due to remodeling, inflammation, and ASMC responsiveness but is also increased by exudate, mucus abnormality and reduced surfactant (86). Indeed, mucus plugs were observed in at least four lung segments, in 67% of subjects with asthma showing FEV_1 < 60% of theoretical value (160), and 82% of subjects with asthma have mucus plugs persisting for at least 3 years, usually in the same segment of airways (161). Mucus plugs are more evident during exacerbations with obstruction of at least 40% of airways (162) and are associated with regional ventilation defects (163).

In conclusion, all these observations confirm that AHR is the result of many mechanisms, which may have different relevance in individuals, resulting in clinical types with possible different therapeutical needs.

Challenge tests for AHR assessment

As described below, AHR in asthma comprises both variable and fixed components, each contributing to the overall sensitivity and reactivity of the airways. Understanding these components is crucial for characterizing the dynamic nature of AHR in individuals with asthma.

The variable component of AHR refers to the reversible and transient narrowing of the airways in response to various stimuli. This component is characterized by acute bronchoconstriction that can be triggered by factors, such as allergens, exercise, cold air, or respiratory infections. The degree of variability in airway narrowing is typically assessed through bronchoprovocation tests, such as the methacholine challenge or exercise challenge tests (164, 165).

The variable component reflects the dynamic nature of asthma symptoms, where individuals may experience fluctuations in airflow obstruction in response to different environmental or physiological triggers. Pharmacological interventions, such as bronchodilators (*e.g.*, short-acting beta-agonists), are often effective in rapidly reversing this component, providing relief from acute bronchoconstriction, and improving lung functions (166).

In contrast, the fixed component of AHR refers to persistent and irreversible structural changes in the airways, leading to increased baseline airway resistance. This component is associated with air-

way remodeling, epithelial damage, and alterations in the extracellular matrix (165). Unlike the variable component, the fixed component is less responsive to bronchodilator therapy and is indicative of long-term changes in the architecture of the airways. These structural alterations contribute to a heightened baseline airway resistance, even in the absence of acute triggers. The fixed component is considered a more permanent aspect of AHR, reflecting the chronic and progressive nature of asthma in some individuals (166). Understanding the interplay between the variable and fixed components of AHR is crucial for tailoring asthma management strategies. While bronchodilators effectively target the variable component, addressing the fixed component may require anti-inflammatory therapies aimed at modifying the underlying inflammatory and remodeling processes. This comprehensive approach is essential for achieving optimal asthma control and improving long-term outcomes for individuals with asthma.

Assessment of bronchial hyperresponsiveness is commonly utilized in both clinical practice as well as in research settings and provides clinicians with objective measures to assess bronchial hyperresponsiveness and guide treatment decisions in asthma management. Regular monitoring of bronchial hyperresponsiveness is crucial for optimizing asthma treatment and adjusting therapeutic strategies based on individual patient responses. The choice of specific tests depends on factors such as patient age, clinical presentation, and the availability of testing facilities. Moreover, not all tests are indicated in severe forms of asthma, especially in case of uncontrolled disease, with difficulties in assessing AHR presence or grading in a specific patient.

Direct and indirect challenge tests

AHR may be assessed by direct and indirect challenge tests based on the type of stimulus and based on reflective components that one would like to evaluate (6, 167).

Direct bronchoprovocation challenges (*e.g.*, methacholine) act directly on specific airway smooth muscle receptors, M3 or H1 (6) and are more sensitive and less specific than indirect challenges. In subjects with clinically current symptoms (within a few days) who inhale methacholine without deep inhalations, a normal methacholine test (provocative concentration causing a 20% fall in FEV_1 [PC20] >16 mg/mL) rules out asthma with reasonable certainty. Arbitrary cut points have been set for predictive values. A positive test in the moderate or greater range (PC20 < 1 mg/mL) has high specificity and positive predictive value, comparable to the indirect challenges (6).

Indirect challenges, by physical or pharmacological stimulus, cause the release of endogenous bronchoconstrictor mediators from epithelial cells, mastocytes, and eosinophils and stimulate nerve terminals (4, 167). The indirect challenges commonly used in pulmonary function laboratories include exercise voluntary hyperpnea, hypertonic (4.5%) saline, and mannitol (167). All these indirect challenges are associated with the release of mast cell mediators (*e.g.*, prostaglandins, leukotrienes, and histamine). Although hyperresponsiveness to indirect challenges is frequently associated with sputum eosinophilia, it is not a prerequisite because the mast cell is the most important source of mediators (167). Airway sensitivity to indirect challenges is reduced or even totally inhibited by treatment with inhaled corticosteroids (ICS), so a positive response to an indirect stimulus is believed to reflect active airway inflammation. Indirect challenges are appropriate to inform further on both the pathogenesis of asthma and the role of anti-inflammatory agents in its treatment (167).

Direct challenge tests use standardized protocols based on the administration of growing concentrations of the agonist using a breath-actuated or continuous nebulizer or by deep inhalation in the dosimetric method (6, 168). The methacoline test is considered positive when the provocative concentration (PC20) and the provocative dose (PD20) result in 20% decrease in Forced Expiratory Volume in the first second (FEV_1) (164, 168, 169-174).

Other functional tests

AHR may also be assessed by other functional tests, for example, by lability in peak asthma flow (PEF), which some studies have correlated with direct and indirect AHR (175). The most useful index of PEF lability in the management of asthma (stable although either controlled or uncontrolled) was found to be the minimum morning prebronchodilator PEF over a week (expressed as percent recent best or percent predicted) because it strongly correlates with AHR (176). In this context, PEF variability could be used as an index of disease activity.

Symptoms correlated to AHR

Several authors found a positive correlation between airway responsiveness and some of the symptoms investigated by questions from the standardized asthma questionnaire (177-183). Symptoms like wheezing, shortness of breath, cough and history of dyspnea episodes were significantly correlated with methacholine responsiveness. These symptoms can vary in severity and frequency from person to person and can be triggered by various factors such as allergens, exercise, cold air, or respiratory infections. Since some symptoms may be considered surrogate clinical markers of AHR, is it possible to speculate that this can be useful tools to recognize AHR in patients with severe asthma, when testing with direct or indirect challenges is not suitable?

Potential surrogate markers of AHR useful to monitor during normal clinical practice are listed in **table I**.

Indirect AHR challenges may be represented by scalable chemical stimuli (*i.e.*, mannitol, hypertonic or hypotonic solutions, adenosine) or single bouts of high-intensity hyperventilation (*i.e.*, high-intensity physical activity, eucapnic voluntary hyperventilation (EVH) (44, 59, 184-188). The indirect tests are considered positive when FEV_1 is reduced by 15% *vs* baseline in the stress testing and by 10% in the EVH test (189).

Interpretation and indications of tests for AHR

The main indication of direct AHR tests is confirmation of asthma diagnosis in subjects with normal spirometry (18, 190, 191). No gold standard is available for the diagnosis of asthma; therefore, diagnosis is based on clinical data (mainly the probability of asthma pre-test) and function tests demonstrating a variable respiratory function. Among these tests, the AHR assessment has the best diagnostic performance. However, this performance is dependent on the pre-test probability and cut-off used to define positivity (6, 168, 190-192). The direct AHR test with methacholine, if the whole range of positivity is considered (*i.e.*, a PC20 between 0.0625 and 8 or 16 mg/ml methacholine, corresponding to 0.1425 to 190 or 380 µg methacholine), has high sensibility and positive predictive value (PPV) but has a low specificity. False positivity is not rare, as in subjects with asymptomatic hyperreactivity or subjects with atypical symptoms and low pre-test probability, while negativity is a reliable result and may rule out current asthma in symptomatic subjects (6, 7, 174, 192, 193). A moderate-high AHR response (PC20 < 1m/ml methacholine, corresponding to PD20 < 23.75 µg methacholine) is highly spe-

Table I *- Surrogate markers of airway hyperresponsiveness.*

Test type	Bronchial hyper-reactivity and associated symptoms	Reference
	Direct tests	
Methacholine challenge test	• Wheeze, • Wheeze with dyspnea, • Cough • History of chronic bronchitis, pneumonia, and acute bronchitis	Dales RE, Ernst P, Hanley JA, Battista RN, Becklake MR. Prediction of airway reactivity from responses to a standardized respiratory symptom questionnaire. Am Rev Respir Dis. 1987;135(4):817-21. doi: 10.1164/arrd.1987.135.4.817.
	• Wheezy chest • Attacks of shortness of breath with wheezing • Dry cough at night	Remes ST, Pekkanen J, Remes K, Salonen RO, Korppi M. In search of childhood asthma: questionnaire, tests of bronchial hyperresponsiveness, and clinical evaluation. Thorax. 2002;57(2):120-6. doi: 10.1136/thorax.57.2.120.
	• Wheezing, • Shortness of breath • Cough • History of episodes of dyspnea and wheeze	Yurdakul AS, Dursun B, Canbakan S, Cakaloğlu A, Capan N. The assessment of validity of different asthma diagnostic tools in adults. J Asthma. 2005;42(10):843-6. doi: 10.1080/02770900500370981.
	• Cough • Cough from chest • Shortness of breath • Chest tightness	Shin B, Cole SL, Park SJ, Ledford DK, Lockey RF. A new symptom-based questionnaire for predicting the presence of asthma. J Investig Allergol Clin Immunol. 2010;20(1):27-34.
Histamine challenge test	• Shortness of breath or wheezing, or both to irritants like cold air, smoky atmospheres, traffic fumes, and common household chemicals (hair sprays, perfumes, bleach, *etc*.) • Bronchial irritability • Nocturnal dyspnea • Morning tightness	Mortagy AK, Howell JB, Waters WE. Respiratory symptoms and bronchial reactivity: identification of a syndrome and its relation to asthma. Br Med J (Clin Res Ed). 1986;293(6546):525-9. doi: 10.1136/bmj.293.6546.525
	• Wheeze • Shortness of breath • Tightness in the chest on coming into contact with animals, dust or feathers	Burney PG, Chinn S, Britton JR, Tattersfield AE, Papacosta AO. What symptoms predict the bronchial response to histamine? Evaluation in a community survey of the bronchial symptoms questionnaire (1984) of the International Union Against Tuberculosis and Lung Disease. Int J Epidemiol. 1989;18(1):165-73. doi: 10.1093/ije/18.1.165.
	Indirect tests	
Inhaled procaterol	• Wheeze • Breathlessness • Chest tightness • Cough[7]	Tomita K, Sano H, Chiba Y, Sato R, Sano A, Nishiyama O, *et al.* A scoring algorithm for predicting the presence of adult asthma: a prospective derivation study. Prim Care Respir J. 2013;22(1):51-8. doi: 10.4104/pcrj.2013.00005.

cific. It has a high PPV but has little sensibility and may result in many false negative responses.
Its performance is like that of indirect tests, and a positive test may be used to confirm the diagnosis of asthma (6, 174). As a result, the higher the pre-test probability, *i.e.*, if reported symptoms are recent and characteristic of asthma, and the lower the PC20 and PD20, the higher the probability that a positive methacholine test is associated with asthma (6, 168,174).
In conclusion, the indirect tests have higher specificity and lower sensibility for asthma diagnosis than direct tests and do not detect subjects with mild or borderline AHR, which the methacholine test can show (6, 100, 194-196). Several studies confirmed the low sensibility and high specificity of indirect tests, which are indicated to confirm a diagnosis of asthma more than to rule it out (33, 197-200).
Another clinically relevant characteristic of indirect tests is their correlation with eosinophilia of airways, measured as number of eosinophils in the sputum and by expired NO (31, 33), and with mastocyte infiltrate in airways (201). The response to hypertonic or hypotonic stimuli is associated with exercise-induced bronchoconstriction (202, 203) and responses to hyperpnea (100), while test with mannitol is less sensible for exercise – related asthma (101). It results that indirect tests are rarely positive if direct tests are not, but they can also be negative in the presence of a positive AHR with methacholine test, confirming that asthma may not always be associated with inflammation (33, 185, 198).

AHR and biologic treatments: focus on tezepelumab

Treatment strategies targeting the abnormal responsiveness to bronchoconstrictors featuring direct and indirect AHR attained good outcomes in patients with mild-moderate asthma, including reducing the risk of exacerbations and remodeling of airways. Inhaled corticosteroids (ICS), with or without long-acting beta-agonists (LABA), with the possible addition of long-acting muscarinic antagonists (LAMAs), have been the cornerstone of asthma management for decades (204). Nevertheless, not all patients are controlled due to the heterogeneity of the disease, and patients with severe asthma still have unsatisfactory outcomes (5, 205). Recently, many biological therapies have been licensed for severe asthma, demonstrating positive clinical effects on exacerbations, symptom control and lung functions. They have different mechanisms of action and target components mainly belonging to inflammatory pathways response of the airways (206). Many studies confirmed the positive effect on inflammation or on common clinical parameters of these therapeutic options, but little evidence is available on the potential effect on the second hallmark of asthma, AHR. The latter is particularly important in the field of severe asthma since this resistant form of the disease does not respond in terms of protection to bronchoconstriction stimuli.
Effect of omalizumab (anti-IgE mAb) on AHR was assessed in 9 studies (207-215) but only in three studies the drug showed slight reduction in AHR to challenges as methacholine, acetylcholine, and AMP (207, 209, 215), and mainly in moderate allergic asthmatics (nevertheless studies were not consistent for dose, route of administration, asthma severity and type of test). No studies assessed omalizumab's effect on mannitol testing.
Only three pieces of evidence are available on mepolizumab, but without any effect on AHR displayed by the IL-5 antibody (51, 216, 217).
On the contrary, Chan *et al*. showed that benralizumab-induced eosinophil depletion is associated with attenuated mannitol AHR in severe uncontrolled eosinophilic asthma (24).
To date, there are no published *in vivo* studies relating to dupilumab and AHR.
More convincing evidence is derived from tezepelumab, the anti-TSLP monoclonal antibody. Three different studies showed that TSLP inhibition induced by tezepelumab reduced AHR to methacholine and to mannitol (47, 218, 219). In addition, it was shown that tezepelumab reduced both early and late allergic responses (218-220). In a mouse model of respiratory allergy to house dust mites, resulting in AHR to methacholine, the administration of tezepelumab inhibited inflammation, preventing the overexpression of IL-4, IL-13, TSLP, and TGF-β1. Control of airway inflammation was associated with inhibition of structural remodeling and reduced AHR to methacholine (221).
The first double-blind, randomized clinical trial with tezepelumab, was conducted in 31 patients with mild allergic asthma (218). It demonstrated that treatment was effective on early and late allergic responses, reduced AHR to methacholine, allergen-induced bronchoconstriction, FEV_1 decline, and eosinophil count in blood and sputum. The PC20 to methacholine challenge was significantly increased on day 83, compared to the group receiving placebo ($p = 0.004$).
The multicenter, exploratory, double-blind, randomized, placebo-controlled, phase 2 CASCADE study assessed the effect of tezepelumab on airway inflammatory cells, airway remodeling, and AHR in adult patients with moderate to severe uncontrolled asthma (219). Overall, 116 patients receiving inhaled corticosteroids were randomized either to tezepelumab 210 mg or placebo, subcutaneously every 4 weeks. Patients in the tezepelumab group had a significantly greater reduction in AHR to mannitol *versus* placebo ($p = 0.030$). A larger proportion of patients in the tezepelumab group had a negative AHR to mannitol at the end of treatment (13/30, 43% *vs* 7/28, 25% in the placebo group).
UPSTREAM was a double-blind, placebo-controlled, randomized trial designed to evaluate whether tezepelumab decreases AHR and airway inflammation in patients with symptomatic asthma resistant to inhaled corticosteroids (220). It enrolled adult patients with asthma and AHR to mannitol, who received either 700 mg tezepelumab or placebo intravenously every 4 weeks. At week 12, AHR to mannitol was more reduced by tezepelumab than by placebo (mean reduction of PD15 was 1.9, 95%CI 1.2-2.5 *versus* 1.0,

95%CI 0.3-1.6, in the placebo group). The test was negative in 9 (45%) tezepelumab and 3 (16%) placebo patients (p = 0.04). This improvement was especially evident in patients with eosinophilic asthma. Eosinophils in airway tissue and BAL decreased by 74% (95%CI -53 to -86%) and 75% (95%CI -53 to -86%), respectively, with tezepelumab, while they increased by 28% (95%CI -39 to 270%) and decreased only by 7% (95%CI -49 to 72%), respectively, with placebo (p = 0.004 and p = 0.01). The total mast cells in airway mucosal biopsies decreased by 25% (95%CI -47 to 6%) in the treated group and increased by 18% (95%CI –18 to 69%) in the placebo group (p = 0.07). These results demonstrated that tezepelumab efficacy in patients with asthma may be obtained both with type 2 inflammation and non-type 2 inflammation (220). In this trial, tezepelumab improved allergen induced broncoconstriction, in contrast with results obtained with anti-IL-5 biologics (24, 51, 216, 217). This data supports the hypothesis that inhibition of TSLP-related mast cell activation contributes to attenuation of AHR by tezepelumab. Treatment of this smooth muscle cell component of asthma would be the mechanism of tezepelumab benefit on non-type 2 asthma. A larger effect size might have been observed if the study had recruited only patients with severe AHR (PD15 < 35 mg) to mannitol. These data suggest that the primary mechanism by which tezepelumab improves asthma clinical and physiological outcomes is suppression of airway eosinophilia.

In a prespecified exploratory analysis of the Phase III NAVIGATOR trial, tezepelumab resulted in early and sustained improvements in morning and evening PEF compared with placebo, with effects observed at the first week of administration and continued over the 52-week observation. Clinically meaningful improvements from baseline in morning and evening PEF were observed with tezepelumab as early as week 2 (222).

The clinical results obtained with tezepelumab confirm the relevance of preclinical data demonstrating the pivotal role of epithelial damage and TSLP in the pathophysiology of inflammation and airway remodeling associated with AHR. Indeed, TSLP is central in the development of inflammation in response to epithelial damage, resulting in eosinophilia activation of MCs and AHR. Additionally, TSLP is involved in airway remodeling and AHR through increased epithelium permeability, osmolarity changes, loss of ciliary function, mucus hypersecretion, angiogenesis, and direct activation of ASMC by damaged epithelial cells. Tezepelumab acts on several components of AHR, including closure that is often found in patients with severe asthma (15). This activity could be mainly linked to an improvement of baseline ventilation heterogeneity (155), and contributes to reducing airways remodeling, and to prevention of airways closure (15, 141). It must be remembered that the tezepelumab trials (219, 223) enrolled patients with asthma not controlled by medium- or high-dose ICS but with a relevant AHR to mannitol challenge. Inflammation and development of AHR were not inhibited by ICS in these subjects, showing the presence of mechanisms inducing resistance to corticosteroids, possibly correlated to TSLP production, as this factor reduces the response to steroids (224), and indeed, AHR was blocked by tezepelumab in most of these patients. These data show that tezepelumab acts on top of ICS both in T2-type and non-T2-type asthma and opens new therapeutic perspectives. The definition of the non-reversible or non-modifiable component of asthma considered when only high-dose ICS were available must be reappraised; the new tool acting on AHR, the unifying mechanism of all asthma manifestations, provides new options to obtain severe asthma remission. In conclusion, current evidence from preclinical and clinical studies suggests that inhibition of type 2 inflammation only is unsatisfactory for AHR, as modulation of this pathway alone does not necessarily induce broncho reactivity. Inhibition of epithelial pathways and cross-relationship of TSLP involving structural cells, inflammatory cells, mast cells, and remodeling mediators may be cardinal, as demonstrated by targeting the epithelial-derived cytokine TSLP. This opens a new perspective in the clinical consideration of new monoclonal antibodies like tezepelumab.

Conclusions

AHR is a complex trait of asthma, induced by the concurrence of many pathophysiological factors and related to different clinical manifestations. This review of the literature shows that the phenomenon has been investigated for many years, unveiling many contributors and inter-relationships of inflammatory and remodeling processes through immune and structural cells. Recent evidence demonstrates the important role of airway epithelial damage and TSLP production in many of these events.

Nowadays, the assessment of AHR in clinical practice may improve knowledge and therapeutic perspectives for severe asthma. Such assessment is based on conventional challenge tests, but the identification of AHR through morning pre-bronchodilator PEF or suggestive symptoms as surrogate markers of AHR could be an innovative, convenient and patient-oriented approach.

A therapeutic response based on AHR control could be considered as a condition of disease remission and seems a promising new goal for the management of patients with severe asthma. In this perspective, the recently approved biologic agent for severe asthma acting on TSLP is introducing a new way of managing severe asthma beyond inhibiting inflammation and preventing exacerbations.

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Contributions

All authors: conceptualization. AV: literature analysis and revision, writing - original draft, writing - review & editing.

Conflict of interests

The authors declare that they have no conflict of interests.

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