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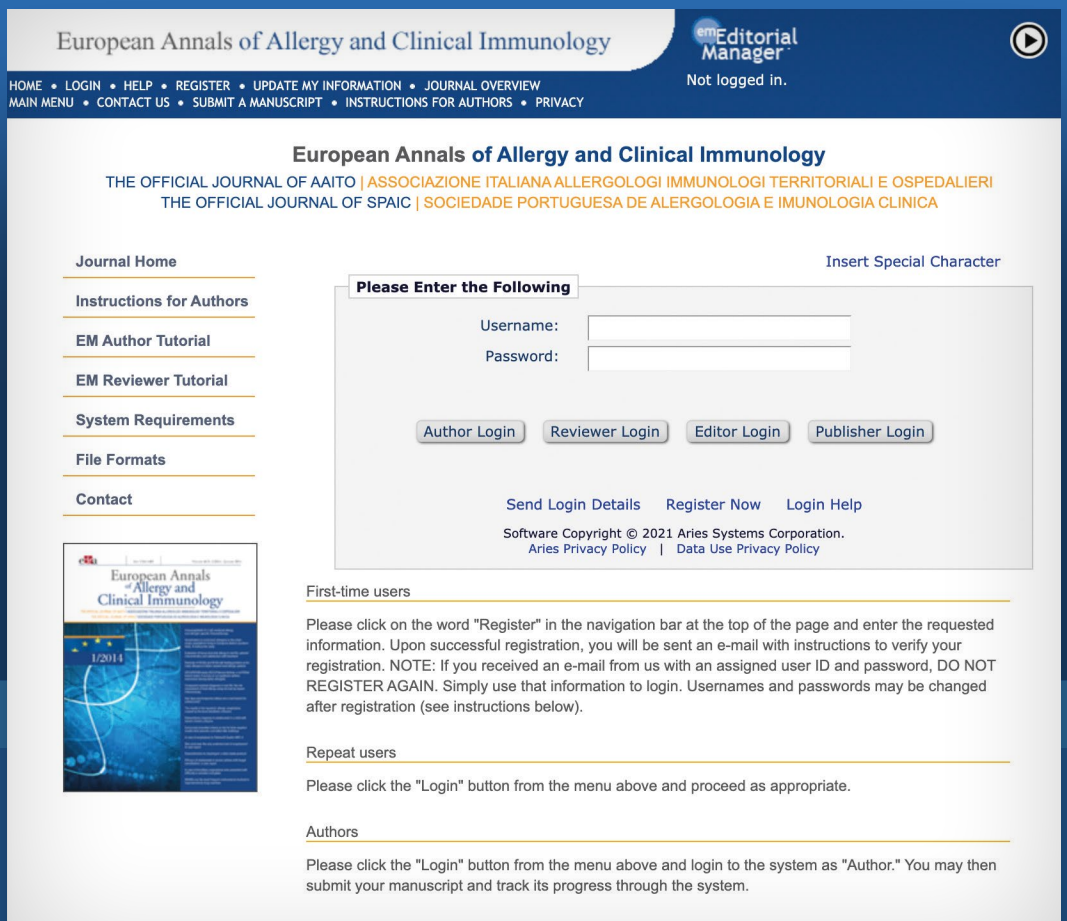
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EDRA SpA

Via G. Spadolini, 7
20141 Milano - Italy
Tel. 0039 (0)2-88184.1
Fax 0039 (0)2-88184.301
www.edizioniedra.it

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Adriano Vaghi[1], Maria Beatrice Bilò[2,3], Francesco Bini[4], Lorenzo Cecchi[5], Claudio Micheletto[6], Antonino Musarra[7]

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

[1]Former Head of Pneumology and Chief of the Department of Medicine and Rehabilitation, Guido Salvini Hospital-ASST Rhodense, Garbagnate Milanese, Milan, Italy
[2]Department of Clinical and Molecular Sciences, Università Politecnica delle Marche, Italy
[3]Allergy Unit, Department of Internal Medicine, University Hospital delle Marche, Ancona, Italy
[4]Pneumology Unit, UTIR ASST-Rhodense, Milan, Italy
[5]Allergy and Immunology Unit, San Giovanni di Dio Hospital, USL Toscana Centro, Florence, Italy
[6]Respiratory Unit, Integrated University Hospital, Verona, Italy
[7]Allergy Unit, Casa della Salute di Scilla, Scilla, Reggio Calabria, Italy

Key words

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

Corresponding author
Adriano Vaghi
Pulmonology Unit
Ospedale Guido Salvini
ASST Rhodense
viale Carlo Forlanini 95
20024 Garbagnate Milanese, Milan, Italy
ORCID: 0009-0001-1896-2852
E-mail: adriano.vaghi@aiporicerche.it

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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

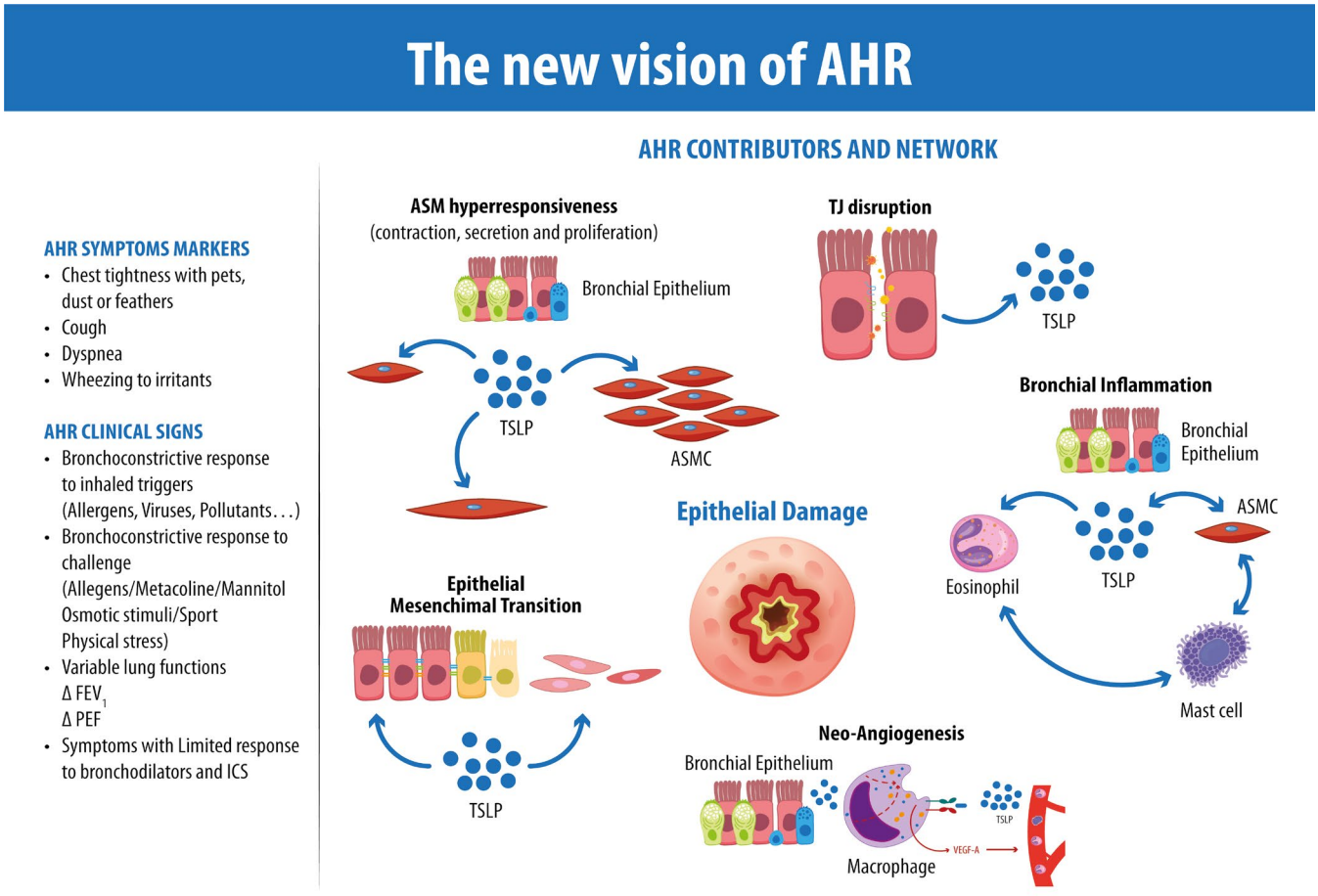
and are related to the severity of indirect AHR and T2 inflammation, in contrast with previous studies showing a correlation of indirect AHR with eosinophils under the mucosa (55). Intraepithelial eosinophils may be easily stimulated by external factors, explaining the reactivity of patients with asthma (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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ORIGINAL ARTICLE

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Sreenivasulu Reddy Boreddy[1], Christina Mary Mariaselvam[1], Benita Nancy Reni Micheal[1], Kommoju Vallayyachari[1], Sree Nethra Bulusu[1], Molly Mary Thabah[1], Mahesh Padukudru Anand[2], Thirumurthy Madhavan[3], Vir Singh Negi[1]

Functional characterization of complete and immunodominant epitopes of a novel pollen allergen from *Parthenium hysterophorus*

[1]Department of Clinical Immunology, Jawaharlal Institute of Postgraduate Medical Education and Research, Puducherry, India
[2]Department of Respiratory Medicine, JSS Medical College, JSSAHER, Sri Shivarathreeshwara Nagar, Mysore, Karnataka, India
[3]Department of Biotechnology, School of Bioengineering, SRM University, Kattankulathur Tamilnadu, India

Key words

Parthenium hysterophorus; allergic rhinitis; bronchial asthma; Skin Prick Test; basophil activation test.

Corresponding author
Vir Singh Negi
Department of Clinical Immunology
Jawaharlal Institute of Postgraduate Medical Education and Research (JIPMER)
Dhanvantari Nagar
Puducherry - 605006 India
ORCID: 0000-0003-1518-6031
E-mail: vsnegi22@yahoo.co.in

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Impact statement

The allergenic epitopes deduced from 40 kDa pectin methylesterase allergenic protein of P. hysterophorus was found to induce prompt phenotypic responses by SPT and cellular immune responses comparable with that of the 40kDa allergenic protein and crude pollen extract.

Summary

Background. *Parthenium hysterophorus pollen induces chronic clinical conditions such as allergic rhinitis and bronchial asthma. Among the plethora of proteins in the pollens, only few were reported to induce allergy. Currently sensitization to P. hysterophorus pollen allergen is diagnosed by skin prick test (SPT) using the entire pollen extract instead of using the specific allergen.* ***Methods.*** *In P. hysterophorus sensitized patients, SPT was done using the crude pollen extract, 40kDa allergenic pollen protein and two commercially synthesized allergen epitopes (17 and 24) of P. hysterophorus. Dot-blot of allergen epitopes was done using P. hysterophorus sensitized sera. Crude pollen extract (1, 1.25, 2.5, 5 and 10µg/mL), 40kDa allergenic protein (3µg/mL), and allergen epitopes (3µg/mL) were used to perform Basophil Activation Test (BAT).* ***Results.*** *Crude pollen extract at 2.5, 5, 10 µg/mL and 40kDa allergenic protein at 3 µg/mL concentrations induced wheal and flare reaction by around 15 minutes, whereas commercially synthesized allergen epitopes at 3 µg/mL induced wheal and flare reactions in <10 minutes. Allergen epitopes (3µg/mL) revealed strong reactivity with sensitized patient's IgE in dot-blot analysis. Basophil activation Test using crude pollen extract (2.5, 5, 10 µg/mL), 40 kDa allergenic protein (3 µg/mL), and allergenic epitopes (3 µg/mL) indicated significant basophil activation (as measured by CD63 expression) in sensitized patients.* ***Conclusions.*** *The 40 kDa allergenic protein and its allergenic epitopes (17 & 24) induced phenotypic and cellular immune responses in P. hysterophorus sensitized individuals. The tested allergenic epitopes (17 and 24) induced faster wheal and flare reactions in comparison with the crude extract and the 40kDa allergenic protein. The novel 40kDa allergenic protein and its allergen epitopes identified here may be useful for the development of component-resolved diagnosis (CRD) while also serving as a potential therapeutic lead for desensitization treatment for P. hysterophorus pollen induced allergy.*

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Introduction

Allergy is one of the leading illness, affecting more than 20% of the Indian population (1). Allergic rhinitis and asthma are the common and serious manifestations of allergy, causing considerable distress and burden by being chronic in nature, with remissions and relapses in the affected population but are rarely fatal (2). In the absence of specific treatment, palliative measures using epinephrine, antihistamines, and corticosteroids for symptom relief are usually offered to the patients during clinical exacerbations of allergy (3).

As a diagnostic procedure for allergies, skin prick test (SPT) is commonly used to confirm allergic sensitization to established allergens. Although, SPT is minimally invasive, economical, and provides immediate results (4, 5) some patients might develop anaphylactic reactions (6). The crude allergenic extracts used for SPT can lead to cross reactivity between related allergens. Besides, crude allergic extracts are heterogenous and contain undefined nonallergenic materials and contaminants (7). Batch to batch and manufacturer associated variations in the major and minor components of the allergens in the extracts used for SPT affect the sensitivity and specificity of the test. Variable responses are observed in patients based on their sensitization to different determinants, making precise standardization of methods essential for diagnosing clinical allergies (8, 9). Therefore, the use of well-standardized allergens is recommended for diagnosis. Improved standardization of allergens using allergen epitopes helps to discriminate between cross reactivity, enhancing the specificity of the diagnostic assay and to assess disease severity (10). In 2001, a project funded by the European Union, CREATE, introduced the idea of standardizing and optimizing allergenic extracts based on the content of the major allergens (11). The development of recombinant allergens has also contributed to the standardization of allergenic extracts for use in diagnosis (4). In India, the data on the specific allergens from the source is very sparse and dose dependent allergenic extracts are not commonly used in clinical practice for allergy diagnosis. In India, allergic respiratory disorders are common and pollen aeroallergens from various plant sources were implicated as etiologies (12). *P. hysterophorus*, a ubiquitous and invasive weed of global significance, is abundant in more than 30 countries. Though *P. hysterophorus* is not included in the panel of respiratory allergens routinely tested in Europe, it has been identified as the leading cause of allergic rhinitis and asthma in India, including Puducherry over the last three decades, reaching epidemic proportion (13). Earlier studies conducted on *P. hysterophorus* did not provide information on allergen concentration used for SPT and cell-specific immune response by basophil activation test (BAT). Therefore, in this study, *P. hysterophorus* pollen crude extract, 40kDa allergenic protein and its in-silico predicted allergen epitopes were subjected to in-vitro BAT and SPT to obtain quantitative and qualitative conclusions on allergen specific effector cell responses.

Materials and methods

Study subjects

Patients with allergic rhinitis fulfilling Allergic Rhinitis and its Impact on Asthma (ARIA) guidelines (14) and allergic asthma fulfilling Global initiative for Asthma (GINA) guidelines (15) and who tested positive to *P. hysterophorus* allergens by SPT, were enrolled from the Clinical Immunology, Otorhinolaryngology and Pulmonary Medicine outpatient clinics from 2014 to 2018 between May and September. A panel of 26 allergens (16 plant pollens, 3 fungal, 4 insects, 3 animal dander) were tested by skin prick test (SPT) as a part of routine diagnosis (**supplementary table I**) to identify the allergen specific sensitization in the patients. The patients who developed wheal and flare reaction (> 3 mm diameter) within 15 minutes after SPT were considered to be sensitized to the particular allergen. Histamine dihydrochloride (Sigma-Aldrich, USA) at 5 mg/mL and sterile PBS (Sigma-Aldrich, USA) were used as positive and negative controls respectively.

Patients with chronic obstructive pulmonary disease (COPD), autoimmune diseases such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), dermatomyositis, metabolic diseases such as diabetes mellitus, dyslipidemia, thyroid dysfunction, hypertension, diseases of the skin, such as psoriasis, vitiligo and those on long term immunosuppression drugs were excluded from the study. Healthy controls were individuals without any family history of chronic infections, allergic or autoimmune diseases.

After the initial screening, patients who tested allergic to *P. hysterophorus* by SPT were included after obtaining a written informed consent. Ten milliliters of peripheral venous blood were collected (5 mL in plain sterile vials, 5 mL in heparinized tubes). Heparinized blood sample was used for BAT. Serum was separated from the clotted blood and stored at -80 °C until further use. The study was approved by JIPMER Ethics Committee (Human Studies), Protocol No. JIP/IEC/2014/10/482 dated January 30, 2015. As a negative control for all the functional assays, heparinized blood sample and serum obtained from healthy individuals who tested negative by SPT to all the 26 allergens was used.

The total IgE level in the serum of *P. hysterophorus* sensitized patient was measured using the commercial IgE kit (N Latex IgE mono kit, Siemens, Germany) by Nephelometry (BN ProSpec® System, Siemens, Germany). Subjects with ≥ 100 IU/mL of total IgE were considered as "sensitized" while those tested < 100 IU/mL were considered as "unsensitized". The serum and heparinized blood samples thus obtained from SPT-sensitized patients with total IgE ≥ 100 IU/mL were subjected to functional analysis.

Characterization of specific allergenic protein from pollens of P. hysterophorus

The inflorescences from the *P. hysterophorus were collected from various locations in Puducherry between 2014-2018. From the inflorescence, pollen collection and extraction of pollen proteins were* performed following the published protocol (16). The pollen protein extract was lyophilized (ModulyoD Freeze Dryer, Thermo Scientific, USA) and stored at -80 °C until further use. When needed, the lyophilized pollen protein extract was reconstituted in sterile Milli-Q water, and its protein concentration was measured using a UV-visible spectrophotometer (Picodrop, PICOPET 01, UK). The proteins in the pollen extract were resolved on 12.5% SDS-PAGE and transferred onto a nitrocellulose membrane (Sigma Aldrich, USA) by semi-dry blot method (Trans-Blot SD Semi Dry Transfer Cell, Bio-Rad, USA). The unbound sites in the membrane were blocked using 5% bovine serum albumin (BSA). Following three washes using Phosphate Buffered Saline with Tween 20 (PBST), the membrane was incubated overnight at 4 °C, with the diluted serum (1:500) containing IgE from the sensitized patient. After washing the membrane was incubated at 37 °C for 3 hours with diluted anti-human IgE antibody HRP conjugate (1:500) (Abcam, USA) (17, 18). The membrane was incubated with Clarity Western peroxide reagent and Clarity Western Luminol/Enhancer reagent (Clarity Western ECL blotting substrate, Bio-Rad, USA). Images were acquired using the Chemi-Doc™ XRS⁺ system (Bio-Rad, USA).

After identifying the reactive allergenic pollen protein by immunoblotting, the protein was isolated from the SDS-PAGE gel by excising and protein stripping by cold acetone method (19). Protein precipitate was treated with cold acetone (1:4 v/v) and sample was incubated at -20 °C for 1 hour and centrifuged for 10 min at 10,000 g. Precipitated protein free of SDS was then dissolved in 500 µl of 1x PBS by vortexing and was subjected to ultra-performance liquid chromatography (UPLC) using the Acquity Ultra Performance LC system (Waters, USA) in the reversed phase mode and protein was separated on the Acquity UPLC BEH300 C4 column (Waters, USA). The concentration of the purified protein was quantified (Picodrop, PICOPET 01, UK) and was stored at 4 °C for further analysis. The amino acid sequence of the identified protein was analyzed using commercial service (Sandor proteomics, Hyderabad, India).

Allergen epitope identification

Immune epitope database (http://tools.immuneepitope.org/bcell/) and analysis resource tools were used to predict epitopes from the 40 kDa allergenic protein (20). Various immune epitope database tools were used to analyze peptide parameters such as solubility (Parker Hydrophilicity Prediction), flexibility (Karplus and Schulz flexibility scale), accessibility (Emini surface accessibility scale), Beta-turns (Chou and Fasman Beta-Turn prediction), antigenicity (Kolaskar and Tongaonkar antigenicity scale), and linear epitopes (Bepipred 1.0 and 2.0) (20-22). NetSurfP 2.0 server was used to predict the surface accessibility, and secondary structure of peptides (23). The peptides that exhibited high flexibility, hydrophilicity, antigenicity, and surface accessibility were selected as candidate molecules for further analysis. The total net charge of peptides and their binding potential (Boman index) was also calculated using the antimicrobial peptide database (https://aps.unmc.edu/prediction) (24). Based on the data derived from Immune Epitope Database tools, NetSurfP 2.0 server, and antimicrobial peptide database, two peptides (17 and 24) were selected.

In vitro peptide synthesis

The selected peptides were synthesized using a commercially available service ('S' BioChem company, Kerala, India) and the peptides were synthesized by Solid Phase Peptide Synthesis (SPPS) method using Specific Automated Peptide Synthesizer Autopep-001A (CS Bio, California). Briefly, 4-(2',4'-Dimethoxyphenyl-Fmoc-aminomethyl) phenoxy resin 100-200 mesh was used to provide a C-terminus free carboxyl group to the peptide. Deprotection of peptide was performed using 20% piperidine in dimethylformamide. The resin was removed by filtration and washed with hexane, dimethylformamide, chloroform, and methanol, and dried. The synthesized peptide was isolated from the solution using excess peroxide free pure cold diethyl ether (25, 26). After isolation, crude peptide was dissolved in 5% acetonitrile solution and purified using reverse phase HPLC on a RPC18 column (M/s Shimadzu Corporation, Japan). The molecular mass of the synthesized peptide was determined using ESI-MS (Waters, USA).

Dot blot analysis

The commercially synthesized peptides (allergen epitopes) were diluted from the stock to a final concentration of 3 µg/µL in sterile PBS and 10 µL of peptides were separately blotted onto the 0.2 µm nitrocellulose membranes (Sigma Aldrich, USA). The membranes were blocked using 5% BSA and then incubated at 4 °C for 2 hours with 10 mL of diluted serum (1:500) containing IgE from *P. hysterophorus* sensitized patient and then washed twice using 1x Tris-buffered saline with 0.1% Tween 20 detergent (TBST). The membranes were then incubated with anti-human IgE antibody (Abcam, USA) HRP conjugate (1:500) at 37 °C for 3 hours (27). Post washing with PBST, clarity western peroxide reagent and clarity western Luminol/Enhancer reagent was added to the membrane (Clarity Western ECL blotting substrate, Bio-Rad, USA) and images were acquired using ChemiDoc™ XRS⁺ system (Bio-Rad, USA). As a negative control, serum from an apparently healthy person, non-reactive by SPT was used.

Evaluation of reactivity of pollen allergenic extract, 40kDa allergenic protein and in vitro synthesized peptides using SPT

To optimize the diagnostic dose for SPT, the crude pollen extract (1, 1.25, 2.5, 5, 10 µg/mL), 40 kDa allergenic protein and aller-

gen epitopes at 3 µg/ml were used for SPT. The time taken to develop wheal and flare reactions for the respective test preparation was recorded and measured respectively and compared with the positive control (5 mg/mL Histamine dihydrochloride, Sigma Aldrich, USA).

Basophil degranulation test

Basophil degranulation assay was carried out using the Fast Immune™ CD63/CD123/Anti–HLA-DR reagent kit (BD Biosciences, California, USA). Variable concentrations of crude pollen extract, 40 kDa allergenic protein, and commercially synthesized peptides were used for the assay. In this exploratory study, we used 1, 1.25, 2.5, 5 and 10 µg/mL of crude pollen extract to perform SPT. A crude pollen extract of 2.5, 5 and 10 µg/mL was found to induce the wheal and flare reactions in sensitized individuals. Based on this observation, the minimal concentration of 3 µg/mL of 40 kDa protein and 3 µg/mL of allergen epitopes (17 and 24) was considered to be sufficient for SPT and basophil activation test. Heparinized blood samples were collected from patients tested positive by SPT to *P. hysterophorus* and healthy donors. Briefly, 100 µL of blood was mixed with 20 µL of basophil stimulation buffer 20 µL of crude pollen extract (1, 1.25, 2.5, 5 and 10 µg/mL), 40 kDa allergenic protein (3 µg/mL) and allergen epitopes (3 µg/mL) was added separately to the above tube and incubated at 37 °C in a water bath for 15 min. N-Formylmethionyl-leucyl-phenylalanine (fMLP) and basophil stimulation buffer (BSB) were used as positive and negative controls respectively. Degranulation was stopped by chilling the tubes with the addition of 1 mL of ice-cold PBS with 10 mmol/L EDTA on ice and were centrifuged for 5 min. The CD63 FITC/CD123 PE/Anti–HLA-DR PerCP antibody cocktail (20 µL) was added to each tube and incubated in the dark on ice for 20 min. Samples were then lysed using 1X BD FACS™ lysing solution at room temperature for 15 min and centrifuged. Supernatants were analyzed by BD FACS™ flow cytometer with a 488-nm laser to detect the $CD63^+$ basophils. Data was acquired with a threshold to eliminate most of $CD123^-$ cells and at least 500 $CD123^+$ cells were acquired per sample. Basophils were identified as low side scatter (SSC), $CD123^+$ and $HLA\text{-}DR^-$ cells. The quantitative determination of activated basophils was measured on CD63 FITC.

Statistical analysis

Descriptive variables are represented as mean and standard deviation (SD) or the median with interquartile range (IQR). Kruskal-Wallis test was used to compare the difference between the percentages of activated basophils in patients. A P-value < 0.05 was considered statistically significant.

Results

A total of 484 patients were screened in this study. Among them, only 18 patients (mean age 37.9 ± 13.5 years) tested reactive to *P. hysterophorous* allergens by SPT. Of these 18 patients, five (mean age 39.6 ± 10.5 years) had a history of direct exposure by virtue of their profession. In addition to *P. hysterophorus*, these five patients were also tested positive to *Ambrosia artemisiifolia* (short ragweed), *Casuarina equisetifolia,* and *Dermatophagoides pteronyssinus* or *Dermatophagoides farinae* aeroallergens and had elevated total serum IgE levels (> 100 IU/mL). Five individuals (mean age 32.8 ± 1.6 years) tested negative by SPT to all the 26 allergens, were recruited as healthy controls. The serum from the healthy control was used as negative control for all the downstream assays.

Prediction and in vitro synthesis of potential allergen epitopes from 40kDa allergenic protein of P. hysterophorus

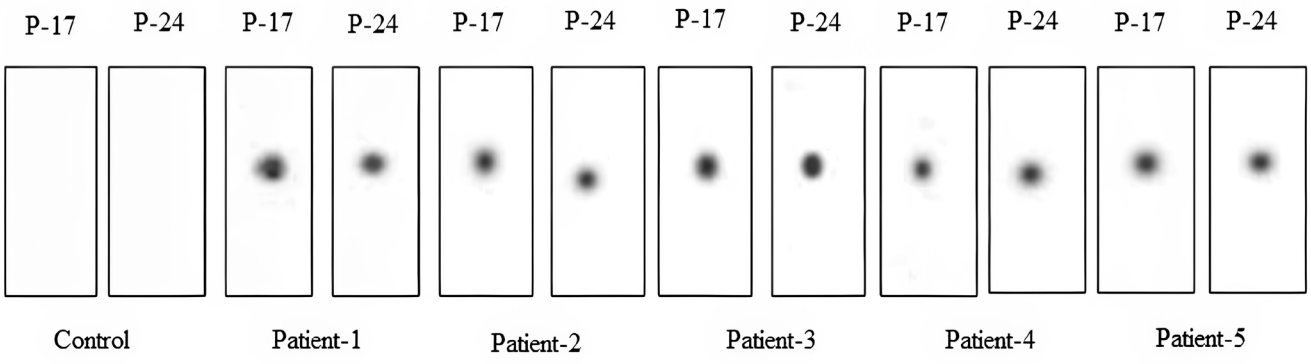
SDS-PAGE and immunoblotting analysis of *P. hysterophorus* pollen extract using sensitized sera revealed binding of IgE to a 40 kDa pollen protein. The 40 kDa protein was further characterized by amino acid sequencing as pectin methylesterase (data not shown). Using bioinformatic tools, a total of 48 peptide sequences from 40 kDa pectin methylesterase were identified. The peptides that exhibited high flexibility, hydrophilicity, antigenicity, and surface accessibility were selected. Peptides 17 and 24 fulfilled the required physicochemical features such as length, molecular weight, and protein binding potential to be considered as potential allergen epitopes (**supplementary figures 3-5**).

The predicted allergen epitopes were commercially synthesized. The matrix assisted laser desorption ionization-time of flight (MALDI-TOF) analysis of peptide-17 showed the ESI mass spectrum of 2.78e8 detected four charge states of the peptide: m/z 965.25 [M+2H]2+, m/z 482.98 [M+4H]4+, m/z 796.00 [M+5H]5+, m/z 663.50 [M+6H]6+ and the molecular mass of the peptide was found to be 1929.18 daltons (**supplementary figure 6A**). The HPLC analysis of the peptide showed a single sharp peak with a retention time of 7.626 min indicating a peptide with > 99% purity (**supplementary figure 6B**). The MALDI-TOF analysis of peptide-24 revealed the ESI mass spectrum of 5.72e7 detected four charge states of the peptide: m/z 1330.25 [M+2H]2+, m/z 665.70 [M+4H]4+, m/z 796.00 [M+5H]5+, m/z 663.50 [M+6H]6+ verifying a molecular mass of peptide to be 2660.12 daltons (**supplementary figure 7A**). The HPLC analysis of peptide-24 showed a single sharp peak with a retention time of 10.770 min representing peptide with > 99% purity (**supplementary figure 7B**).

Evaluation of the reactivity of the synthesized peptides with specific IgE

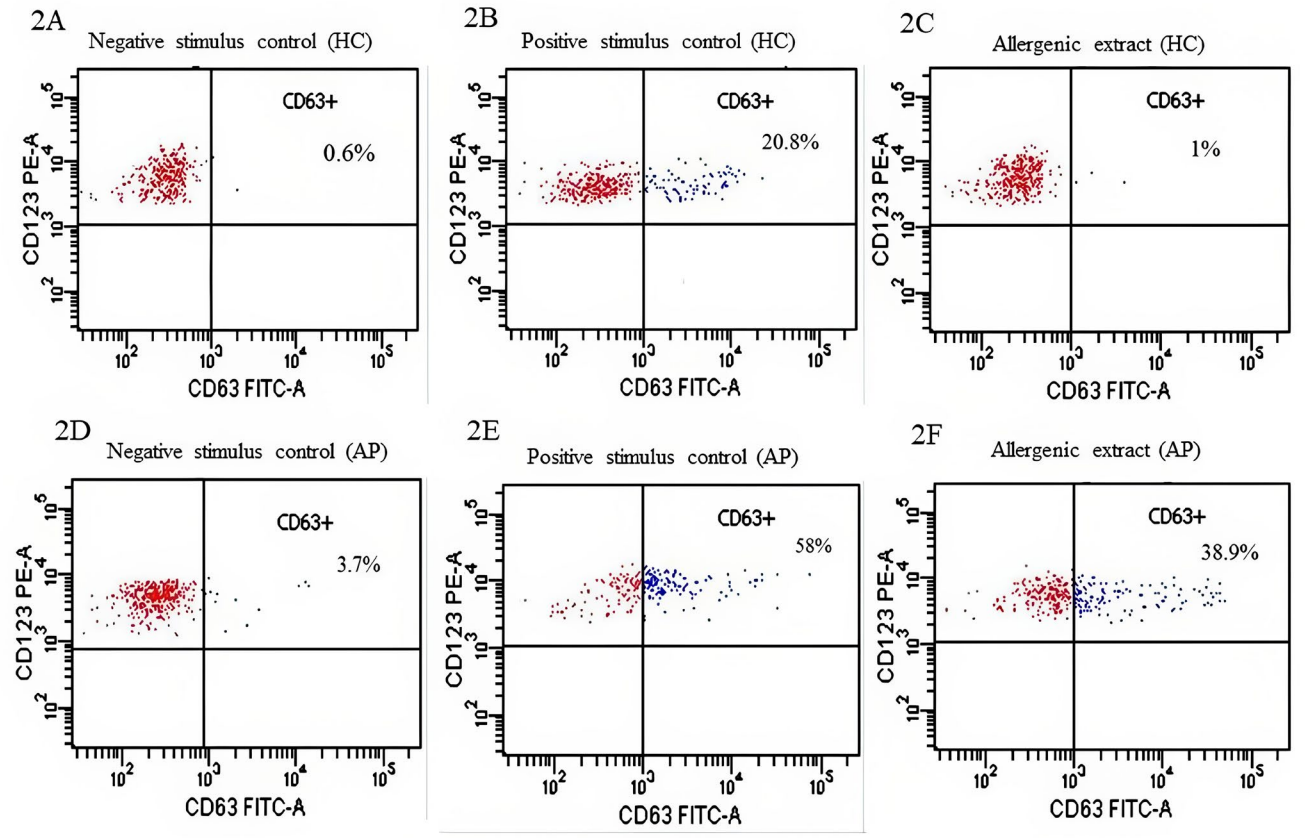
The *in vitro* synthesized peptides (17 and 24) were diluted from the stock to a final concentration of 3µg/µL and 10µL of peptides (17 and 24) blotted on nitrocellulose membranes. The *P. hysterophorus* sensitized patient's sera exhibited strong IgE (1:500) reactivity with the peptides (17 and 24) by dot-blot analysis, whereas no reaction was observed with the negative control (healthy individ-

Figure 1 *- Dot-blot analysis of peptide-17 and 24 (3 µg/mL) using P. hysterophorus sensitized sera (1:500).*



Control: Dot-blot analysis of peptide-17 (P-17) and peptide-24 (P-24) using a healthy control serum. Patient's-1-5: Dot-blot analysis of peptide-17 and 24 using *P. hysterophorus* sensitized patient sera of allergic rhinitis and bronchial asthma patients.

Figure 2 *- Effect of negative and positive stimulus controls and test (crude allergenic extract) on basophils of healthy control and allergic patients.*



(A-C) Percentage of activated basophils from healthy control stimulated with negative control (BSB: basophil stimulation buffer), positive control (fMLP: N-Formylmethionine-leucyl-phenylalanine), and test (allergenic extract: 2.5 µg/mL); **(D-F)** Percentage of activated basophils from allergic patient stimulated with negative control (BSB), positive control (fMLP) and test (allergenic extract: 2.5 µg/mL).

ual serum) which clearly indicates that peptides reacted with specific IgE of sensitized patients and the details are given in **figure 1**.

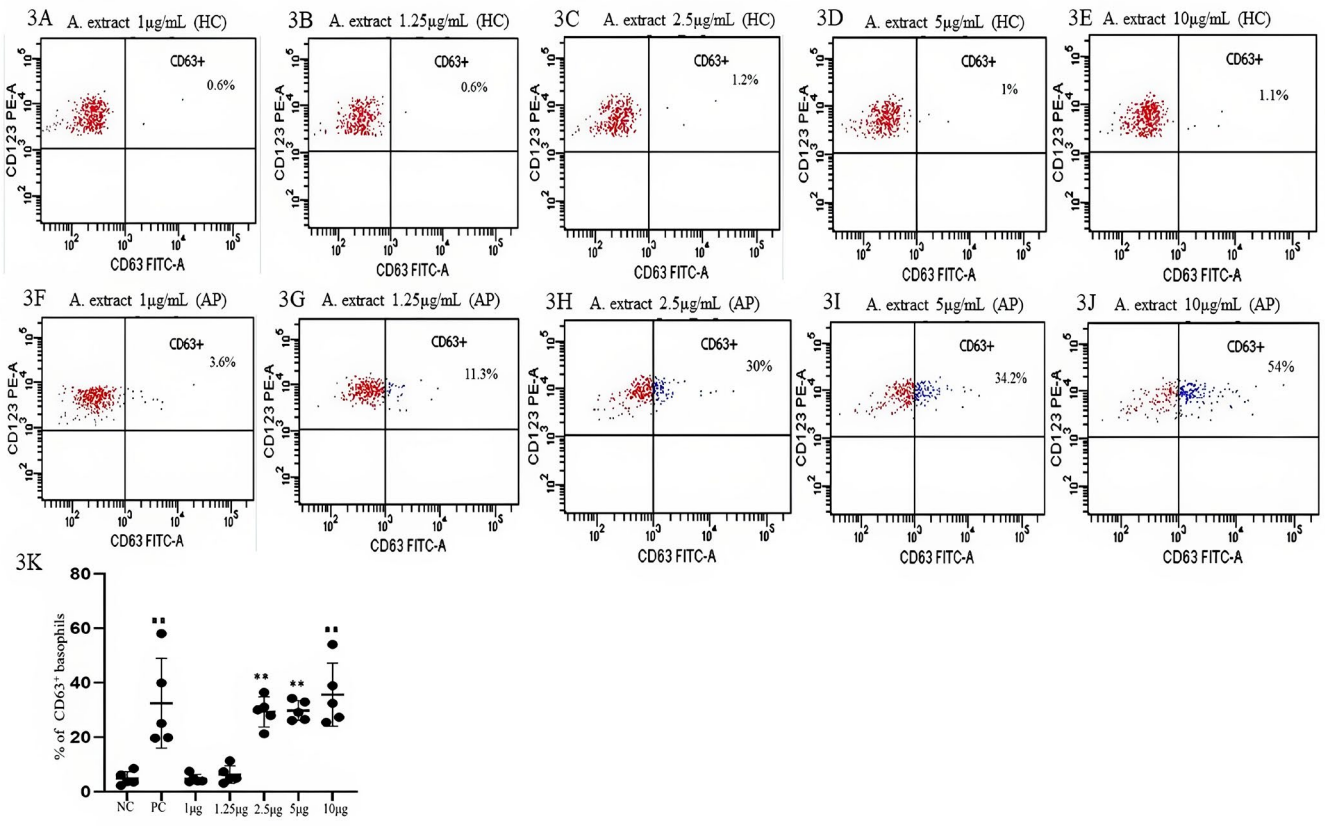
SPT reactivity of pollen crude extract, 40kDa allergenic protein and allergen epitopes

SPT performed in sensitized patients (n = 5), using different concentrations of (1, 1.25, 2.5, 5 and 10 µg/mL) antigenic extract, 40 kDa allergenic protein (3 µg/mL) and allergen epitopes 17 and 24 (3 µg/mL) showed that tested patients exhibited significant reactivity with the crude antigenic extract at concentration ranging from 2.5, 5 and 10 µg/mL. Sensitized patients also exhibited strong reactivity to 40 kDa allergenic protein and commercially synthesized allergen epitopes (3 µg/mL). In sensitized patients, strong wheal and flare reactions were observed within 10 minutes with the allergen epitopes, while wheal and flare reactions were observed after 15 minutes using the crude pollen extract and 40 kDa protein. The results of SPT carried out using crude antigenic extract, 40kDa allergenic protein and allergenic epitopes and their wheal and flare reactions are given in **supplementary table II**.

Basophil activation test

The gating strategy for basophils is described in **supplementary figures 1** and **2**. Effect of negative and positive stimulus controls and 2.5 µg/mL crude pollen extract on basophils of healthy control and allergic patient was tested. In sensitized patients a higher percentage of basophil activation ($CD63^+/CD123^+/HLA\text{-}DR^-$) was seen using positive control compared to healthy individuals (58% *vs* 20.8%) and with crude pollen extract at 2.5µg/mL concentration, 38.9% basophil activation was noted in sensitized patients compared to 1% in healthy controls (**figure 2A-F**). On testing different concentrations of crude pollen extract in controls no activation of basophils were noted (**figure 3 A-E**) while a dose dependent rise in the basophil activation was observed with 2.5 (30%, p = 0.05), 5 (34.2%, p = 0.05) and 10µg/µL (54%, p

Figure 3 *- Effect of 1, 1.25, 2.5, 5 and 10 µg/mL of P. hysterophorus pollen allergenic extract on basophils of healthy control and allergic patient.*



(A-E) Percentage of activated basophils of healthy control stimulated with 1, 1.25, 2.5, 5 and 10 µg/µL of allergenic extract; **(F-J)** Percentage of activated basophils of allergic patient stimulated with 1, 1.25, 2.5, 5 and 10 µg/µL of allergenic extract; **(K)** The percentage of activated basophils between negative, positive controls and different allergenic extract concentrations (1, 1.25, 2.5, 5 and 10 µg/mL) were and tested using Kruskal-Wallis test. $p < 0.05$ is considered significant.

= 0.05) concentrations in patients (**figure 3 F-J**). Likewise, the observed basophil activation frequency with 40kDa allergenic protein, allergenic epitopes (17&24) at 3µg/µL each was 47.4% (p = 0.05), 28% (p = 0.05) and 42.3% (p = 0.05) respectively in patients compared to controls (**figure 4**).

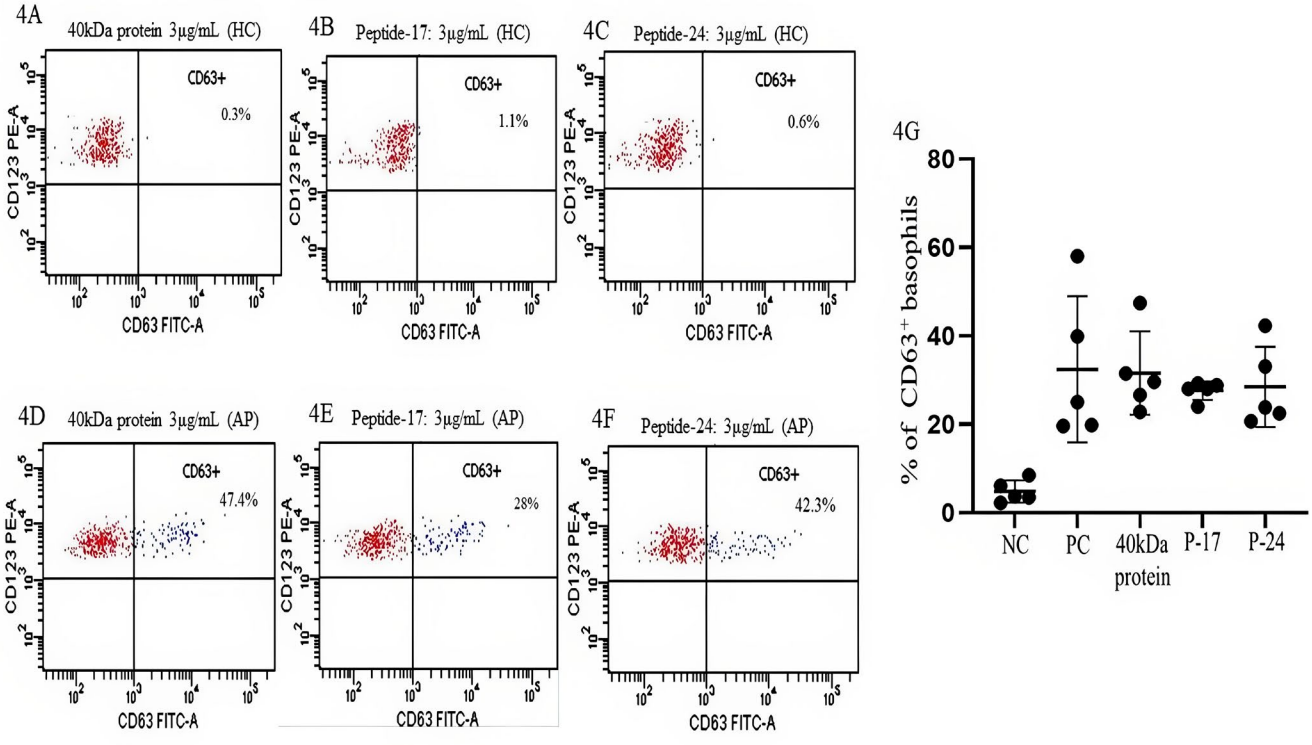
Discussion and conclusions

In this study, we assessed the immune response induced by the 40 kDa allergenic protein of *P. hysterophorus* pollen and two immunodominant allergenic epitopes identified from the 40 kDa allergen in allergic rhinitis and asthma patients.

Immunoblotting analysis of *P. hysterophorus* pollen protein extract using sensitized sera revealed 40 kDa protein to be allergenic and in silico studies revealed that it is a member of the pectin methylesterase family (data not shown). Pectin methylesterase family members from pollen and other sources have been reported to induce allergy (28, 29). Salamanca *et al.* reported a 37.4 kDa Ole e 11 as a pectin methylesterase from olive tree, had 57% and 54% similarity with pectin methylesterase of *Arabidopsis thaliana* and Sal k 1 of *Salsola kali* (Russian thistle) pollens respectively (28). Barderas *et al.* reported a 43 kDa pectin methylesterase from Russian thistle to be highly allergenic with significant sensitization rates in the Spanish population (30). Pectin methylesterase of Japanese hop pollen, has 23.2-50.2% of sequence similarities with Ole e 11, and Sal k 1 (31). These data highlight the importance of cross-reactive amino acids in pectin methylesterase family members and their impact in susceptible individuals which necessitates the characterization of clinically important allergen epitopes for accurate allergy diagnosis.

Defensins are antimicrobial glycoproteins, that plays a critical role in the plant immune system and have been reported to induce allergy (32, 33). A diverse number of defensins were reported in the members of the Asteraceae family members, especially from the *Artemisia sp., Ambrosia* sp., and *P. hysterophorus* (33). Par h 1 a defensin-polyproline-linked protein from *P. hysterophorus* has

Figure 4 *- Effect of 3 µg/mL of 40 kDa protein, peptide-17 and 24 on basophils of healthy control and allergic patient.*



(A-C) Percentage of activated basophils from healthy control stimulated with 3 µg/µL of 40 kDa protein and peptides (17 and 24); **(D-F)** Percentage of activated basophils from allergic patient stimulated with 3 µg/µL of 40 kDa protein and allergen epitopes (17 and 24); **(G)** The percentage of activated basophils between negative, positive controls and 40kDa allergenic protein and allergen epitopes (17 and 24) were tested using Kruskal-Wallis test. $p < 0.05$ is considered significant.

high sequence similarity with Amb a 4 and Art v 1, defensins of *Ambrosia artemisiifolia* and *Artemisia vulgaris* respectively (33). In *P. hysterophorus* Gupta *et al*. identified 28, 31, and 45 kDa proteins of which only 31 kDa Par h I was allergenic based on their reactivity to sera from patients with allergic rhinitis and bronchial asthma (17). Our findings are different to the above studies, and it could be due to differences in the geographical regions and the climate/environment induced changes in pollen protein composition. Pollen protein component variations collected from variable geographic regions in India and differences in their ability to cause disease severity has already been reported (34-36). Although all the *P. hysterophorus* sensitized patients were polysensitized to other aeroallergens, resource limitations precluded us from evaluating the cross-reactivity of *P. hysterophorus* specific pectin methylesterase with the other allergens. Nevertheless, specific 40 kDa allergenic protein characterized in this study, may be useful for in-vitro and *in vivo* diagnosis of *P. hysterophorus* induced allergy in future.

Immune epitope prediction database tools were used to predict the specific IgE binding epitopes from the 40 kDa protein. A total of 48 peptides were predicted out of which only two peptides (17 and 24), possessed the required criteria such as length, molecular weight, and protein binding potential. The selected peptides were also shown to form the alpha-helical structure. Hence, these two peptides were used for downstream SPT and cellular assays.

Using immune epitope database tools, Carrera *et al.* had identified the B-cell epitopes from the major fish allergens beta parvalbumins (37). Using BepiPred 1.0, Chen *et al*. reported seven B-cell epitopes from the major cockroach allergens Per a 6 of *Periplaneta americana* and Bla g 6 of *Blattella germanica* (38). Similarly, three B-cell IgE binding epitopes were identified from the osmotin protein of tobacco (*Nicotiana tabacum*). The B-cell epitopes of osmotin displayed higher reactivity with allergen-specific IgE by dot-blot analysis (39). Molecular analysis of sesame allergen, 14 kDa β-globulin revealed two IgE binding epitopes, which exhibited strong reactivity in dot-blot analysis using sensitized patient sera (40). T and B cell epitopes of pectin methylesterase from Russian thistle were predicted using immunoinformatic tools. Molecular docking studies of Sal k 1 with MHC-II identified Sal k 1 as a promising molecule for allergen specific immunotherapy as it revealed strong and stable interactions (41). From the major Sal k 1 allergen, two isoforms Sal k 4.03 and Sal k 4.02 were identified using immunoinformatic tools. IgE binding assay of these isoforms revealed that the Sal k 4.03 bound better to specific IgE than Sal k 4.02, indicating a hypoallergenic nature useful to devise desensitization therapy (42).

In our study, immunoblotting assays confirmed that the sensitized patient's IgE specifically reacted with the 40 kDa allergen. Dot-blot assay using allergen epitopes (17 and 24) displayed stronger binding with the IgE of *P. hysterophorus* sensitized individuals. Our study results are in parallel with the above reports of IgE binding epitope identification and characterization.

In our study, varied concentrations of *P. hysterophorus* pollen extract (1, 1.25, 2.5, 5, and 10 µg/mL) induced strong wheal and flare reactions in sensitized patients by SPT. In contrast, a 40 kDa allergenic protein and allergenic epitopes elicited skin reactions at a standard concentration of 3 µg/mL. However, the time to develop wheal and flare reactions was slightly different. While SPT was performed using allergen epitopes (17 and 24), we observed development of wheal and flare reaction within 10 minutes. However, a delay in the development of responses by 5 minutes was observed when the crude pollen extract and purified allergenic 40 kDa protein was used. Peeters *et al*. studied the effect of peanut-specific purified allergens (Ara h 1, Ara h 2, Ara h 3, and Ara h 6) in eliciting skin reactions by SPT. It was shown that the sensitized patients with severe symptoms developed significant reactions with the low concentrations (0.1 µg/mL) of Ara h 2 and Ara h 6 and with higher concentrations of Ara h 1 and Ara h 3 (100 µg/mL) (43). In our study, we found that 40 kDa allergenic protein and allergen epitopes induced the visible skin reaction at 3 µg/mL, a slightly higher concentration. The salient finding of our study is the faster immune response elicited by the allergen epitopes compared to the 40 kDa protein. This could be due to instantaneous recognition of the allergenic epitopes by high affinity allergen-specific IgE in the sensitized patients and activation of allergen-specific mast cells (44). Ebo *et al*. reported that purified Mal d 1 (a major apple allergen) could activate basophils even at 1 µg/ml (45). Likewise, a marked increase in the percentage of CD63 expressing basophils was reported using 1µg/mL of wasp recombinant allergens (Ves v 1, Ves v 2, Ves v 3, and Ves v 5) (46). In our study, the allergenic crude extract at lower concentrations failed to induce basophil activation, while at higher concentrations, a dose-dependent increase in the activation of basophils was noted. Compared to the crude extract, a significantly higher percentage of basophils were activated by 40 kDa allergenic protein and allergenic epitopes (17 and 24) in sensitized individuals. Although the above studies have reported basophil activation with lower concentrations, we did not carry out the basophil activation assays using variable concentrations which is a limitation of our study. Resource limitations and lack of data from the published literature precluded us from using variable concentrations of 40 kDa allergenic protein and allergen epitopes (17 and 24) of *P. hysterophorus* to analyze their use in *in vivo* and *in vitro* assays. Therefore, we used a standard concentration of 3 µg/mL of 40 kDa allergenic protein and allergenic epitopes. However, 3 µg/mL of 40 kDa allergenic protein and allergenic epitopes significantly induced phenotypic and cellular immune responses. Future studies would help to optimize the minimum concentration of these molecules required for activating basophils as well as diagnosis of *P. hysterophorus* pollen allergy.

Although there are reports of other protein components in the *P. hysterophorus* pollen being allergenic from various geographi-

cal locations in India (17, 47), we for the first-time report that a 40 kDa Pectin methylesterase protein induced allergic responses among patients from Puducherry. Both the complete allergen and the predicted epitopes were tested to elicit cellular and phenotypic responses in sensitized individuals indicating its enhanced specificity. As these predicted allergen epitopes are specific and unique for the allergen, use of them for diagnosis would negate the cross reactivity with the similar allergens to a greater extent. However, we could not perform further *in silico* and *in vitro* studies to assess its cross reactivity with the other allergens and test its use to diagnose *P. hysterophorus* pollen sensitization. Also assessing cellular and phenotypic responses by using various concentrations would have helped us to arrive at the effective concentrations to be used for the effector functional studies. A major limitation of this study is its smaller sample size and hence these data should be validated in a larger cohort to confirm its clinical utility. Likewise, provocation studies and chemical modification of the peptides in future, might help to identify and develop allergen epitopes with poor avidity to IgE, which could be tested for its use in desensitization therapy to treat the sensitized patients thereby reducing their allergic symptoms, anaphylaxis related complications, and associated costs to the individual and society in future. In summary, the 40 kDa allergenic protein and its allergenic epitopes (17 and 24) were demonstrated to induce phenotypic and cellular immune responses in *P. hysterophorus* sensitized individuals. The allergenic epitopes identified here may also be tested in a larger cohort for validating its use in the rapid diagnosis of *P. hysterophorus* pollen induced allergy.

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Contributions

VSN, SRB, MPA, CMM: conceptualization. VSN: resources. SRB: investigation. SRB, VSN, MMT: data curation. BNRM, KV, SNB: data curation, formal analysis. SRB, TM: formal analysis. CMM, MMT, TM, MPA, VSN: writing – review & editing. SRB, CMM: writing – original draft. VSN: funding acquisition.

Conflict of interests

The authors declare that they have no conflict of interests.

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ORIGINAL ARTICLE

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Carlo Lombardi[1], Giovanni Passalacqua[2], Francesco Menzella[3], Rikki Frank Mauritz Canevari[4], Giovanni Danesi[5], Alessandro Maria Pusateri[5], Mauro Carone[6], Carlo Vancheri[7], Fabiano Di Marco[8], Claudio Micheletto[9], Giuseppina Manzotti[10], Mario Di Gioacchino[11], Beatrice Bilò[12,13], Matteo Gelardi[14], Gianenrico Senna[15], Giorgio Walter Canonica[16], on ARIA Italy Panel[*]

Chronic rhinosinusitis with nasal polyposis and biological agents: the ARIA-ITALY survey

[1]Co-Chairman ARIA ITALY; Unit of Allergology, Clinical Immunology and Respiratory Diseases, Fondazione Poliambulanza, Brescia, Italy
[2]Co-Chairman ARIA-Italy; Allergy and Respiratory Diseases, IRCCS Policlinico S. Martino, University of Genoa, Genoa, Italy
[3]UOC Pulmonary, "S. Valentino" Hospital, AULSS 2 Marca Trevigiana, Montebelluna, Treviso, Italy
[4]Department of DISC, Otolaryngologist Clinic-IRCCS Policlinico San Martino, University of Genoa, Genoa, Italy
[5]Department of Otorhinolaryngology and Skull Base Microsurgery-Neurosciences, Papa Giovanni XXIII Hospital, Bergamo, Italy
[6]Fondazione Salvatore Maugeri, IRCCS, Istituto Scientifico di Telese Terme, Benevento, Italy
[7]UOC Respiratory System Diseases, Università degli Studi di Catania, Catania, Italy
[8]UOC Respiratory System Diseases, Papa Giovanni XXIII Hospital, Bergamo, Italy
[9]UOC Pulmonary, Azienda Ospedaliera Universitaria Verona, Verona, Italy
[10]Unit of Allergology, Casa di Cura B. Palazzolo, Bergamo, Italy
[11]Società Italiana di Asma Allergologia e Immunologia Clinica, University of Chieti, Chieti, Italy
[12]Department of Clinical and Molecular Sciences, Università Politecnica delle Marche, Italy
[13]Allergy Unit, Department of Internal Medicine, University Hospital delle Marche, Ancona, Italy
[14]ENT Clinic, University of Foggia, Foggia, Italy
[15]Asthma Center and Allergy Unit, University of Verona and General Hospital, Verona, Italy
[16]Personalized Medicine, Asthma and Allergy, Humanitas Research Hospital, Rozzano, Milan, Italy
[*]Stefania Arasi, Diego Bagnasco, Ilaria Baiardini, Simona Barbaglia, Fabiola Barosso, Roberto Bernardini, Alvise Berti, Beatrice Bilò, Luisa Brussino, Frank Rikki Canevari, Giorgio Walter Canonica, Cristiano Caruso, Antonio Caviglia, Lorenzo Cecchi, Pasquale Comberiati, Lorenzo Cosmi, Marcello Cottini, Mariangiola Crivellaro, Danilo Di Bona, Alessando Fiocchi, Sandra Frateiacci, Matteo Gelardi, Giuseppe Guida, Enrico Heffler, Cristoforo Incorvaia, Massimo Landi, Amelia Licari, Luca Malvezzi, Manlio Milanese, Eustachio Nettis, Gianni Pala, Vincenzo Patella, Diego Peroni, Silvia Peveri, Francesca Puggioni, Alessandro Maria Pusateri, Erminia Ridolo, Oliviero Rossi, Eleonora Savi, Jan Schroeder, Veronica Seccia, Gianenrico Senna, Massimo Triggiani, Marzio Uberti, Gilda Varricchi, Maria Teresa Ventura, Maria Teresa Zedda

Key words

Chronic rhino sinusitis with nasal polyposis phenotype; CRSwNP; bronchial asthma; multidisciplinarity; biological agents.

Impact statement

The results of this survey obtained from an extensive number of Italian specialists allow some important concluding remarks about biologicals and the treatment of CRSwNP and its impact on asthma.

Corresponding author
Francesco Menzella
UOC Pulmonary
"S. Valentino" Hospital
AULSS 2 Marca Trevigiana
via S. Ambrogio di Fiera 37
31100 Treviso, Italy
ORCID: 0000-0003-3950-5789
E-mail: francesco.menzella@aulss2.veneto.it

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Summary

Background. *Chronic rhinosinusitis (CRS) is an inflammatory disease that affects the nasal mucosa and the paranasal sinuses. CRS can be associated by nasal polyposis (CRSwNP phenotype) in up to 30% of patients and it is frequently associated with bronchial asthma. CRSwNP shows predominantly an underlying activation of type 2 inflammatory pathways with the involvement of eosinophils, IgE, interleukin (IL)-4, IL-5 and IL-13. Biological drugs that target these inflammatory cytokines are currently a therapeutic option recognized by guidelines for the treatment of uncontrolled form of the disease.* ***Methods.*** *As part of the activity of the "ARIA-Italy" working group, a panel of 255 Italian Ear, Nose and Throat (ENT) specialists, pneumologists and immuno-allergologists actively participated in this national survey and answered a series of questions geared toward understanding the main criteria for patient characterization and therapeutic decision, highlighting multidisciplinarity, and the implementation of the management of CRSwNP patients, as a part of the precision medicine concept and the appropriate use of the biologicals.* ***Results.*** *Two hundred and fifty-five experts and specialists participated in the survey.* ***Conclusions.*** *The results of this survey obtained from an extensive number of active specialists throughout Italy allow some important concluding remarks to be drawn. The main points of agreement were that multidisciplinary care teams provide many benefits but that, once the team is established, meetings and communication between members must be coordinated. Finally, the dissemination of national disease registries and the continuous updating of guidelines and position papers related to CRSwNP and comorbidities should be encouraged.*

Introduction

Chronic rhinosinusitis (CRS) is an inflammatory disease affecting the nasal mucosa and paranasal sinuses, with prevalence varying in different geographical areas. In Europe, it is estimated that CRS may affect more than 10% of the adult population (1). The prevalent signs and symptoms that define CRS are nasal obstruction and congestion, anterior/posterior rhinorrhea, facial pain, hypo/anosmia, and sleep disturbances. CRS can present without (chronic rhinosinusitis without nasal polyposis, CRSsNP) or with nasal polyposis (chronic rhinosinusitis with nasal polyposis, CRSwNP). Polyps are semi-transparent, light gray lesions resulting from inflammation and remodeling of the mucosa of the sinuses or nasal cavity (2). Up to 30% of patients with CRS may present with the phenotypic form with nasal polyposis (3). From the patient's perspective, CRSwNP has a significant impact on the quality of life (QoL) (4). Patients with CRSwNP experience higher symptom scores and greater severity of the clinical disease if compared with patients with CRSsNP. From a pathophysiological point of view, CRSwNP is characterized by the activation of specific inflammatory pathways that define its endotype and influence its severity, course and response to treatments (1). In the majority of patients, the CRSwNP is associated with the activation of type 2 inflammatory pathways, with an increase in the concentration of eosinophils (systemic and/or local), IgE (systemic or even just local) and interleukin (IL)-4, IL-5 and IL-13 (5). Patients with CRSwNP frequently present with comorbidities, such as bronchial asthma, including late-onset and often severe forms, also characterized by a type 2 inflammation pattern, suggesting the existence of common immunological pathways between the two diseases (6). The chronicity characteristic of the disease and comorbidities imply frequent treatments to control recurrent symptoms including medical therapies (intranasal corticosteroids, oral steroids, antibiotics) and surgical approach (7). The high frequency of the use of systemic corticosteroids, however, is associated with complications and adverse events and that make the management of these patients complex (8). Today, only about 35-40% of patients with CRS are well controlled after conventional treatment (9). Comorbidities also require patients to be followed by different specialists, with an increasing need to coordinate interventions, to optimize their timing and effectiveness. In light of what has been highlighted on the diagnostic and treatment clearly emerges the importance of multidisciplinarity as the most appropriate tool for the management of the complex patient with CRSwNP. The introduction of biologic agents (monoclonal antibodies directed against molecules involved in inflammatory mechanisms such as IgE, IL-5, IL-4 and IL-13) as a therapeutic option for the treatment of CRSwNP has helped to improve significantly outcomes in patients with uncontrolled disease, improving QoL, and providing the basis for the achievement of personalized treatment targeted to the peculiar phenotypic and endotypic characteristics of each patient. However, the introduction of the new therapies raises new questions in clinical practice, such as the correct definition of the target patient type, the timing of intervention and the definition of the best biological agent for the specific patient phenotype/endotype, to ensure a personalized therapy while optimizing the cost/effectiveness of treatment (6). In particular, for the use of biologic drugs, there is a need for skills appropriate specialists who take into account the different components of the pathology (involvement of the upper and/or lower airways, allergies, drug hypersensitivity, recurrent infections, assessment of nasal structures and QoL of the patient). In real life clinical experience, complex situations are common, with patients with a long-standing history of pathology, undergoing different treatments including for the comorbidities, for whom the therapeutic decision is complicated and not clearly defined by the national and international Guidelines. For these patients, the multidisciplinary approach is crucial and mandatory.

Materials and methods

As part of the activities of the ARIA-Italy working group, a survey was organized with the participation of experts and specialists in allergology-immunology, pulmonology, and otolaryngology active throughout the Italian country. The survey was based on the completion of a questionnaire consisting of 17 items (**table I**). The questions focused on the following points: 1) management of the patient with CRSwNP in clinical practice; 2) factors to be considered for therapeutic decision-making (comorbidities, previous surgery, *etc.*); 3) criteria for characterizing the patient to undergo the treatment and choice of biological agent; and 4) role of multidisciplinarity for personalized patient management. Starting from literature evidence and the indications for treatment reported in the Guidelines, the participants answered the questions anonymously and taking into account the clinical practice in relation to the different regional realities. The opinions were collected during the period 2022-2023 and were discussed in a webinar coordinated by the authors of this article.

Results

Two hundred and fifty-five experts and specialists (age range: 26-77 years; M: 56%; F: 44%) participated in the survey. Participants came from all regions of Italy, with predominance for those from Lombardy – this region is the most populous in Italy with about 10 million people. Regarding the type of activity performed, the following distribution was observed: 130 hospital practitioners (51%); 84 freelancer practitioners (33%), 41 university professors and researchers (16%). Regarding the participant's specialty branch the distribution was as follows: 80 ENT specialists (31%), 71 immuno-allergologists (28%), and 104 pneumologists (41%) (**figure 1**). Although a wide distribution of responses was found, more than 64 of the respondents (25%) believed that the presence of asthma in their patients with CRSwNP was between 20 and 30% of the total cases; while on the other hand, more than 30% of the respondents believed that the presence of CRSwNP in patients with asthma was between 20 and 30% (**figure 2**). Finally, 250 participants (98%) thought it was important to assess the

Table I *- Survey ARIA CRS with polyposis and biologics: questionnaire.*

(1)	Age (yrs) Sex (M/F)
(2)	Specialty: 1) Allergology 2) Pneumology 3) ENT 4) Internal Medicine 5) Pediatrics
(3)	REGION of your Country (Italy)
(4)	Employment status: 1) University 2) Hospital Physician 3) Freelancer Practitioner
(5)	Approximately in how many of the patients with nasal polyposis do you find asthma? 5-10% 11-20% 20-30% 30-50% > 50%
(6)	Approximately in how many of the patients with asthma do you find nasal polyposis? 5-10% 11-20% 20-30% 30-50% >50%
(7)	To patients with nasal polyposis, do you make endoscopic surgery the first choice? YES NO
(8)	In patients with nasal polyposis, do you use systemic steroids? YES, in cycles YES, continuously NO, never
(9)	In case of using biological agent (according to indications) which one do you give preference to? Dupilumab Mepolizumab Omalizumab
(10)	When choosing a biologic agent to treat CRSwNP, do you take into account the presence of asthma comorbidity? YES NO
(11)	Do you think it is important to assess the presence of atopy in patients with CRSwNP? YES, always NO, never
(12)	For patients with N-ERD, who are difficult to treat and frequently have recurrence of polyposis, do you consider them suitable for therapy with biologic agents? YES NO
(13)	Where there is an indication, do you always initiate biologic agent therapy after polypectomy? YES, always NO, not necessary
(14)	How long after starting therapy with biological agent to treat CRSwNP do you consider the patient responder or non-responder? 3 months 6 months 9 months 12 months
(15)	In case you are an ENT specialist or pulmonologist/allergist, do you always have the referring counterpart specialist? YES NO
(16)	Does the facility where you work have a multidisciplinary team for the management of patients with CRSwNP? YES NO
(17)	In case of nasal polyposis, which of these tests do you use for monitoring over time? NPS SNOT-22 VAS total symptoms all of the above

Figure 1 - *Typology of work activity and specialty branch of the Survey participants.*

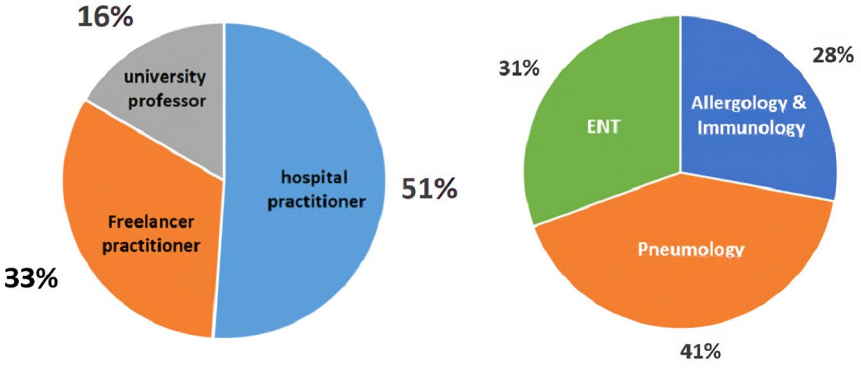
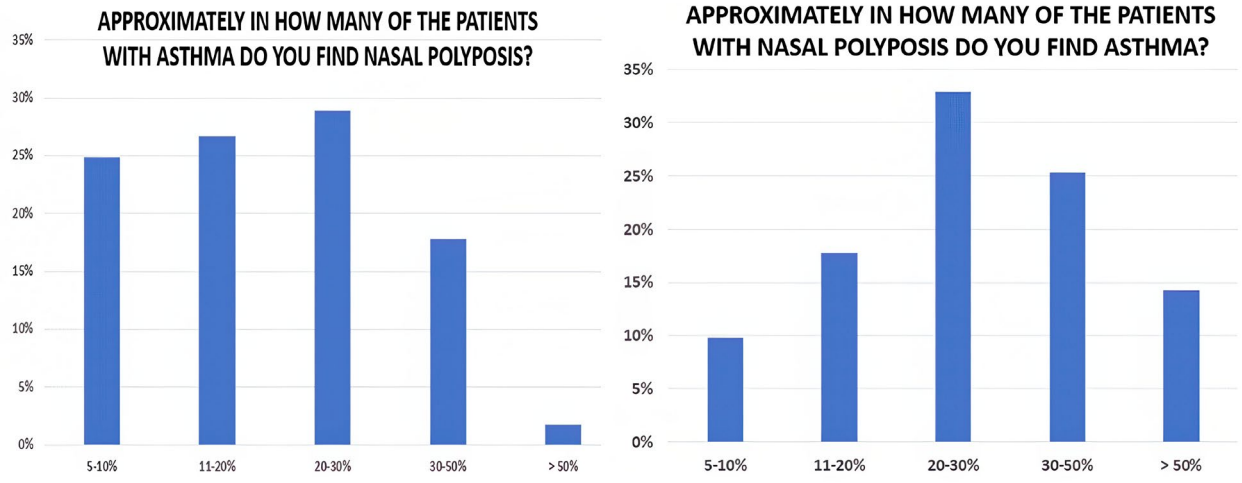
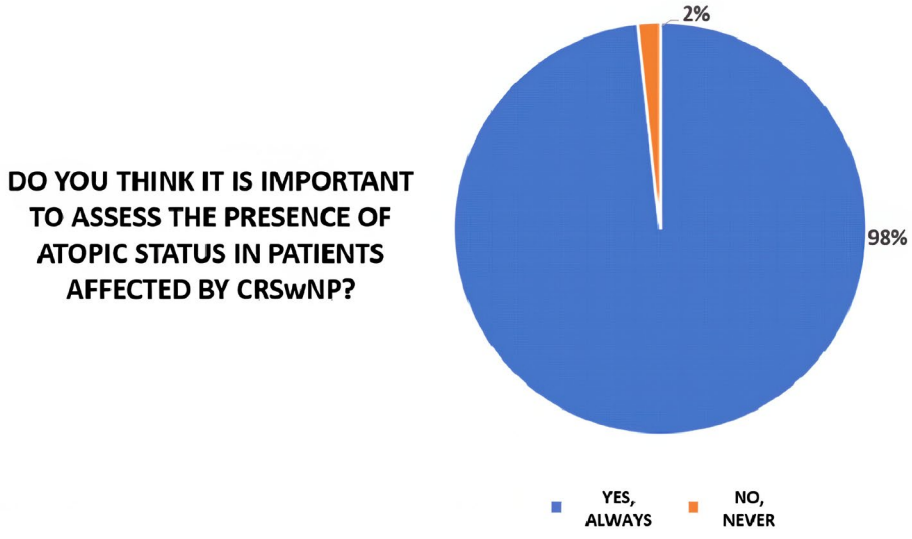


Figure 2 - *Items regarding the presence of comorbidities in the SRCwNP setting.*





presence of an atopic condition in CRSwNP patients (**figure 2**). Regarding CRSwNP therapeutic aspects, only 82 participants (32%) believe that endoscopic surgery should be the first choice in the treatment of CRSwNP today (**figure 3**). Regarding the use of systemic steroids in the treatment of CRSwNP, 68% of participants use them in cycles, 31% never use them, and only 2% use them continuously (**figure 3**). Some questions were specifically asked to assess participants' treatment behavior regarding the use of biological agents in CRSwNP. As can be seen from the results shown in **figure 4**, the participants believe that the preference among the various biological agents available in Italy today for the therapy of CRSwNP should be given to dupilumab (75% of responses); however, it should be pointed out that dupilumab was the first to be introduced for the treatment of polyposis and experience with omalizumab and mepolizumab in Italy was limited at the time the survey was conducted. When choosing the biological agent for the treatment of polyposis, asthma comorbidity is largely (98% of responses) taken into account. The use of biological agents is also being considered in other complex diseases

Figure 3 *- Items regarding the choice of endoscopic surgery and the use of systemic steroids.*

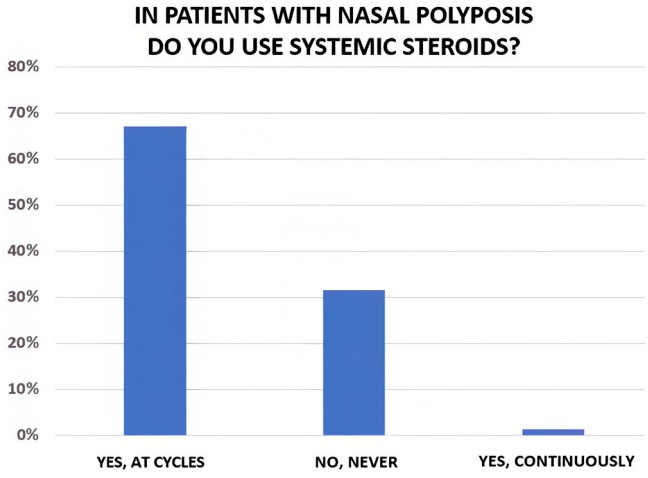
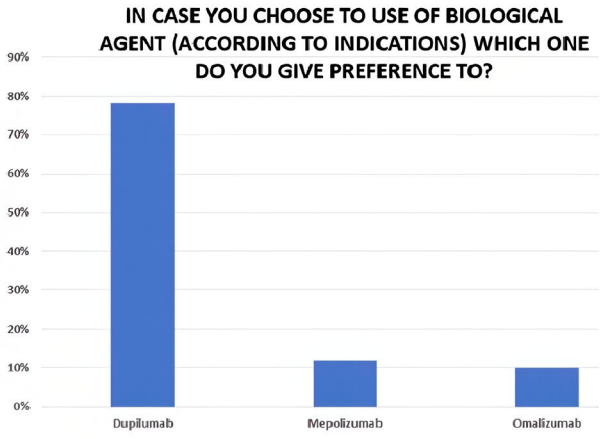
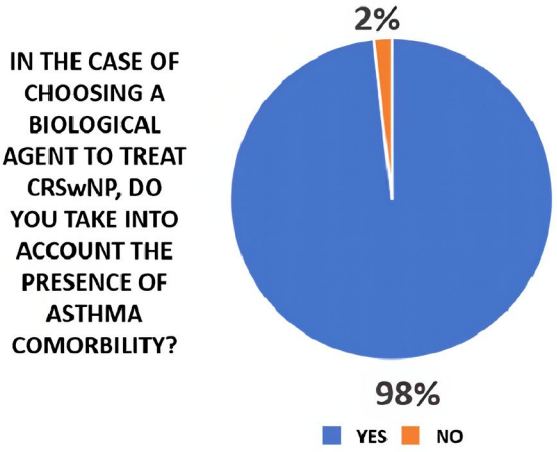
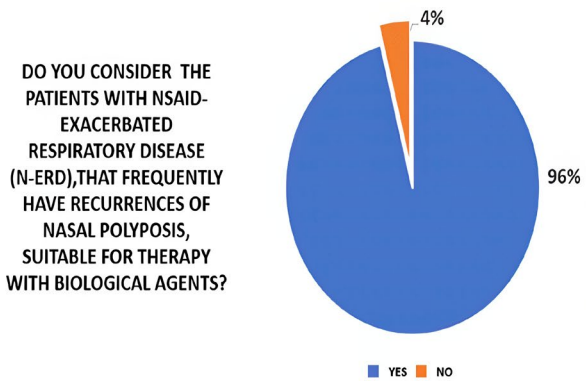


Figure 4 *- Specific questions and answers (%) about the approach to use biological agents in patients with CRSwNP.*







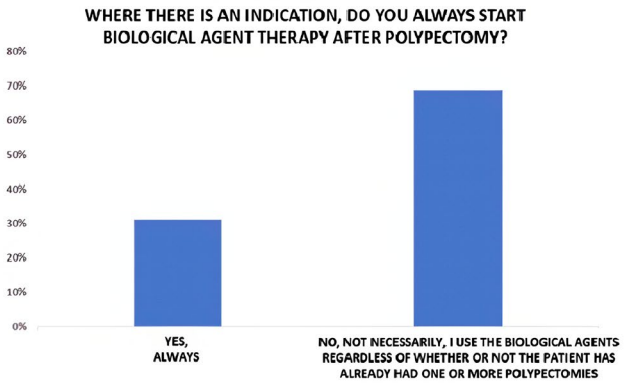
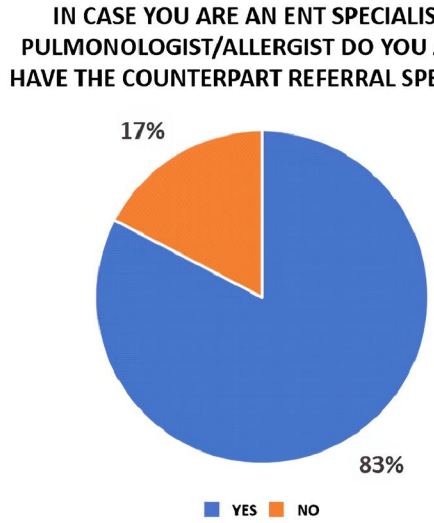
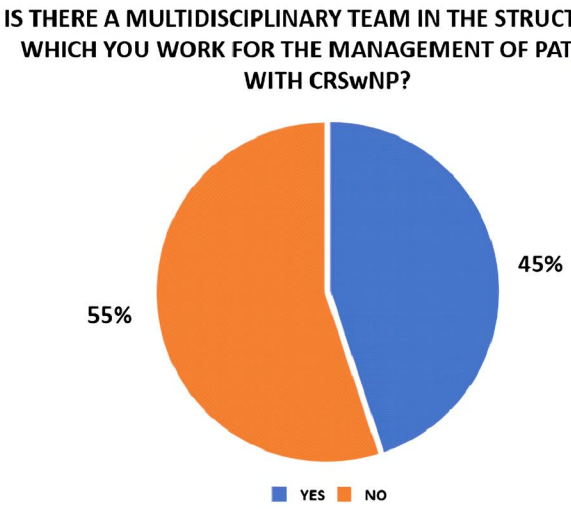


Figure 5 *- Specific questions and answers (%) about the organizational and multidisciplinary dynamics in patients with CRSwNP.*





characterized by the presence of comorbidities, such as N-ERD. Seventy percent of respondents believed that the use of biological agents in CRSwNP should not necessarily be postponed to polypectomy. Regarding the specific question "*After starting therapy with biological agent to cure CRSwNP when do you consider the patient responder or non-responder?*" participants answered: 3 months, 6 months, 9 months, and 12 months in 13%, 59%, 9%, and 19%, respectively; therefore, most of the participants believe that a 6-month observation is the most appropriate for evaluating the efficacy of CRSwNP biological therapy. The following question was then formulated: "*In case of nasal polyposis, which of these tests (SNOT-22, VAS, NPS) do you use for monitoring the response to therapy with biological agents over time?*" and participants answered 22% SNOT-22, 6% VAS, 5% NPS, and 67% all of the above, respectively. From this response can be inferred the focus on making the assessment of response to biological agents using multiple rating scales at the same time. The last part of the survey focused on opinions regarding the multidisciplinary approach to CRSwNP. While it is true that almost all participants (83%) confirm that they relate to other specialists in the management of this pathology, particularly when it is associated with other comorbidities (such as asthma); it is also true that only in a limited number of Centers (45%) has a multidisciplinary working group been established with facilitated diagnostic-therapeutic pathways for patients (**figure 5**).

Discussion and conclusions

The results of this survey obtained from an extensive number of active specialists throughout Italy allow some important concluding remarks to be drawn. The course of the patient with CRSwNP is made complex by the numerous symptoms and comorbidities that contribute to the definition of disease severity. The current availability of biological agents represents a potential improvement in the treatment and QoL of patients; but the use and choice of the biologic agents need to be optimized in clinical practice through discussion among specialists, so that it can be targeted to those patients who can benefit most from it, to reduce therapeutic inappropriateness and economic burden. In the context of CRSwNP and comorbidities the patients' point of view or patient perspective can be viewed through two different but related lenses: 1) the individual's perspective as it relates to each patient's individual situation and 2) the aggregate perspective of the CRSwNP population, *i.e.*, a perspective of common denominators despite unique individual variations. Recognition of the importance of the individual patient's perspective regarding their experience of CRSwNP is exemplified by the evolving patient/healthcare providers clinical interaction. Indeed, increasing recognition of the complexity of CRSwNP and comorbidities diagnosis and its treatments requires a "bidirectional exchange" of opinions and objectives between patients and healthcare providers, in order to promote integration of the patient perspective into the patient/healthcare providers relation-ship. Treatment focused on the underlying disease often fails to address the ripples of impact provoked by CRSwNP with comorbidities which may become the main source of concern to the patient. For the patient perspective to be valid, it must be informed by an adequate comprehension by the patient of the facts of the clinical situation (10, 11). Furthermore, the application of narrative medicine methodology could prove useful (12). Because patients with

CRSwNP have had only limited occasions to unite to have their voices heard, hence missing the opportunity to contribute to the improvement of CRSwNP care, it was recently published a Patient Advisory Board Statement of the European Forum for Research and Education in Allergy and Airways diseases (EUFOREA) (13). The aim of this initiative was to identify unmet needs in CRSwNP from the perspective of CRSwNP patients. Semi-structured interviews were conducted individually with European patients with CRSwNP and a panel of 30 members of the Patient Advisory Board reviewed the interview report and provided further input. Along with a loss of smell and continuous nasal secretions, most patients reported poor sleep quality and psychological impact as the most bothersome symptoms. Patients' frustrations relate primarily to the underestimation of the disease burden, the lack of coordination of care and the limited treatment options available to them. Treatment options with systemic steroids and/or nose surgery both have positive and negative aspects, including the lack of long-lasting efficacy. Better coordination of care, more patient-centered care, greater public awareness, increases in the disease mechanisms and better therapeutic options would be warmly welcomed by CRSwNP patients. The multidisciplinary approach, organization into networks, and the use of registries are identified as the key strategies for establishing a common language between the specialists and the patient, to implement the connection between specialist centers and the territory, diagnosis and management of the patient, with the goal of personalization of care. CRSwNP is certainly a "cross-cutting" condition that needs, in both the diagnostic and therapeutic phases, the contribution of multiple specialized expertise (14). Pharmacotherapy often may fail to treat CRSwNP and endoscopic sinus surgery (ESS) is often required. However, the synergistic use of pharmacotherapy and surgery often does not achieve disease control in the most severe cases. Furthermore, CRSwNP is associated with greater morbidity compared with CRSsNP, due to repeated exposure to OCS and surgery. The results of the present survey highlighted these contradictions. In particular, the response to question 8 concerning the use of OCS in CRSwNP, prompts a noteworthy observation: one-third of the surveyed specialists refrain from utilizing OCS, despite its established utility in controlling CRSwNP and assessing disease severity, along with its implications for biological therapy eligibility and for the potential excessive OCS use on CRSwNP management. These contradictory behaviors also emerge from the answers to question 13 about the sequencing of surgery and biological therapy; the striking revelation that 70% of respondents initiate biological therapy irrespective of prior surgical intervention suggests a prevailing inclination toward a medically-oriented approach to CRSwNP. This deviation from established guidelines advocating surgical intervention as the cornerstone of CRSwNP management, invites scholarly discourse and collaborative exploration. Furthermore, the significant economic and clinical burden of CRSwNP highlights the need for better treatment options and reorganization of the current care pathways (13). In this context, a multidisciplinary approach may improve CRSwNP management in patients with comorbidities, but currently there are only sparse examples of shared management models. Recently, an Italian panel of clinicians with different clinical expertise (pulmonologists, ear, nose and throat specialists, immunologists and allergy physicians) identified three different profiles of patients with coexisting asthma and nasal symptoms and discussed the specific tracks to guide a comprehensive approach to their diagnostic and therapeutic management: 1) Patient with severe asthma who needs to start a biologic therapy at the Allergy/Pulmonary Unit complaining about nasal symptoms; 2) Patient with severe asthma with ongoing biologic therapy at the Allergy/Pulmonary Unit complaining about nasal symptoms; and 3) Patient with Severe CRSwNP at the ENT Unit Complaining about Asthma Symptoms (15). Based on these different types of patients with comorbidities and different clinical and therapeutic presentation characteristics, it seems clear that there is a need to define a multidisciplinary approach by at least ENT specialist, allergist-immunologist and pulmonologist in order to evaluate symptoms and clinical history, confirm diagnoses and to identify the best treatment strategy aimed at controlling both diseases and preventing clinical exacerbations. Regarding the preponderance of respondents' choice of dupilumab (question 9), it should be pointed out that, because the opinions in the present survey were collected in the period 2022-2023, the use of mepolizumab and omalizumab is probably underestimated because these biologics have been introduced in Italy for the treatment of CRSwNP as of March 2023. To improve the management aspects of this clinical-pathological area, a study was recently published that has summarized the outcomes of a Delphi process involving a multidisciplinary panel of ENT specialists, pulmonologists, and allergist-immunologists involved in the management of CRSwNP, who attempted to reach consensus on key statements relating to the diagnosis, endotyping, classification and management (including the right placement of biologic agents) of CRSwNP patients (3). On the following points, we think we can agree that there are many theoretical benefits of a multidisciplinary approach, which include the reduced need for documents to make referrals, access to services and treatments that would otherwise be inaccessible (*e.g.*, radiological examinations, new biological treatments), optimized flow of patients from primary to secondary to tertiary care, management of adverse events, and obtaining a detailed overview of the management of multiple therapies for more than one pathology (16). Indeed, multidisciplinary care teams assure patient centrality, improvement of direct and indirect outcomes, cost reduction, and more appropriate therapeutic decisions (17-19). Once a multidisciplinary team is created, there is a need for coordination of meetings and communication between the various members. Among the effective and efficient planning tools capable of linking all phases of

diagnosis-care-assistance are, along with the Individual Therapeutic Plan (ITP) and Individualized Care Plan (ICP), the Diagnostic Therapeutic Care Pathways (DTCP). Other additional factors were considered to be useful as theoretical-practical multidisciplinary training events on diagnosis and therapy, which will attract considerable interest from ENT specialists, pulmonologists and immuno-allergists. Educational events were also considered to be important since the approach to CRSwNP and comorbidities is evolving rapidly, and the number of treatment options is expanding. Finally, the use and dissemination of national disease registries and the continuous updating of guidelines and position papers related to CRSwNP and comorbidities should be encouraged.

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Contributions

CL, GP, FM, GWC: conceptualization, data curation, formal analysis, writing – original draft, writing – review & editing; the other authors participated in the survey, reviewed and approved the manuscript.

Conflict of interests

The authors declare that they have no conflict of interests.

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Rita Barbosa Silva[1], Ângela Moreira[1], Beatriz Pimenta[1], Inês Pádua[1-3]

Allergens weaning: what is missing from commercial baby food?

[1]University Institute of Health Sciences (IUCS), CESPU, Gandra, Portugal
[2]UCIBIO - Applied Molecular Biosciences Unit, Translational Toxicology Research Laboratory, University Institute of Health Sciences (1H-TOXRUN, IUCS-CESPU), Gandra, Portugal
[3]Associate Laboratory i4HB - Institute for Health and Bioeconomy, University Institute of Health Sciences, CESPU, Gandra, Portugal

Key words

Food allergy; food allergen; complementary feeding; weaning; prevention.

Corresponding author
Inês Pádua
University Institute of Health Sciences (IUCS), CESPU
Rua Central da Gandra 1317
4585-116 Gandra, Portugal
ORCID: 0000-0003-1100-9441
E-mail: maria.silva@iucs.cespu.pt

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Impact statement

This study highlights the scarcity of major food allergens in commercial baby foods and their frequent ultra-processing, emphasizing the need for healthier, allergen-inclusive products to support food allergy prevention.

Summary

Background. *Current recommendations for infant weaning suggest introducing common food allergens by the age of 12 months. While homemade meals are advisable, there is a notable demand for commercially available complementary foods (CACF). Furthermore, emerging evidence suggests a potential link between the consumption of ultra-processed products and the incidence of allergic diseases. This study aimed to examine the presence of the fourteen main food allergens in CACF ingredients through label analysis and evaluate their extent of processing.* ***Methods.*** *Between January and February 2024, labels of all CACF found in infant feeding sections of 10 Portuguese grocery retailers were analyzed. CACF were categorized based on the NOVA food classification system's processing levels. Milk formulas, products for children over 15 months, and those for children with food allergies or intolerances were excluded.* ***Results.*** *Of the 492 products analyzed, 132 contained wheat and 112 contained milk. 16 products included fish and 6 contained eggs. Soy was listed as an ingredient in 11 products, mainly as soy lecithin. Only 2 products contained nuts, and 1 product contained peanuts. None of the products contained the remaining six allergens. The majority of milk- and wheat-containing products were classified as ultra-processed and contained added sugars and/or sweeteners.* ***Conclusions.*** *Despite the current guidelines, commercial baby foods often lack major allergens, namely nuts and peanuts, eggs, and shellfish. Our results underscore the need for healthy, age-appropriate, minimally processed products that incorporate rather than exclude major food allergens.*

Introduction

The introduction of allergenic foods during complementary feeding has been a topic of significant research interest in the context of preventing food allergy in infants. Studies have indicated that the early introduction of allergenic foods, such as peanut and egg, during the complementary feeding period may reduce the risk of developing food allergies, even in infants at high risk of food allergy (1, 2). This approach represents a shift from previous recommendations of food allergen avoidance to the promotion of deliberate and regular dietary intake of these allergens during the introduction of complementary feeding (3).

Although it is advisable for parents to introduce home-prepared meals (4, 5), there is a strong consumer demand for commercially available complementary foods (CACFs), and the choice in supermarkets is vast and driven for many reasons, such as convenience, portability and food safety (6). Accordingly, although scientific evidence on infant consumption trends is still scarce, a study conducted on a cohort of infants and children from sev-

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eral European countries demonstrated that the majority consume CACFs during the first two years of life (7).
The main objective of this study was to evaluate the presence of the eight main allergens (cow's milk, egg, wheat, soy, peanut, nut, fish and shellfish) as an ingredient in CACFs through the analysis of their labelling.

Materials and methods

From January to February 2024, a cross-sectional study of product labels within sections intended for infant feeding, encompassing both physical and digital retail platforms, was conducted across ten Portuguese grocery retailers/companies and infant food manufacturers. The CACFs were categorized into five distinct classes: snacks, meals, fruit pots and pouches, porridges, and yoghurt/veggie-based yoghurt pouches. Milk formulas were excluded, as well as products intended for children older than 15 months and for children with food intolerances or allergies. Ingredient lists were assessed for the presence of the fourteen substances or products causing allergies or intolerances, according to Reg EU nº 1169/2011 (cow's milk, soy, egg, wheat, peanut, tree nuts, fish, shellfish, sesame, lupine, mustard, celery, and sulfites). The content of sugar, sweeteners and additives was also analyzed, and food products were classified by degree of processing based on the groups defined by the NOVA food classification system (8).

Results

We have identified 492 CACFs for infants aged less than 15 months. Among these products, 41.5% (n = 204) were fruit pots and pouches, 20.3% (n = 100) were porridges, 13.8% (n = 68) were categorized as finger food snacks, 13.2% (n = 65) as prepared meals, and 11.2% (n = 55) as yoghurt/vegetable-based yoghurt pouches.

Food allergen presence

The food category that presented the highest presence of allergens was yoghurt/veggie-based yoghurt pouches (87%) followed by porridges (86%) whereas fruit pots and pouches was the category with the lowest presence of food allergens.
Concerning food allergen presence, the most common food allergens in CACFs were wheat, reported in 132 CACFs (26.8%), and cow's milk, reported in 121 (24.6%). Soy was identified as an ingredient in 11 products (2.2%); however, in the majority of them (10 products), it was in the form of soy lecithin for emulsifying properties. Fish was reported as an ingredient only in 16 products (3.3%), and in 3 of these was in the form of fish oil. Egg was found in 6 CACFs (1.2%), nuts in 2 (0.4%), and peanuts in only one product (0.2%). None of the products contained shellfish, sesame, lupine, mustard, celery, and sulfites.
Allergens were described and highlighted in accordance with current regulations, mostly with the whole food name, even if they were non-natural ingredients for which more terminology was required, such as hydrolyzed wheat or soy lecithin.
In this study, 168 (34.1%) CACFs had allergens listed in the first three ingredients of their labels. For all CACFs, these allergens were wheat and/or cow's milk, except for those containing fish. None of the products listed the specific percentage of milk, wheat, soy, fish, egg, nut or peanut protein present, not enabling an estimation of the quantity in grams of food allergen present per serve.

Precautionary allergen labelling

Precautionary allergen labelling, which is voluntary and not standardized following the legislation issued by the European Union (Reg EU nº 1169/2011), was found in 17.7% of products (n = 87). The most frequently reported allergen in labelling warnings was soy (n = 60), followed by milk (n = 48) and nuts (n = 23).

Sugar content and degree of processing of the CACFs containing major food allergens

The analysis also included an assessment of added sugar, free sugars, and artificial sweeteners content in CACFs. Among products containing cow's milk and wheat, 86.8% (n = 105) and 72.0% (n = 95), respectively, were found to contain sugars and/or sweeteners. All soy lecithin-containing products also contained sugars and/or sweeteners, and similarly, the three fish products containing fish oil were found to be sweetened. Regarding products containing eggs, half of them also contained sugar/sweeteners. No products with nuts and peanuts contain sugar or sweeteners.
Food products were also classified by degree of processing, based on the groups defined by the NOVA food classification system (8). The NOVA system classifies all foods and food products into four groups, according to the nature, extent, and purpose of industrial food processing applied. Group 4 corresponds to ultra-processed foods (UPF), defined as formulations of ingredients (as oils, fats, sugars, starch, protein isolates), primarily designed for industrial applications, that are submitted to various sequences of industrial processes, often necessitating high-tech equipment. These processes include the fractioning of whole foods, use of techniques such as extrusion, molding and pre-frying, and the use of additives at various stages of manufacture (9). In this sample, 253 of the total CACF were classified as UPF, 76 as processed food (PF), and 163 as minimum processed food (MPF). The CACF class with the most products classified as UPF were fruit pots and pouches (99 products), followed by porridges (n = 81) and yoghurt/veggie-based yoghurt pouches (n = 30). The results also showed that most products containing milk (n = 110; 90.9%) and wheat (n = 97; 73.5%) were UPF. 2 of the 6 egg-containing products were also UPF.

Discussion and conclusions

The results of our study reveal that CACFs in Portugal have a generally low presence of major food allergens, not reflecting the

current infant feeding and allergy prevention guidelines that the prioritize inclusion of food allergens in order to foster oral tolerance and diminish the likelihood of food allergy development.
The latest Portuguese national recommendations for complementary feeding date from 2019 (10), and despite advising that the introduction of potentially allergenic foods not be delayed, they are still silent regarding the imperative of introducing these allergens in terms of allergy prevention. Notably absent from these guidelines is explicit guidance on introducing tree nuts, peanuts and shellfish, potentially influencing both household attitudes and product development by the food industry, notwithstanding the broader context provided by international guidelines.
Few studies exist on the prevalence of food allergies in Portugal. Two studies in pediatric age reported a prevalence of food allergies of 1% in children and adolescents (11, 12), and for adults, the reported prevalence was between 1% and 4% (13, 14). However, considering the study period or the studies' geographical specificity, the results may not be fully representative.
Nevertheless, data from these studies (11-14) show that most foods implicated in allergic reactions are included in the so-called "big eight allergens". Likewise, the Portuguese Anaphylaxis Registry reported that food is the leading cause of anaphylaxis in the pediatric population, with cow's milk, tree nuts, shellfish, egg, fresh fruits, fish, and peanut being the main elicitors (15). These data reinforce the importance of concerted strategies regarding food allergy prevention, particularly for major food allergens.
Different studies in different countries have focused on nutritional analysis of CACF (16-19), however there is a paucity of works that address the allergen content of weaning foods. In this context, our results are in line with previous results reported in Australia (20) and United Kingdom (21), where low availability of CACF with food allergens is also reported. Although the legal, commercial and epidemiological contexts differ between Portugal and these two countries, the results taken together highlight the need for greater effort in developing and accepting CACF with allergens for infants.
We found that in addition to the low allergen content of CACF, those that contain them are mostly UPF and contain sugar and/or sweeteners, making them not nutritionally compliant to be widely recommended. Recommendations for complementary feeding have been consistent in recommending not to introduce/limit sugars and sweeteners (22). For UPF, emerging evidence suggests that the consumption of ultra-processed products could be positively associated with the occurrence of food allergic diseases and may affect allergy prevention, possible mainly due to the presence of advanced glycation end products (AGEs) (23, 24), emulsifiers (25) and changes in gut microbiome composition (26).
Apart from the limited presence of allergen-containing products and their nutritional quality, it is crucial to highlight that the recommended age ranges specified by manufacturers may also not align with allergen weaning guidelines. For instance, despite the recommendation to introduce nuts and peanuts from 6 months onwards (3, 22), the available products are marketed for children aged over 9 and 12 months, respectively. This point also deserves some reflection, considering consumption trends in Portugal, which reflect a growing presence of nuts in the population's diet (27), and the fact that peanuts are one of the allergens associated with anaphylactic reactions (15).
Our study has limitations such as the fact that we analyzed a small number of products that can be introduced into children's diets, despite having analyzed practically all of those that are marketed to them. Despite these limitations, our study allows us to characterize the national supply in terms of CACFS considering its use for the allergens weaning. It is also, to our knowledge, the first work that specifically relates the content of allergenic ingredients with the content of added sugar and sweeteners and the degree of processing.
Our results reinforce the need for more significant investment in developing healthy, age-adapted, minimally processed products that include, rather than avoid, major food allergens. At the same time, continual public health messaging strategies are essential for effectively encouraging caregivers to safely introduce major food allergens into home-prepared meals and also nationally adapted, scientific and practical guidance that meets the potential for preventing allergic disease.

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Contributions

RBS: conceptualization, methodology, formal analysis, investigation, writing – original draft. AM, BP: conceptualization, methodology, formal analysis, investigation. IP: conceptualization, methodology, writing – review & editing, visualization, supervision.

Conflict of interests

The authors declare that they have no conflict of interests.

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LETTER TO THE EDITOR

Eur Ann Allergy Clin Immunol Vol 57, N.6, 282-285, 2025

Jorge Sanchez[1], Margarita Velásquez[2], María Fernanda Ordoñez[3,4]

Baricitinib for atopic dermatitis in real life: effectiveness, safety profile, and adherence

[1]Group of Clinical and Experimental Allergy, Hospital "Alma Mater de Antioquia", University of Antioquia, Medellín, Colombia
[2]Dermatological Research Center, University of Antioquia, Medellín, Colombia
[3]Division of Dermatology, Department of Internal Medicine, Hospital "Militar Central", Bogotá, Colombia
[4]Atopic Dermatitis Clinic, Cayre Risk of Fracture, Bogotá, Colombia

Key words

Baricitinib; atopic dermatitis; real life; lifestyle; SCORAD.

Corresponding author
Jorge Sanchez
Group of Clinical and Experimental Allergy
Hospital "Alma Mater de Antioquia"
University of Antioquia
Cra 27, n37 B Sur 60, Medellín, Colombia
ORCID: 0000-0001-6341-783X
E-mail jorgem.sanchez@udea.edu.co

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To the Editor,

in the last decade new molecules has been offered for the treatment of severe atopic dermatitis (1, 2). Baricitinib has proved its efficacy in different clinical trials (2), but there is lacking information in real life (3, 4) and little has been studied on aspects such as adherence, tolerance, and the time needed to evaluate the clinical response before considering continuing or changing therapy. In this study we present new information in real life about baricitinib in atopic dermatitis.

Twenty-seven patients from two different centers were included and follow-up for one year. The ethics committee of the "Alma Mater de Antioquia" Hospital gave its approval for this study (protocol IN41-2022). In the first six months, a bimonthly follow-up was carried out to evaluate the response to therapy and adherence to treatment. At the beginning of the second semester, based in clinical control, patients continued or not with baricitinib, and follow-ups were carried out every 3 months. Due to local regulations, all patients who received baricitinib were over 18 years of age, had previously received at least one immunosuppressant without adequate clinical response or with serious adverse events, and had an Atopic Dermatitis score (SCORAD) greater than 20 points. Clinical response was defined as SCORAD ≤ 14 points and a change from baseline of one minimum clinically important difference of the SCORAD (≥ 9 points). Recruited patients with SCORAD less than 30 points had to have an Atopic Der-

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matitis Control Test (ADCT) greater than 12 points and Dermatology Life Quality Index (DLQI) over 15 points. All patients received the same dose of Baricitinib: 4 mg/day. In those patients who did not achieve clinical response after six months, baricitinib was discontinued.

Table I presents the characteristics of the patients as well as the clinical changes during the first six months. A total of 14 mild adverse events were presented in 8 patients with a median duration of 14 days and none of them suspend the therapy. Thirteen (48.1%) of the patients achieved clinical control in all the scales used (DLQI, ADCT, SCORAD); clinical control was achieved in the first 2 months in 12 of the 13 patients. Among them, none experienced moderate or severe relapse during the one-year follow-up (**figure 1A**); additional 3 patients achieved pruritus control but low change in eczema extension and severity; 11 patients did not show improvement after six months with baricitinib, so it was suspended. No severe effects were reported. Adherence to treatment was calculated according to the number of days with treatment *versus* number of days not taking it and expressed as a percentage *[(Days treatment taken / total treatment days prescribed) × 100]*. The median adherence was 86.7% and it was not significantly different between patients who had clinical response and patients without it.

When comparing the characteristics of patients who had clinical control *versus* those who did not have clinical control, we observed that those with a clinical response had a lower SCORAD at the beginning of the treatment (**figure 1B**). Other factors were not associated with differences in clinical response.

For some national health systems baricitinib is less expensive than other JAK-inhibitors or biologics. In our study, less than 50% of patients achieved an adequate response to treatment with Baricitinib but among these patients, control was nearly complete and in least than two months which is enough time to evaluate the clinical response.

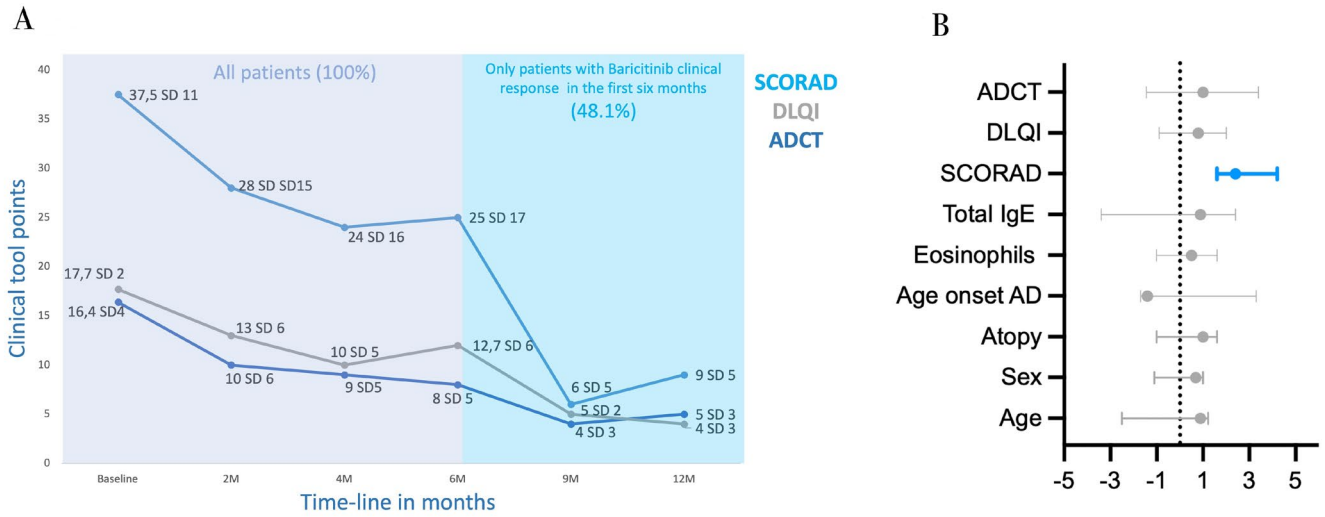
According to our results, in general, adherence was high, perhaps due to the Hawthorne effect (5). Different factors affect adher-

Table I *- General characteristics.*

Characteristics	Baseline	After six months
Age (mean, SD)	30.19 (9.14)	30.69 (9.75)
Male sex	14 (51.9%)	
Atopy	27 (100%)	
AD onset in years (mean, SD)	3.3 (1.4)	
Eosinophils (mean, SD)	262 (302)	234 (332)
Total IgE (mean, SD)	536 (53)	539 (58)
DLQI (mean, SD)	17.7 (2.9)	12.7 (6.48) *
DLQI ≤ 6 points	0	13
ADCT (mean, SD)	16.4 (4.6)	8.89 (5.98) *
ADCT ≤ 6 points	0	13
Pruritus	8 (3.4)	5 (2.3) *
SCORAD (mean, SD)	37.5 (11.71)	25 (17.2) *
Patients SCORAD ≤ 20 points	0	13
Patients SCORAD75%	N/A	13
Patients SCORAD90%	N/A	10
Adverse events	N/A	14
Severe	N/A	0
Gastrointestinal	N/A	4
Respiratory infections	N/A	6
Other	N/A	4

Clinical and sociodemographic characteristics of patients before and after six months with baricitinib. Atopy was defined as one positive specific IgE. Pruritus was defined according to a subjective scale from 0 (no pruritus) to 10 (intense pruritus). SCORAD: Score atopic dermatitis; DLQI: Dermatology life quality index; ADCT: Atopic dermatitis control tests; N/A: No apply. *$p < 0.05$.

Figure 1 *- Clinical response to Baricitinib and factors associated.*



(A) Score obtained according to the SCORAD (Score atopic dermatitis), DLQI (Dermatology life quality index), and ADCT (Atopic dermatitis control test) scales. In the first six months, follow-up is presented for all patients (n = 27) but from month 6 onwards, only those who had clinical control with Baricitinib are presented; **(B)** Exploration of the variables associated with clinical control with Baricitinib according to odds ratio.

ence such as patient education, support systems, and socio-economic status. In our study, none of these factors seemed to be associated with adherence failures, perhaps because the health system in Colombia covers the full cost of the therapy. However, 5 of the 13 patients who had clinical control forgot sometimes to take the medication, indicating that treatment tolerates some interruptions. AD has a major impact on mental health, unfortunately we did not assess this aspect in the study. Including additional measures to evaluate the psychological and emotional well-being of patients would provide a more holistic assessment of the treatment's effects. Finally, as an exploratory analysis, we observed that the most appropriate profile to start baricitinib therapy are patients with SCORAD lower than 40 points. Although severe skin pruritus is one of the most important clinical targets of JAK-inhibitors, it did not appear to be a determining factor to predict clinical response with baricitinib.

In conclusion, this study provides valuable insights into the use of baricitinib for severe atopic dermatitis. While the findings are promising, such as the rapid clinical response and good adherence, significant limitations, including no control group, a small sample size, and lack of long-term data, should be noted. Future research should focus on larger, more diverse populations and include detailed analyses of cost-effectiveness and long-term outcomes. This study lays the foundation for understanding the potential role of baricitinib in treating severe atopic dermatitis and emphasizes the need for ongoing investigation.

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Contributions

JS: conceptualization. MV, MFO: writing – original draft, writing – review & editing, investigation, formal analysis.

Conflict of interests

JS, MV, and MFO have been advisors and speakers for Lilly, Pfizer, Sanofi, and Abbvie laboratories. The conflict of interests are not related to this work.

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LETTER TO THE EDITOR

Eur Ann Allergy Clin Immunol Vol 57, N.6, 286-288, 2025

Monica Colque-Bayona[1], Daniel Laorden[2], David Romero[2], Santiago Quirce[1,3,4], Javier Domínguez-Ortega[1,3,4]

Anti-IL5/5R in the treatment of chronic eosinophilic pneumonia and severe asthma

[1]Department of Allergy, La Paz University Hospital, Madrid, Spain
[2]Department of Pulmonology, La Paz University Hospital, Madrid, Spain
[3]La Paz Institute for Health Research (IdiPAZ), Madrid, Spain
[4]CIBER of Respiratory Diseases (CIBERES), Madrid, Spain

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Chronic eosinophilic pneumonia; severe asthma; mepolizumab; reslizumab; benralizumab.

Corresponding author
Monica Colque-Bayona
Department of Allergy
La Paz University Hospital
Paseo de la Castellana 261
Madrid, Spain 28046
ORCID: 0000-0003-1534-5573
E-mail: monica.colque@salud.madrid.org

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To the Editor,

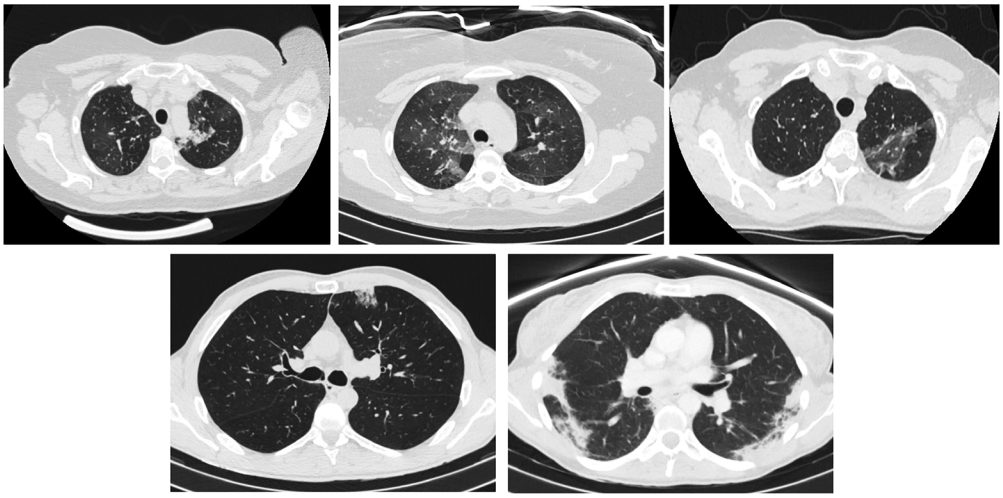
chronic eosinophilic pneumonia (CEP) is a rare disease among the diffuse parenchymal lung diseases characterized by significant eosinophil infiltrations in the pulmonary parenchyma and the alveolar spaces (1). Patients with CEP frequently have history of asthma and atopy, therefore it may occur predominantly in patients who are prone to develop a T-helper-2 response. Currently, it diagnosis is based on the presence of respiratory symptoms for at least two weeks, chest radiologic findings (diffuse pulmonary alveolar consolidation and/or ground glass opacities, especially with peripheral predominance), the presence of eosinophilia at bronchoalveolar lavage (BAL) and/or peripheral blood (a BAL cell count differential > 25% or blood eosinophils > 1,000/μL), and the absence of other known causes of eosinophilic lung diseases (2). Although oral corticosteroids (OCS) are the mainstay treatment with usually a good respond, relapses frequently occur while decreasing or stopping OCS, thus requiring prolonged treatment with the risk of long-term side effects (1, 2).

In last years, the knowledge of eosinophil biology has led to the development of several biologics targeting eosinophils such as biologics targeting interleukin (IL)-5 (mepolizumab and reslizumab) and IL-5 receptor (benralizumab) (3). These therapies have revolutionized glucocorticoid sparing treatment of eosinophilic respiratory diseases (4). Due that eosinophils play a primary role in the pathophysiologic of CEP and the association with asthma (2), eosinophil-specific biologics may be alternative candidates for the treatment. Recent data in case series (5-7) and case reports (8-10) show their potential benefit effect in this disease. Here, we present an additional case series of patients with diagnosis of CEP and concomitant severe eosinophilic asthma treated successfully with anti-IL-5/5R biologics.

We retrospectively analyzed the clinical records of patients with diagnosis of CEP and severe asthma treated with anti IL5/5R therapy in our department from 2010 to 2023. We evaluated the effect of biologic therapy on the daily dose of OCS, number of annual asthma exacerbations, asthma control assessed by

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Figure 1 *- Chest computed tomography at the moment of diagnosis of chronic eosinophilic pneumonia.*



the Asthma Control Test (ACT) and peripheral blood eosinophil counts at baseline and after one year of treatment.

Six patients were included (five women and one man). The mean age at diagnosis of CEP was 39.6 years (from 21 to 49 years). Five had concomitant diagnosis of severe uncontrolled asthma and allergic rhinoconjunctivitis, and one asthma-chronic obstructive pulmonary disease overlap. Two were former smokers. CEP was diagnosed based on the criteria described before (2) and other causes of eosinophilic lung diseases were excluded. Five patients had compatible findings on the lung computed tomography (CT) (**figure 1**) with marked eosinophilia at BAL in three patients (mean of 38% of eosinophils, range 30-49%) and the other two patients presented peripheral blood eosinophilia (1,730 and 4,400/μL). One patient was diagnosed by transbronchial lung biopsy. In this patient we could not collect the CT images nor the laboratory data at the moment of the diagnosis. In addition, all the patients underwent screening tests for eosinophilic granulomatosis with polyangiitis (EGPA) and had negative results for proteinase 3 antineutrophil cytoplasmic antibodies (PR-3 ANCA) and myeloperoxidase antineutrophil cytoplasmic antibodies (MPO-ANCA).

Anti-IL-5/IL5R were principally prescribed because of severe uncontrolled asthma and the prolonged glucocorticoid treatment. Prior the biologic therapy, all patients were treated with at least high-dose inhaled corticosteroids plus long-acting β-agonists with poor control of their asthma (mean of ACT 16.6, range from 16 to 18). Five patients were receiving OCS with a mean daily dose of prednisone of 12 mg/day (from to 5-30 mg). One patient presented avascular necrosis of the femoral head and shoulder and developed diabetes related to corticosteroid treatment.

Reslizumab was prescribed in two patients (200 and 337 mg every 4 weeks according to the patient's weight), two received mepolizumab (100 mg every 4 weeks) and two benralizumab (30 mg every 8 weeks). One of them had received omalizumab previously. One patient reported headaches associated to mepolizumab. No other adverse effects of biologics were recorded.

After one year of treatment with anti IL5/IL5R, among the five patients with OCS, three could discontinued the corticosteroid treatment; in one patient the daily dose of prednisone was dropped from 30 to 10 mg, and one continued with the same dose (5 mg/day). All patients had reached asthma control according to the ACT (mean 23.3, range 21 to 25) and we found a decrease in the mean of asthma annual rate of exacerbations (from 2.5 to 0.6). Regarding the blood eosinophils count, we found a decrease from a mean 1,316.6/μL (400-3,970/μL) to 60/μL (0-150/μL). No relapses of CEP have been observed since the introduction of anti IL-5/5R. No changes in the spirometry values had been observed. The summary of our findings is shown in **table I**.

Although there is clear evidence of the efficacy and safety of anti-IL-5/IL5R in severe asthma that led their approval for its treatment by the European Medicines Agency (EMA) and the Food and Drug Administration (FDA), there is still scarce data of their efficacy on CEP. In the present study, we found that anti-eosinophil biologics were effective in the treatment of both CEP and severe asthma, especially in terms of reducing or discontinuing the OCS therapy and controlling both diseases decreasing asthma exacerbations and CEP relapses. Recently published case series described similar findings: Delcors *et al.* (5) reported a case of series of 29 patients treated with mepolizumab and benrazilumab; after a median duration of 13 months, no CEP relapse was reported, the median annual rate of severe asthma exacerbations decreased from 0.15 to 0, and 72% of the patients were eventually weaned from oral corticosteroids. Moreover, Brenard *et al.* (6) reported a case series of

Table I *- Clinical and laboratory outcomes after one year of Anti-IL5/5R treatment.*

	All patients (n = 6)	Reslizumab (n = 2)	Mepolizumab (n = 2)	Benralizumab (n = 2)
Blood eosinophil count (cells/μL)	1,316.6 (400-3,970)	1,180 (520-1,840)	585 (530-640)	2,185 (400-3,970)
Prior treatment	60 (0-150)	65 (60-70)	105 (150-60)	10 (0-20)
After one year				
ACT (mean, range)	16.6 (16-18)	(16-17)	(16-17)	(16-18)
Prior treatment	23.3 (21-25)	(21-24)	(23-25)	(23-24)
After one year				
Number of annual asthma exacerbations (mean, range)	2.5 (1-5)	(1-3)	(3-5)	(1-2)
Prior treatment	0.6 (0-3)	0	(1-3)	0
After one year				
Patients treated with OCS (n)	5	1	2	2
Prior treatment	2	0	1	1
After one year				
Daily dose of prednisone mg/día (mean, range)	12 (5-30)	5	(5-10)	30
Prior treatment	7.5 (5-10)	0	5	10
After one year				

ACT: Asthma Control Test; FEV_1: forced expiratory volume in 1 second; FVC: forced vital capacity; OCS: oral corticosteroids.

10 patients with CEP treated with mepolizumab, after a median follow-up of 9 months, the treatment was associated with a significant annual rate of relapse (from 0.8 to 0), a lower consumption of corticosteroids (tapered from 5 to 0 mg) and also a remission of lung lesions on follow-up high resolution CT.

In conclusion, based on our findings and the previous literature, anti-IL-5/5R can be a safe and effective treatment in steroid-dependent patients with CEP and severe asthma.

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Contributions

All authors: conceptualization, data curation, formal analysis, methodology, writing - original draft, writing - review & editing.

Conflict of interests

The authors declare that they have no conflict of interests.

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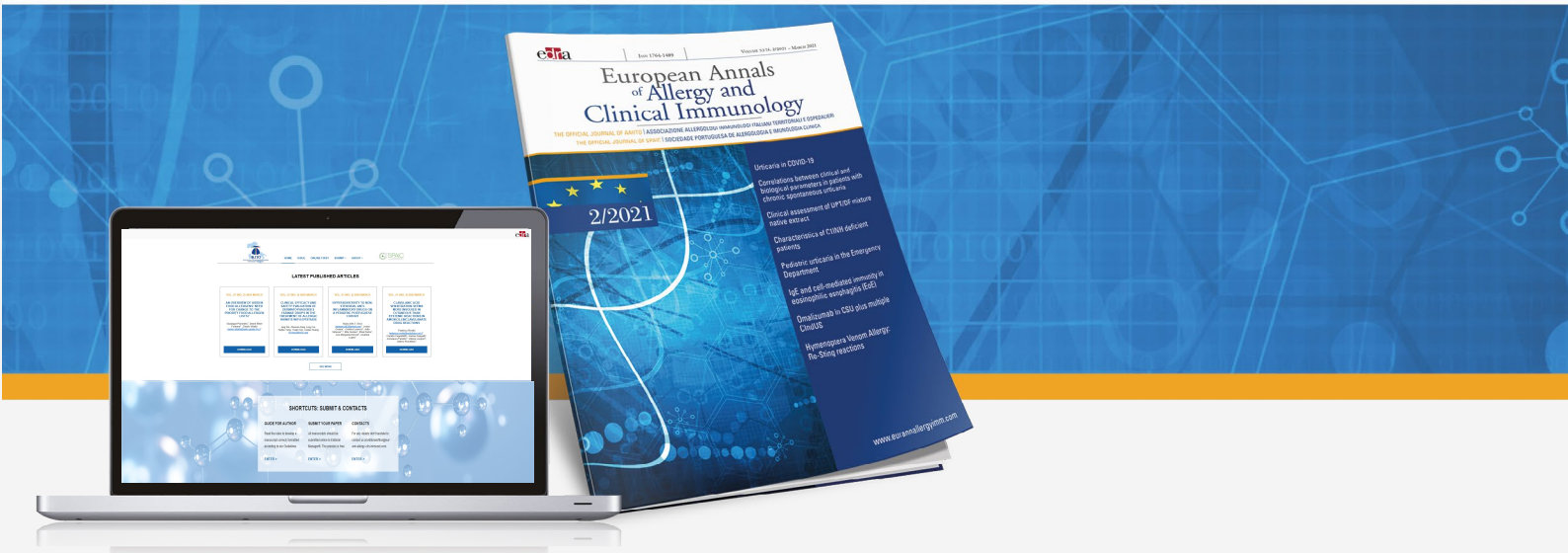
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