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Nasal cytology identifies allergic rhinitis phenotypes for managing allergen immunotherapy in clinical practice

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Abstract
Backgrounds Allergic rhinitis (AR) and non-allergic rhinitis with eosinophils (NARES) share type 2 inflammation characterized by nasal eosinophilic infiltrate. Allergen immunotherapy (AIT) is the unique specific treatment for AR, but some patients do not respond. AIT failure may depend on possible comorbidity, mainly concerning NARES.
Methods In all, 33 patients (15 men, mean age 44 years) with AR due to house dust mites allergy were enrolled and treated with sublingual AIT using a monomeric allergoid (LAIS). AIT lasted 3 years. Symptom perception was assessed by visual analog scale (VAS). Symptoms included nasal obstruction, rhinorrhea, sneezing, cough, and olfaction. Nasal cytology evaluated the presence of eosinophils. Patients were evaluated at baseline, after 6 months, and after 1, 2, and 3 years.
Objective The current study aimed at investigating the role of nasal cytology in identifying non-responders to AIT.
Results A total of 28 patients significantly ($p<0.001$) improved already after 6 months and showed a progressive reduction of eosinophilic infiltrate ($p<0.001$). The 5 non-responder patients continued to experience symptoms, and consistent nasal inflammation did not disappear.
Conclusion Nasal cytology is a fruitful tool to identify non-responder to AIT and phenotype mixed rhinitis, such as AR associated with NARES. Therefore, nasal cytology is useful in AIT management, mainly in non-responders.

Keywords NARES · Mixed rhinitis · Immunosuppression · Desensitization, immunologic · Non-responders

Abbreviations

AIT	Allergen immunotherapy
AR	Allergic rhinitis
CRSwNP	Chronic rhinosinusitis with nasal polyps
MGG	May-Grünwald-Giemsa
NAC	Nasal allergen challenge
NARES	Non-allergic rhinitis with eosinophils
NC	Nasal cytology
SEM	Standard error of mean
SLIT	Sublingual immunotherapy
VAS	Visual analog scale

Introduction

Allergic rhinitis (AR) is very common and significantly burdens the patients, family, and society [1]. Allergic rhinitis symptoms may be very bothersome so that the quality of life is poor [2]. A type 2 high inflammation characterizes AR and typically includes eosinophilic infiltrate and sensitization, such as allergen-specific IgE production [3]. Exposure to the causal allergen elicits symptoms, including nasal itch-

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ing, sneezing, watery rhinorrhea, and nasal obstruction. The blocked nose is closely dependent on allergic inflammation associated with T helper-2 dependent cytokines, nasal eosinophil count, nasal airflow limitation, and possible bronchial impairment [4].

AR treatment may be preventive, such as avoiding the allergen exposure, symptomatic, using medicines, or specific through allergen immunotherapy (AIT). Allergen avoidance would be ideal but is rarely effective as many allergens are ubiquitous, for example, pollens or mites. Symptomatic drugs, mainly antihistamines and intranasal corticosteroids, are useful relievers, also for nasal obstruction [5], but their effectiveness is short-lived and not decisive. Instead, AIT is a disease-modifier treatment as restores the immunological and clinical tolerance towards the causal allergen [6]. AIT, namely, dampens allergic inflammation, rebalances a physiological type 1 response, and has long-lasting effects [7]. However, AIT is expensive, long, demanding, and some patients are non-responders. The lack of response to AIT may depend on different reasons, mainly including an incorrect diagnosis. The AR work-up usually is based on patient history and documentation of sensitization [8]. At present, there is no reliable predictor of AIT response, even though some biomarkers have been proposed in experimental settings [9].

On the other hand, AR may also be evaluated by nasal cytology [10]. Nasal cytology (NC) is a standardized procedure that allows to detect the presence of inflammatory cells, bacteria, and biofilm [11]. In particular, NC is an easy diagnostic tool to identify non-allergic rhinitis, mainly concerning the non-allergic eosinophilic rhinitis (NARES), as it documents the presence of eosinophils in the nasal mucosa. Patients with NARES present a type 2 high inflammation but a non-allergic endotype, probably due to type 2 innate immunity involvement [12]. The clinical feature of NARES patients is superimposable to AR, but the treatment could be only pharmacological, as NARES patients are not sensitive to AIT. However, AR may be associated with NARES, so constituting the mixed rhinitis [13]. Since mixed rhinitis is common, it could be a reason for AIT failure in some patients. Based on this background, we tested the hypothesis that nasal cytology could be suitable to identify the cause of AIT ineffectiveness in some non-responders. Therefore, the current study aimed to evaluate the role of NC in managing patients with AR and treated with a 3-year course of sublingual immunotherapy (SLIT) in a real-life setting.

Materials and methods

The longitudinal study enrolled 33 patients with AR treated with SLIT for 3 years. The inclusion criteria were adulthood (18–65 years), diagnosis of perennial AR, and allergy to house dust mites. Exclusion criteria were allergy to other allergens, non-allergic rhinitis, chronic rhinosinusitis with nasal polyps (CRSwNP), or any previous immunotherapy.

AR diagnosis was considered if there was a documented sensitization, assessed by skin prick test, and demonstrated a cause/effect relationship between exposure to sensitizing allergen and symptom occurrence [14]. A panel of allergens, including house dust mites, pets (dog and cat), grasses, pellitory, birch, olive tree, cypress, *Alternaria, Cladosporium*, and *Aspergillus* (Lofarma, Milan, Italy), was tested using the skin prick test. Allergy, therefore, was confirmed if sensitization consisted with symptoms after exposure to sensitizing allergen.

Non-allergic rhinitis was diagnosed if the patient referred typical symptoms (watery rhinorrhea, nasal congestion, sneezing, and itching) and had negative skin prick test.

The CRSwNP diagnostic criteria were based on the validated work-up reported in the EPOS document [15]. Nasal endoscopy was performed for this purpose.

All patients gave written informed consent. The ethics committee approved the study (87444).

Patients started sublingual immunotherapy using a monomeric allergoid of mites (LAIS, Lofarma). The schedule was two tablets/week for 3 years. A baseline (T0) and four follow-up visits, such as after 6 months (T1), 1 (T2), 2 (T3), and 3 (T4) years, were planned.

Mometasone furoate nasal spray (two puffs/nostril/twice daily for 20 days/month) was prescribed for the first 6-month period.

The considered parameters included perception of symptom severity by a visual analog scale (VAS) and nasal cytology. Both parameters were evaluated at T0, T1, T2, T3, and T4.

The evaluated symptoms included nasal obstruction, watery rhinorrhea, sneezing, cough, and olfaction. The VAS score ranged from 0 (no symptom) to 10 (very severe symptom) as previously described in detail [16]. A sum of the five VAS scores was calculated. Symptom assessment was performed at every visit.

Nasal eosinophils were counted by nasal cytology, a well-standardized methodology [10]. Nasal cytology included sampling, processing, and microscope reading. Sampling required collecting cells from the surface of the middle portion of the inferior turbinate by a sterile disposable curette. The procedure was performed under anterior rhinoscopy, with an appropriate light source, and it is painless. The sample obtained was immediately smeared on a glass slide, air-dried, and stained with May-Grünwald-Giemsa (MGG) for 30 min. The stained sample was read at optical microscopy, with a 1000× objective with oil immersion. The count of eosinophils was expressed as a mean of 10 microscopic fields.

Intranasal corticosteroids were suspended 10 days before the examination. Data are expressed as a mean of 10 fields. Nasal cytology was performed at every visit.

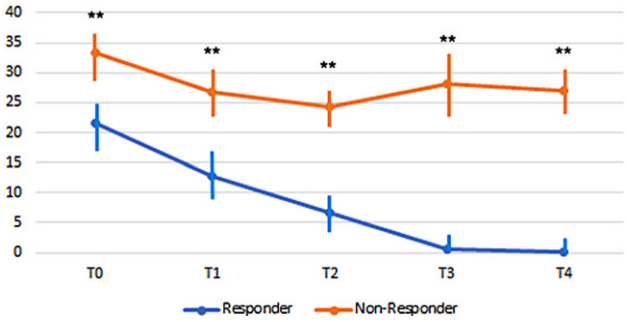

Fig. 1 Total VAS scores in responders (*dark line*) and non-responders (*light line*), measured at baseline (1), after 6 months (2), 1 year (3), 2 years (4), and 3 years (5). Data are expressed as mean ± standard deviation. $^{**}p < 0.001$ for intergroup comparison

Data were calculated as mean and standard error of mean (SEM) of the evaluated observations. Patients were subdivided in two groups: responders and non-responders, on the basis of the clinical response at the 6-month visit. The definition of responder is based on a reduction of at least 30% of symptom severity measured after 6 months [17].

Total VAS scores were calculated at T0, T1, T2, T3, and T4. The independent samples T-test was used. The threshold for statistical significance was set at $p < 0.05$ and all inferential tests were two sided. Statistical analyses were performed using JASP software (version 0.14.1, JASP Team 2020, University of Amsterdam, The Netherlands).

Results

The study included 33 patients (15 men and 18 women; mean age 44 years, range 35–56 years). The patients were subdivided into two groups: responders (28 subjects) and non-responders (5 subjects).

At baseline, the mean total symptom VAS score was 21.68 (SEM 1.12) in the responder group and 33.4 (SEM 1.32) in the non-responder group, as shown in Fig. 1. There was a significant difference between groups ($p = 0.001$).

After 6 months, patients in the responder group had a mean total VAS score 12.71 (SEM 0.63), whereas non-responders had 26.8 (SEM 0.97). There was a significant difference between groups ($p < 0.001$). At T2, responder patients had 6.57 (SEM 0.69) and non-responders 24.2 (SEM 1.77; $p < 0.001$). At T3 responders had 0.5 (SEM 0.13) and non-responders 28.2 (SEM 1.02; $p < 0.001$). At T4 responders had 0.1 (SEM 0.06) and non-responders 27 (SEM 0.44; $p < 0.001$).

In responder group, nasal eosinophils were 18 at baseline, and significantly ($p < 0.001$) decreased to five at 6 months ($p < 0.001$), two after 1 year ($p < 0.001$), and one after 2 years and at the end of AIT course ($p < 0.001$ for both), as reported in Fig. 2.

In the non-responder group, nasal eosinophils were 22 at baseline, 18 at 6 months, 16 after 1 year, and 15 after 2 years and at the end of AIT course. There was no significant difference among visits ($p = 0.44$).

The intergroup analysis showed that non-responders had significantly more eosinophils than responders at T2 ($p < 0.001$), T3 ($p < 0.001$), T4 ($p < 0.001$), and T5 ($p < 0.001$), but there was no significant difference at baseline.

Notably, non-responders perceived relevant nasal obstruction (VAS 8) and impaired smell (VAS 8) at baseline. The perception of nasal obstruction remained constant over time, ranging between 7 and 8. Similarly, the perception of smell impairment remained unchanged over time, ranging between 7 and 8.

The treatment was well tolerated and no clinically significant adverse events were reported.

Discussion

A type 2 inflammation characterizes AR and reflects an eosinophilic infiltrate of the nasal mucosa. However, nasal eosinophilic infiltrate may also occur in non-allergic conditions, mainly including NARES. In effect, eosinophilic inflammation depends on type 2 response, which may involve allergic or non-allergic pathways and innate and/or adaptive immunity [18]. Allergic inflammation mainly recognizes acquired T cell response, sustained by a specific immunological response [19]. On the contrary, innate immunity cells (innate lymphoid cells-2) drive non-allergic eosinophilic inflammation in patients with NARES or chronic rhinosinusitis [20].

Although eosinophilic inflammation is common to allergic and non-allergic disorders, peculiar pathogenic mechanisms identify different immunopathological pathways. As a result, the response to treatments is dissimilar. Typically, AR patients respond to anti-inflammatory drugs and AIT, whereas non-allergic patients may benefit only from pharmacological treatments, even if partially.

Jacobs et al. identified NARES 40 years ago [21]. Furthermore, Settipane and Klein reported overlapping between allergic and non-allergic rhinitis [22]. This study highlighted the concept that mixed rhinitis, such as patients with AR associated with NARES, represents a different condition. However, the relevance of phenotyping patients was recognized further. The advent of biological medications prompted the need to identify responders [23].

Consistently, the requirement to identify non-responder to AIT still represents an unmet need in clinical practice. The definition of non-responder to AIT is presently based on clinical grounds as there is no reliable biomarker in this regard [24]. Moreover, the large AIT trials usually do not report the percent of non-responders but express the results as a mean value concerning all participants.

The current study demonstrated that 15% of enrolled patients did not respond to AIT. This outcome

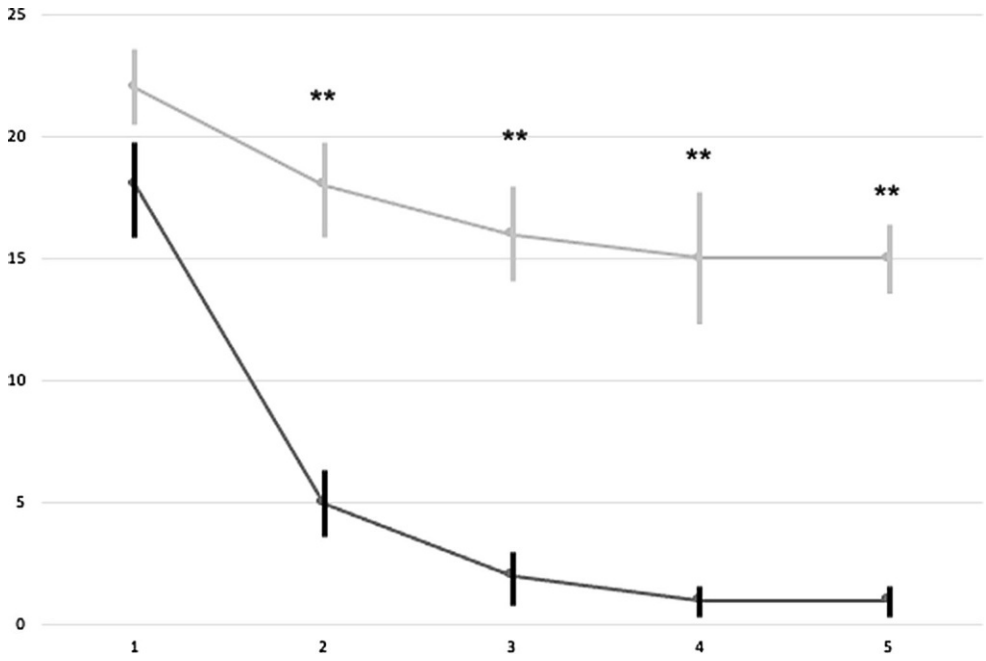

Fig. 2 Nasal eosinophils in responders (*black line*) and non-responders (*grey line*), measured at baseline (1), after 6 months (2), 1 year (3), 2 years (4), and 3 years (5). Data are expressed as mean ± standard deviation. $^{**}p < 0.001$ for intergroup comparison

was consistent with previous studies reporting a percentage of responders to AIT ranging around 80–85% [25, 26]. These experimental studies suggested that increased IL-4 production could have a positive prognostic role, whereas defective IFN-γ production was associated with AIT failure. Certainly, cytokine assessment cannot be performed in clinical practice, while nasal cytology is a standardized and straightforward procedure that any doctor can perform.

Moreover, the non-responder patients had more severe symptoms at baseline, which probably depended on the concomitant presence of allergic inflammation and non-allergic eosinophilic inflammation. This outcome is relevant, but could be demonstrated only *a posteriori*, such as after stratification on the basis of the responsiveness to AIT. As a result, the certainty of diagnosis NARES in patients with AR could be obtained only after AIT. In addition, eosinophilic inflammation intensity persisted in patients with NARES along the AIT treatment.

Therefore, nasal cytology allows the quick—such as after 6 months or more—identification of non-responder patients to AIT as suffering from mixed rhinitis, such as NARES associated with AR. Notably, NARES may be diagnosed only by demonstrating the nasal eosinophil infiltrate by cytology. Consequently, nasal cytology is mandatory to phenotype patients with rhinitis as three main phenotypes may occur: AR, NARES, and mixed.

However, AIT failure may depend on more reasons, including poor adherence, early discontinuation, incorrect diagnosis, low allergen dosage, and low quality allergen extracts.

On the other hand, the present study had some limitations, including the open design, the limited number of participants, and the lack of biomarker assessment. Moreover, from a practical perspective, eosinophilic inflammation documentation before AIT cannot have a prognostic value. Eosinophilic inflammation does not predict AIT response as both AR and NARES share type 2 inflammation. In mixed rhinitis phenotype, NARES could be completely identified only after AIT failure. In addition, nasal allergen challenge (NAC) was not performed to confirm AR at baseline as, usually, it is not carried out in clinical practice. In theory, NAC could not identify non-responder *a priori*, as all patients had AR. However, it has to be verified by an *ad hoc* study.

Moreover, this study was conducted in clinical practice; thus, the outcomes can mirror what happens in real-life settings.

In conclusion, the present study demonstrated that a tiny percentage of patients could be non-responders to AIT. The reason for AIT failure may depend on a defined phenotype, such as mixed rhinitis: AR associated with NARES. Nasal cytology can, therefore, a useful tool in AIT management.

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Declarations

Conflict of interest P. Luperto, S. Masieri, C. Cavaliere and G. Ciprandi declare that they have no competing interests. E. Compalati and F. Frati are employers of Lofarma and only provided statistical analysis of the data.

Ethical standards For this article no studies with human participants or animals were performed by any of the authors. All studies performed were in accordance with the ethical standards indicated in each case.

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