

ORIGINAL ARTICLE

Asthma and Lower Airway Disease

Bronchial asthma triggered by house dust mites in patients with local allergic rhinitis

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Abstract

Background: Over 30% of local allergic rhinitis (LAR) patients self-report bronchial symptoms suggestive of asthma, but the relationship between the allergen exposure and the bronchial symptoms has not been studied.

Objective: To investigate whether a bronchial counterpart of LAR exists.

Methods: Patients were classified by clinical history, skin prick test/serum specific IgE (sIgE), and nasal allergen provocation test (NAPT) into the LAR, allergic rhinitis (AR), and nonallergic rhinitis (NAR) phenotypes. Twenty-eight LAR, 18 AR, and 19 NAR patients self-reporting bronchial symptoms suggestive of asthma and 8 healthy controls (HC) were subjected to a methacholine test (MT) before (Visit 1) and 24 hours after (Visit 3) a bronchial provocation test with *Dermatophagoides pteronyssinus* (BPT-DP) (Visit 2). Induced sputum and peripheral blood obtained after each MT were analyzed for immune cell populations, tryptase, ECP, and sIgE.

Results: A positive MT was found in 50% of LAR, 83.3% of AR, 57.89% of NAR, and 0% of HC individuals ($P = 0.022$ AR vs LAR) at V1. BPT-DP was positive in 8 LAR and 15 AR patients (28% vs 83.3%, $P < 0.001$), with no positive responses in NAR and HC. All BPT-DP+ patients experienced a significant decrease of PC_{20} at V3 vs V1 ($P = 0.016$ LAR, $P \leq 0.001$ AR). BPT-DP+ patients also showed a significant increase of eosinophils, monocytes, and ECP in induced sputum at V3 compared with V1.

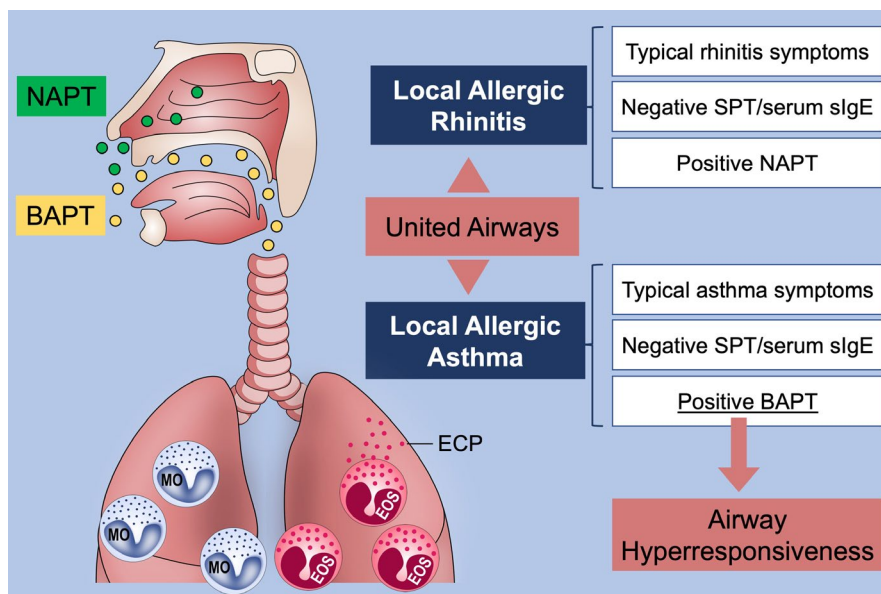
Conclusion: The results suggest the existence of a new asthma phenotype (local allergic asthma) defined by absence of systemic atopy and positivity to BPT with allergen.

KEYWORDS

allergic, asthma, IgE, local, rhinitis

Abbreviations: AA, allergic asthma; AHR, airway hyperresponsiveness; AIT, allergen immunotherapy; AR, allergic rhinitis; BAPT, bronchial allergen provocation test; BPT, bronchial provocation test; DP, *Dermatophagoides pteronyssinus*; ECP, eosinophil cationic protein; FEV1, forced expiratory volume in the 1st second; HC, healthy controls; HDM, house dust mites; ID, intradermal test; LAA, local allergic asthma; LAR, local allergic rhinitis; MT, methacholine test; NAPT, nasal allergen provocation test; NAR, nonallergic rhinitis; sIgE, specific IgE; SPT, skin prick test; V, visit.

Campo, Eguiluz-Gracia, Torres & Rondón equally contributed to this study.



GRAPHICAL ABSTRACT

Twenty-eight percent of patients with house dust mite (HDM)-triggered local allergic rhinitis (LAR) and asthma-like symptoms display positive bronchial allergen provocation test (BAPT) responses. In LAR patients, BAPT induces a significant increase in the airway hyperresponsiveness together with a recruitment and activation of eosinophils and monocytes. Collectively, these findings suggest the existence of differentiated asthma phenotype defined by the absence of atopy and positive response to BAPT. NAPT: Nasal allergen provocation test

1 | INTRODUCTION

In the last decade, a new rhinitis phenotype termed local allergic rhinitis (LAR) has been defined for patients with seasonal or perennial rhinitis symptoms, absence of systemic atopy (negative skin prick test [SPT] and/or serum specific IgE [sIgE]), and a positive nasal allergen provocation test (NAPT).^{1,2} Local allergic rhinitis is an independent rhinitis phenotype not evolving to allergic rhinitis (AR),³ and showing moderate-to-severe symptoms. Recent studies highlight that LAR is a common disease, affecting > 25% of nonatopic patients with rhinitis.^{4,5} The disease often starts during childhood^{6,7} and tends to worsen over time with association of comorbidities such as conjunctivitis and bronchial symptoms suggestive of asthma.³ To date, most studies on LAR have focused on the underlying mechanisms and the definition of the phenotype.

As highlighted by the *Allergic Rhinitis and its Impact on Asthma* (ARIA) document, important pathological links exist between rhinitis and asthma.¹⁰ Of note, the NAPT induces an eosinophilic infiltrate in both the upper and lower airways of atopic rhinitis patients regardless of the presence of asthma.¹¹ Therefore, rhinitis and asthma can be considered the organ-specific expressions of the same chronic airway inflammation (united airways concept). In this regard, the term allergic airway disease was proposed to emphasize this relationship and the relevance of the allergen as trigger of respiratory disease.¹² Rhinitis is a known risk factor for asthma, in both atopic and nonatopic individuals.¹³ Similar to AR, many LAR patients report bronchial symptoms. A recent follow-up study of a LAR cohort showed that the presence of symptoms suggestive of asthma

increased from 18.4% to 24.4% during the first 5 years and to 30.7% after ten years of evolution.³ Though these findings indicate that close to one-third of subjects with LAR will have bronchial symptoms at some point of their disease evolution, the nature of these symptoms remains to be analyzed.

Asthma is a first-order health problem imposing a significant economic burden to health systems.¹⁴ The disease is characterized by chronic airway inflammation and variable airflow obstruction. The *Global Initiative for Asthma* (GINA) and other clinical guidelines recommend the use of objective tests such as the bronchodilator test or methacholine test (MT) to confirm asthma diagnosis.¹⁵ Of note, several studies demonstrate that the mere presence of symptoms overestimates the prevalence of asthma.^{16,17} To the best of our knowledge, this aspect has not been investigated to date in LAR individuals.

Many nonatopic asthma patients display type 2 airway inflammation with increased numbers of eosinophils,¹⁸ and expression of type 2 cytokines such as IL-4, IL-5, and IL-13.^{19,20} This infiltrate is often indistinguishable from that of allergic asthma patients, suggesting shared pathological mechanisms regardless of the atopic status. Importantly, a similar eosinophilic infiltrate was observed in bronchial biopsies from both atopic and nonatopic children with recurrent wheezing.²¹ Collectively, these evidences led investigators to suggest the existence of allergen-triggered asthma in nonatopic patients.^{19,20} Moreover, class switch recombination to IgE has been demonstrated in bronchial biopsies from both atopic and nonatopic asthma patients.²² In a study involving bronchial challenges with house dust mites (HDM) in nonatopic asthma patients,

Dermatophagoides pteronyssinus (DP)-sIgE was found in sputum, and this DP-sIgE was able to mediate the allergen-triggered activation of peripheral basophils from atopic donors.²³ Nevertheless, asthma patients failed to experience a positive bronchial provocation test (BPT) with DP. Yet these results demonstrate that sensitization can occur in the absence of clinical reactivity to allergens, and they also indicate that sIgE can be present in the airway mucosa of some nonatopic individuals. Nevertheless, the link between allergen-triggered asthma and rhinitis in nonatopic patients, and its connection with the united airways concept, has not been addressed to date. Overall, the aforementioned facts highlight the need to investigate the nature and mechanisms of bronchial symptoms in LAR individuals.

In this study, we objectively evaluated the existence of allergen-triggered asthma and its immunological underlying features in a group of subjects with confirmed LAR to HDM, and who reported bronchial symptoms suggestive of the disease. To this end, we evaluated unspecific airway hyperreactivity (AHR) measured by MT, allergen-specific AHR measured by BPT with DP, and the immunological response to BPT in induced sputum and peripheral blood.

2 | METHODS

2.1 | Study population

All patients > 14 years old consulting in our clinic for perennial nasal and bronchial symptoms and who met the inclusion criteria (as described below) were invited to participate in the study. The healthy control group was composed of volunteering medical students. All rhinitis patients had to have also a history of bronchial symptoms suggestive of asthma for at least three years (episodes of cough, dyspnea, and wheezing not related to respiratory infections as defined by GINA). The severity of the suspected asthma was evaluated

following GINA criteria (mild intermittent, mild persistent, or moderate persistent), and symptoms were controlled at the time of the study.¹⁵ Patients were classified in the different study groups according to the following inclusion criteria:

1. **Subjects with LAR:** Subjects 14-65 years old with perennial rhinitis suggestive of allergy to HDM, negative SPT and sIgE (<0.35 kU/L) to a battery of the most prevalent perennial and seasonal aeroallergens in our area, negative intradermal test (ID) to DP, and positive NAPT with DP and negative NAPT with other relevant perennial allergens in our area (*Alternaria alternata*, *Parietaria judaica*, and dog and cat epithelia if the patient was exposed).
2. **Subjects with AR:** Subjects 14-65 years old with perennial rhinitis suggestive of allergy to HDM, positive SPT and sIgE (>0.7 kU/L) to DP, and a positive NAPT with DP.
3. **Subjects with nonallergic rhinitis (NAR):** Subjects 14-65 years old with perennial rhinitis, negative SPT/ID and sIgE (<0.35 kU/L) to a battery of the most prevalent perennial and seasonal aeroallergens in our area including DP, and negative NAPT with DP and with other relevant perennial allergens in our area (*Alternaria alternata*, *Parietaria judaica*, and dog and cat epithelia if the patient was exposed).
4. **Healthy control subjects (HC):** Subjects 14-65 years old, healthy volunteers with no rhinitis or asthma symptoms, with negative SPT/ID and sIgE to a battery of the most prevalent perennial and seasonal aeroallergens in our area, and negative NAPT with DP.

Overall, 65 patients with perennial rhinitis (28 with LAR, 18 with AR, 19 with NAR) and 8 nonatopic HC were included (Figure 1).

A thorough explanation of exclusion criteria and methodology (SPT, NAPT, and sIgE) is available in the Data S1. The study was performed according to the principles of the Declaration of Helsinki and

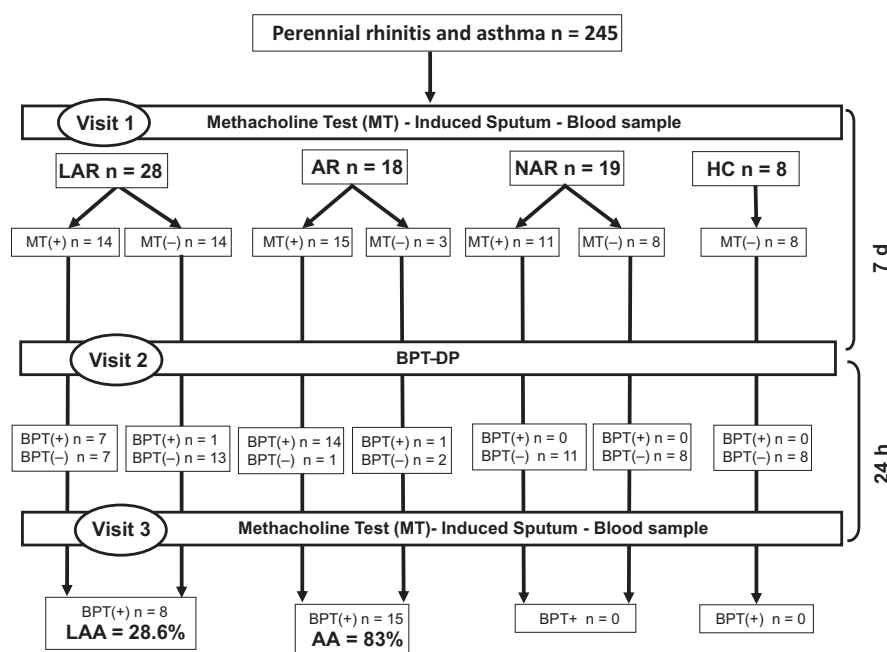


FIGURE 1 Study flowchart with results of methacholine and bronchial provocation test with *Dermatophagoides pteronyssinus* in all groups. AR, allergic rhinitis; BPT-DP, bronchial provocation test with *D. pteronyssinus*; HC, healthy controls; LAR, local allergic rhinitis; MT, methacholine; NAR, nonallergic rhinitis

approved by the Local Ethical Committee of the Hospital Regional Universitario de Malaga (Spain). All subjects over 16 years of age and the parents of the younger individuals signed the corresponding informed consent.

2.2 | Study protocol

All participants underwent a protocol that comprised the following tests:

- Methacholine test: to evaluate the presence of airway hyperresponsiveness (AHR) before and after allergen exposure (positive if $PC_{20} < 4$ mg/mL).
- Bronchial provocation test with *D pteronyssinus* (BPT-DP): to evaluate the bronchial response to allergen exposure (positive if fall in FEV1 $\geq 20\%$)
- Induced sputum and blood test: evaluation of inflammatory response before and after the allergen exposure by measurement of cell populations (eosinophils, monocytes, T lymphocytes, and NK cells) (Figure S1), eosinophil cationic protein (ECP), tryptase, and total IgE and sIgE to DP

Details of the methodology of the different tests and in vitro measurements are described in detail in the Data S1.

The schedule of the study was as follows (Figure 1):

- Visit 1 (V1): A thorough clinical history, MT, and blood and induced sputum samples before allergen exposure were obtained in all participants.

- Visit 2 (V2): One week later and regardless of the results of the MT, a BPT-DP was performed in all participants.
- Visit 3 (V3): Twenty-four hours after the BPT-DP, a second MT and blood and induced sputum samples were obtained in all individuals. This second MT was undertaken with the aim to evaluate the specificity of the bronchial response to the BPT-DP. Those LAR and AR patients with positive response to BPT-DP and increased AHR in the second MT (decrease of PC_{20}) were diagnosed of allergen-induced asthma.

Details of the methodology of the statistical analysis are described in detail in the Data S1.

3 | RESULTS

3.1 | Clinical characteristics

Sixty-five patients (28 with LAR, 18 with AR, 19 with NAR) and 8 HC were finally included in the study. General characteristics of the patients are shown in Table 1.

Most subjects were young females, nonsmokers, with a mean time of disease evolution of 4.5 years for LAR, 10 for AR, and 9 for NAR individuals ($P = 0.253$). There were no differences in the severity of asthma between the three groups ($P = 0.331$). Median dose ($\mu\text{g/day}$) of inhaled corticosteroids (budesonide or equivalent dose) was similar in all patient groups ($P = 0.303$). The median FEV1% in the AR group (75.5%) was significantly lower compared to LAR (90%, $P = 0.002$), NAR (85%, $P = 0.007$), and HC (88%, $P = 0.005$).

TABLE 1 Demographic and clinical data from all the study groups

	LAR n = 28	AR n = 18	NAR N = 19	HC n = 8	P
Gender (% Female)	82.1	77.8	78.9	75.0	0.968
Age (in years) median (IQ range)	31 (20-46)	26 (20.75-34)	36 (30-44)	29 (26-42.75)	0.316
Current or ex-smokers (%)	15.4	25.0	22.2	25.0	0.378
Time of evolution in years (median, IQ range)	4.5 (3-10)	10 (4-14)	9 (3.75-11.25)	n/a	0.253
Total IgE UI/mL (mean, SD)	44.32 (46.42)	45.06 (36.98)	333.88 (266.60)	20.44 (12.58)	<0.001
Positive SPT-ID to <i>D pteronyssinus</i> (%)	0.00	100.00	0.00	0.00	<0.001
Serum sIgE to <i>D pteronyssinus</i> kU/L(mean, SD)	<0.35 (0)	34.14 (33.11)	<0.35 (0)	<0.35 (0)	<0.001
Asthma severity %					
Mild intermittent	42.9	16.7	36.8	n/a	0.331
Mild persistent	17.9	27.8	31.6		
Moderate persistent	39.3	55.6	31.6		
Inhaled corticosteroids $\mu\text{g/day}$ budesonide median (IQ range)	400 (400-800)	400 (200-800)	400 (200-800)	n/a	0.303
FEV ₁ % median (IQ range)	90.0 (77-97)	75.5 (69.50-83.50)	85.0 (80-90)	88.0 (80.75-91.85)	0.003

AR, allergic rhinitis; HC, healthy controls; IQ, interquartile; LAR, local allergic rhinitis; NAR, nonallergic rhinitis; SD, standard deviation; SPT-ID, skin prick test-intradermal test.

3.2 | Evaluation of baseline nonspecific airway hyperresponsiveness (Visit 1)

In order to objectively evaluate the presence of asthma, all participants underwent a MT at V1. In the LAR group, 50% of patients had a positive MT (MT+), 83.3% in the AR group, 57.89% in the NAR group, and 0% in the HC group (Figure 1). Patients with AR had a significantly higher percentage of confirmed asthma than LAR ($P = 0.022$), without significant differences among other groups. Values of PC_{20} were similar among MT+ patients in LAR, AR, and NAR and similar among subjects with negative MT (MT-) (Figure 2).

The bivariate comparative analysis between MT+ vs MT- patients regardless of their rhinitis phenotype showed a significantly longer evolution in MT+ patients (median: 9 years, range: 18 years) than in MT- individuals (median: 6 years, range: 19 years; $P = 0.045$), and a lower basal FEV1% (MT+ median: 81.5, range: 32 vs MT- median: 87%, range: 38; $P = 0.026$). However, no significant differences were detected when the comparison was performed within each rhinitis phenotype (data not shown).

Later on, a forward logistic regression adjusted by time of evolution, smoking habit, rhinitis phenotype, severity of suspected asthma, and FEV1% was performed (MT+ vs MT-). The results showed a positive association between MT(+) and severity of asthmatic symptoms (OR 91.00, IC 95% (8.536-970.071), $P = 0.001$). No confounding variables were detected.

3.3 | Evaluation of responses to bronchial provocation test with DP (Visit 2)

One week later (V2), all participants underwent BPT-DP and were categorized as BPT+ or BPT- (Figure 1). The BPT-DP was positive in 8 LAR patients (28.6%) and 15 AR patients (83.3%), with a similar average fall of FEV1 (LAR: -22.9% vs AR: -23%, $P = 0.142$) (Figure 3A).

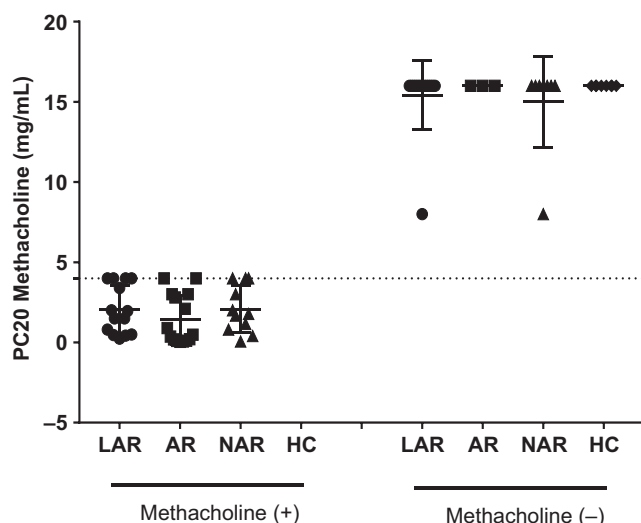


FIGURE 2 PC_{20} methacholine values in methacholine-positive (MT+) and methacholine-negative (MT-) patients. AR, allergic rhinitis; HC, healthy controls; LAR, local allergic rhinitis; MT, methacholine; NAR, nonallergic rhinitis

None of the NAR patients or HC subjects had a positive BPT-DP. In the LAR group, 75% of BPT-DP+ had immediate bronchial responses whereas 25% had dual immediate-late responses (in AR 60% and 40%, respectively). No isolated late responses were observed. An almost perfect concordance was detected between baseline MT and BPT-DP in the AR group (k index = 0.842; $P < 0.001$), a moderate concordance for LAR subjects (k index = 0.521, $P = 0.001$), and a lack of concordance in NAR and healthy control individuals (data not shown). All BPT-DP+ LAR and AR patients at V2 had a baseline MT+ at V1, but one from each group. In the study, 62.5% of LAR patients responded to the highest concentration of allergen in the bronchial challenge (1:1 dilution or 4 μ g/mL of Der p 1 concentration in DP extract), whereas 40% of AR subjects responded to 1:10 dilution (≤ 0.4 μ g/mL of Der p 1) ($P = 0.304$) (Figure 3B).

3.4 | Evaluation of nonspecific airway hyperresponsiveness after BPT-DP (Visit 3)

Twenty-four hours after BPT-DP, all subjects underwent a second MT. All BPT-DP+ patients experienced a decrease of the PC_{20} , as compared with V1 ($P = 0.016$ for LAR and $P \leq 0.001$ for AR; Figure 4A,B, respectively). No BPT-DP- individual had a relevant decrease of the PC_{20} in the second MT (Figure 4C,D). These observations strongly suggest that the BPT-DP did not induce false-positive results and confirmed a specific allergen response.

One MT- LAR patient and one MT- AR patient at V1 (>4 mg/mL) displayed a positive response to BPT-DP at V2. Importantly, both of them showed a decrease higher than 2-fold in the PC_{20} at V3 as compared with V1 (Figure 4A,B). At the end of the study, 28% of LAR patients and 83% of AR individuals had a confirmation of allergen-induced asthma ($P < 0.001$) (Figure 1).

3.5 | Immunological study

Local allergic rhinitis (LAR) and AR patients with a positive BPT-DP+ experienced a significant increase of eosinophils, monocytes, and ECP levels in induced sputum obtained 24 hours after the BPT-DP (V3) compared with V1 (Figure 5A,B,C and Tables S2 and S3). No other significant differences were detected in blood or sputum for cell populations (Tables S1 and S2) or inflammatory mediators (Table S3) in the intragroup comparative analysis (V1 vs V3).

The intergroup comparisons detected significantly higher levels of ECP in sputum from BPT-DP+ AR patients compared to HC at V1 (41.30 ng/mL vs 2 ng/mL, $P = 0.001$), and from BPT-DP+ LAR/AR patients compared to HC at V3 (LAR 16.48 ng/mL vs 2 ng/mL, $P = 0.003$; AR 123 ng/mL vs 2 ng/mL, $P = 0.001$). No significant differences between BPT-DP+ LAR and AR patients were detected (data not shown). Patients testing negative for BPT-DP also had significantly higher levels of ECP in sputum than HC at both V1 (LAR $P = 0.019$, AR $P = 0.098$, and NAR $P = 0.002$) and V3 ($P = 0.005$, $P = 0.098$, and $P = 0.004$).

No significant changes within groups (V1 vs V3) were detected in total IgE and sIgE in serum. As expected, comparison between

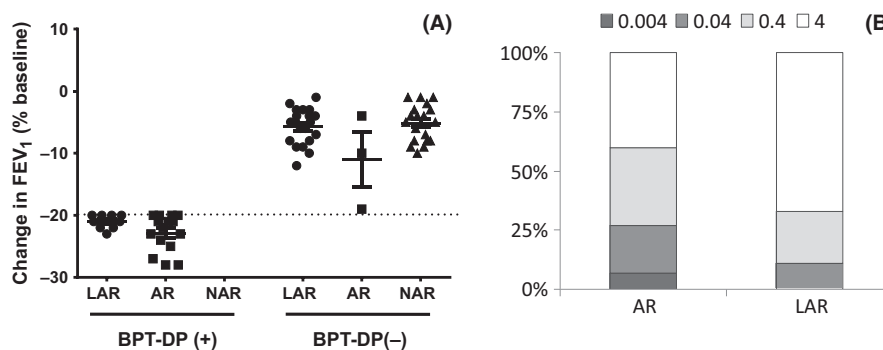
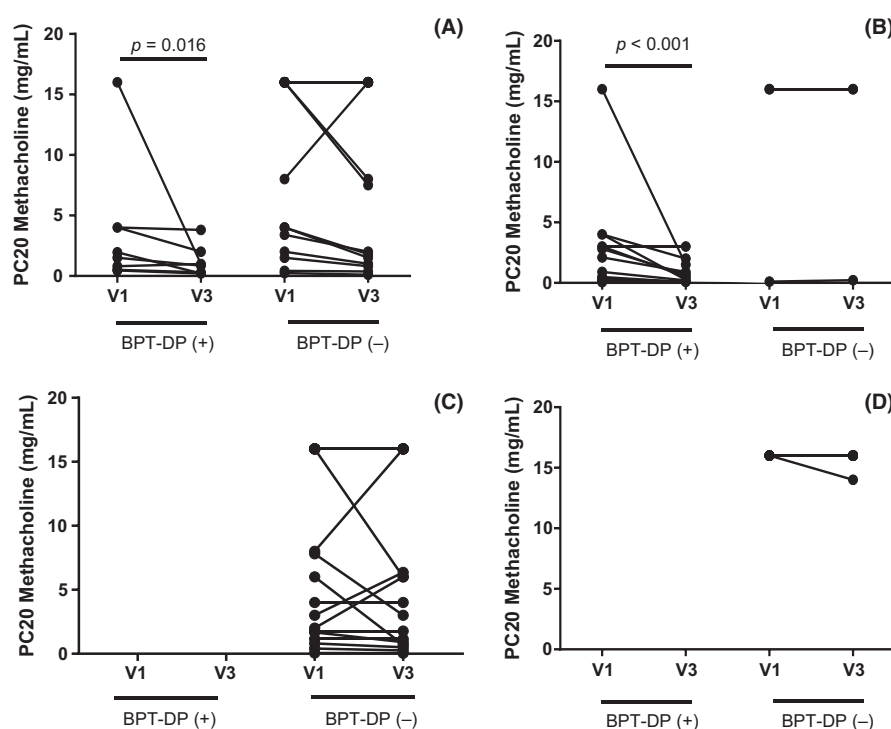


FIGURE 3 A, Graphical representation of FEV₁ fall after bronchial provocation test with *Dermatophagoides pteronyssinus* in patients with positive and negative test result. B, Percentage of responders in bronchial provocation test to each *D pteronyssinus* (Der p 1) concentration in allergic rhinitis and local allergic rhinitis individuals. AR, allergic rhinitis; BPT-DP, bronchial provocation test with *D pteronyssinus*; HC, healthy controls; LAR, local allergic rhinitis; MT, methacholine; NAR, nonallergic rhinitis

FIGURE 4 PC20 methacholine results before (V1) and after (V3) bronchial provocation test with *Dermatophagoides pteronyssinus* in allergic rhinitis (A), local allergic rhinitis (B), nonallergic rhinitis (C), and healthy controls (D). BPT-DP, bronchial provocation test with *D pteronyssinus*



groups showed significantly higher levels of total IgE in serum of patients with AR ($P < 0.001$; Table 1) than in LAR, NAR, and HC individuals (17.11 UI/mL). Similarly, DP-sIgE in serum was significantly higher in AR patients compared with subjects from the rest of study groups at V1 ($P < 0.001$; Table 1) (selection criteria) and V3 ($P < 0.001$). Levels of sIgE in sputum supernatant were undetectable by both ImmunoCAP® and RIA methods (data not shown).

4 | DISCUSSION

Local allergic rhinitis is an independent rhinitis phenotype rapidly evolving toward clinical worsening and the association of symptoms in other mucosal organs.¹ Importantly, 20%-47% of LAR individuals

report bronchial symptoms.^{24,25} Even though large population studies are lacking, a recent systematic review indicated that 25.6% of nonatopic rhinitis patients fulfilled criteria for LAR diagnosis.⁴ This evidence prompted us to investigate the existence of a bronchial counterpart of LAR.

In this study, we demonstrated that 28% of patients with LAR and bronchial symptoms display a positive BPT-DP. Moreover, the bronchial allergen challenge induced in these individuals an increase of eosinophils and monocytes in sputum, a bronchial infiltrate closely resembling that of patients with AR and concomitant allergic asthma (AA). Overall, our results support the existence of an asthma phenotype defined by the presence of AHR, the absence of systemic atopy, and the positivity to the BPT with allergen. Remarkably, this study indicates that inflammatory processes in the upper and lower

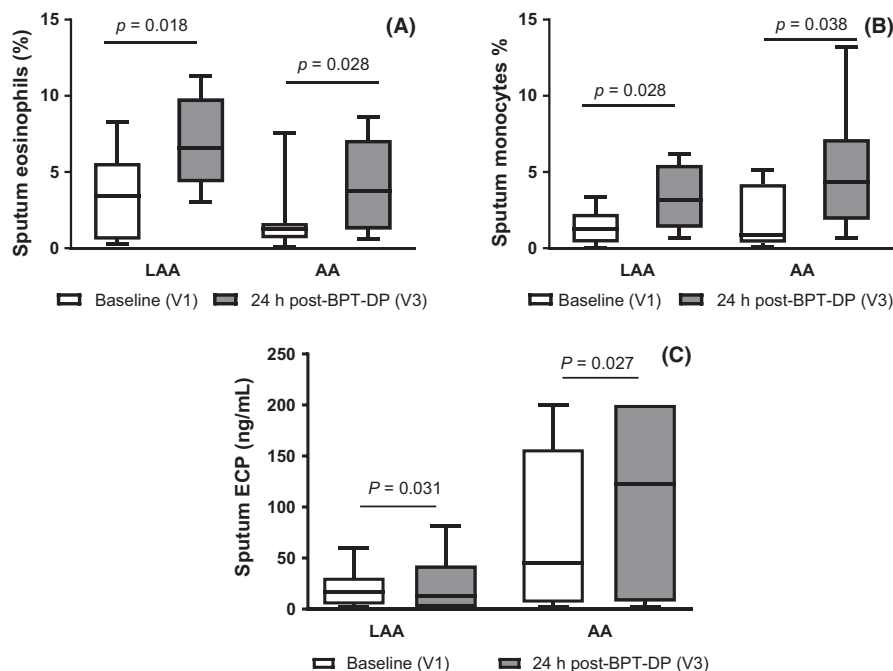


FIGURE 5 A, Percentage of sputum eosinophils before (V1) and after (V3) bronchial provocation test with *Dermatophagoides pteronyssinus* in patients with local allergic asthma (LAA) and allergic asthma (AA). B, Percentage of sputum neutrophils before (V1) and after (V3) bronchial provocation test with *D. pteronyssinus* in patients with local allergic asthma (LAA) and allergic asthma (AA). C, Levels of sputum ECP (ng/mL) before (V1) and after (V3) bronchial provocation test with *D. pteronyssinus* in patients with local allergic asthma (LAA) and allergic asthma (AA)

airways are closely related in the case of local allergy, thus reinforcing the concept of the united airway disease.²⁷

In this study, AHR was assessed by MT, which displays a higher reproducibility and negative predictive value than other methods for bronchial challenge.²⁸ Moreover, MT identifies the AHR related to a broad spectrum of asthma phenotypes, unlike indirect methods displaying a phenotype-specific performance.^{29,30} A cutoff point of MT(+) of <4 mg/mL was selected as recommended by guidelines in order to avoid borderline results.^{31,32}

In our work, AR patients showed a longer disease evolution and a significantly lower FEV1 at baseline than the other groups. Interestingly, in AR patients the diagnosis of asthma was confirmed more frequently than in LAR or NAR subjects. Unfortunately, we cannot determine whether these differences indicate a greater severity for the atopic phenotype, or a more progressed disease state at baseline in this group. Of note, there was a significant correlation between MT positivity and disease evolution.

Allergen-induced asthma (positive BPT-DP) was confirmed in 15 of 18 AR patients, in 8 of 28 LAR individuals, and in none of the 19 NAR subjects. Importantly, in the MT at V3 (24 hours after BPT-DP) PC₂₀ was significantly lower than that observed at V1 (before BPT-DP) in both BPT-DP+ LAR and AR individuals, whereas no difference was observed in the BPT-DP- subjects. This observation indicates the specificity of the bronchial response, strongly suggesting that only in DP-sensitive individuals, regardless of their atopic status, the allergen is able to exert an inflammatory reaction causing a sustained increase of the AHR. Moreover, the clinical condition of allergen-induced asthma in the absence of systemic atopy constitutes a different disease phenotype, for which we propose the term local allergic asthma (LAA). The allergen dose inducing a positive BPT-DP was lower in AA patients than in LAA individuals, which is consistent with previous studies reporting a lower allergen dose tolerated in

the NAPT by AR patients as compared to LAR subjects.^{25,26} Yet this finding suggests a greater severity for the atopic phenotype, and it might also be influenced by the longer disease evolution of AR patients in this study.

Half of the MT+ LAR individuals did not experience a positive BPT-DP, implying that in those patients, the nasal allergen sensitivity coexists with unspecific AHR. This finding is not surprising, as unspecific nasal and bronchial hyperresponsiveness can be present in many respiratory conditions including allergic airway disease.^{31,33} Of note, the vast majority of AA and LAA individuals were MT+ before the BPT-DP.

We also evaluated the changes induced by the BPT-DP in immune cell populations, sIgE, and cell activation markers in sputum and peripheral blood. Interestingly, we did not observe differences at the peripheral level in any of the study groups, suggesting that the main immune mechanisms occurred at the mucosal level. In sputum, we did not find intragroup differences in any examined parameter in the BPT-DP- individuals. Conversely, BPT-DP+ patients experienced significant increases in their sputum eosinophils, ECP, and monocytes at V3 regardless of their atopic status. Airway eosinophilia is a hallmark of AR and AA,³⁴ and accumulating eosinophils produce cytokines, reactive oxygen species, and cytotoxic cationic proteins (like ECP), substantially contributing to tissue damage and remodeling.³⁵ Importantly, both AA and LAA individuals showed increases in sputum ECP, indicating an activated state of accumulating eosinophils. Allergic inflammation induces the recruitment of eosinophil precursors from blood to the airways where they complete their differentiation.³⁶ This fact might explain why after a single allergen administration eosinophils increased in sputum but not in peripheral blood.

Monocyte recruitment is a key feature of both AR and AA and occurs very early after allergen exposure.³⁷ As shown in mouse and

human studies, accumulating monocytes during allergic airway inflammation mature into potent pro-inflammatory cells releasing multiple chemokines and attracting other immune cells such as circulating eosinophils.^{37,38} Furthermore, there is a correlation between mucosal monocytes and eosinophils in AR and AA patients.³⁹ Our results indicate that the recruitment of monocytes accompanies the accumulation of eosinophils also in LAA, further relating both allergic asthma phenotypes. Significant differences in sputum eosinophils or monocytes between AA and LAA individuals were not detected, yet AA patients had higher absolute numbers of both cells and ECP, maybe due to phenotype-specific factors or to the longer disease evolution of AA subjects.

BPT-DP+ individuals did not experience increases of sputum neutrophils, T lymphocytes, NK cells, or sputum tryptase regardless of their atopic status. The allergen-triggered IgE-mediated activation of mast cells induces the release of tryptase, but this phenomenon is not prominently involved in the late-phase reaction of asthma.³⁵ A study of AR patients intranasally challenged during one week reported an increased recruitment of CD4+ T cells from the 3rd day of allergen provocation.³⁷ Furthermore, in mouse models of AA, inflammatory monocytes were shown to recruit and locally reactivate memory Th2 cells.³⁸ The shorter provocation protocol, the different anatomical areas examined, and the lack of quantification of specific T-cell subsets might explain the differences with our study. Moreover, eosinophils and monocytes display a high invasive ability in asthma, being able to migrate through the bronchial walls to the airway lumen,³⁹ whereas T lymphocytes mainly traffic from the secondary lymphoid organs to the mucosa.³⁵ Therefore, the lack of changes in the sputum cannot exclude an effect of the BPT-DP on T cells at the mucosal level.

In this study, we did not find sIgE in the sputum of AA and LAA individuals at any time point. One research group demonstrated the presence of sIgE in the sputum of nonatopic asthma patients.²³ Nevertheless, these patients failed to develop a positive BPT-DP, thus limiting the comparison with our study. Importantly, IgE antibodies bind with very high affinity to FcεRI expressed on immune cells, implying that free IgE will be found in secretions only after the saturation of the whole receptor system.⁴⁰ Therefore, the lack of sputum sIgE does not exclude per se the presence of sIgE bound to the mucosal immune cells. The role of sIgE is still debated in LAR pathophysiology,⁴¹ mainly due to the relatively small proportion of patients with detectable sIgE in nasal secretions.⁴² However, several indirect evidences favor an IgE-mediated mechanism for LAR, including the positivity of the basophil activation test in 30%-50% of patients,⁴³ and the good performance of allergen immunotherapy in 70% of LAR individuals.⁴⁴ Further studies are warranted to elucidate the role of sIgE in LAA.

In conclusion, our study indicates that in a proportion of nonatopic asthma patients, the allergen challenge induces bronchospasm and an allergic-like inflammatory infiltrate. These findings define LAA as a differentiated disease phenotype and reinforce the immune-pathological links between the upper and lower airways.

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

AUTHOR CONTRIBUTIONS

PC, CM, MJT, and CR designed and coordinated the study. PC, IEG, MS, NP, MJT, and CR recruited the study individuals, performed the nasal and bronchial challenges, and obtained the sputum and blood samples. MCPS, MJR, MG, AM, and CM conducted the in vitro techniques using the sputum and blood samples. PC, IEG, CM, MJT, and CR analyzed the data and wrote the manuscript. All authors revised and approved the submitted version of the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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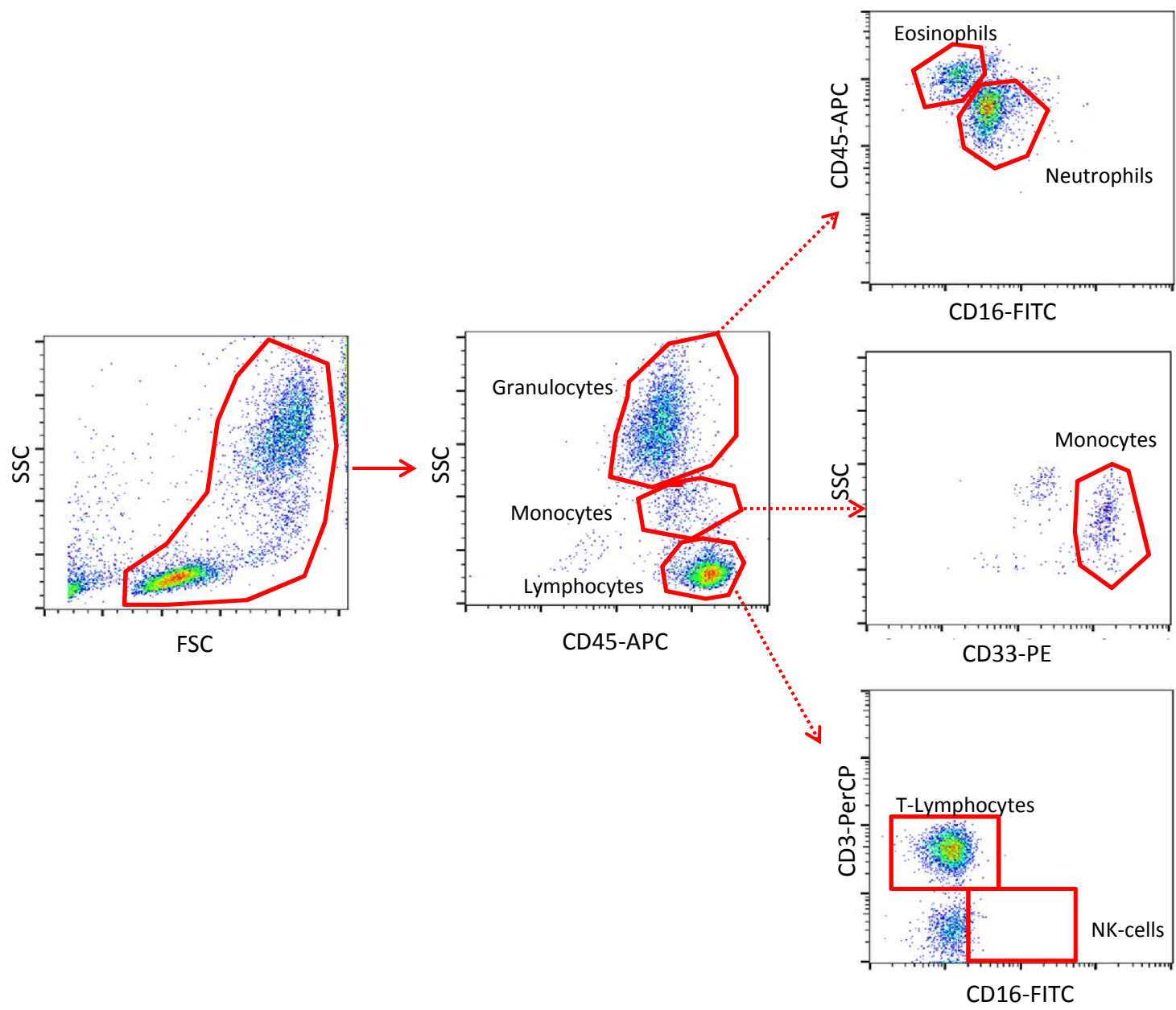


Figure E1

Group	BPT	Visit	N% (mean ± SD)	Eo% (mean ± SD)	Mo% (mean ± SD)	T-Ly% (mean ± SD)	NK% (mean ± SD)
LAR n=28	BPT(+) n=8 (LAA)	V1	27.58±18.72	2.54±3.91	4.77±2.38	33.37±9.87	0.27±0.14
		V3	32.25±21.14	3.48±4.59	4.31±0.78	34.16±10.34	0.27±0.66
		p	0.566	0.243	0.649	0.858	0.226
	BPT(-) n=20	V1	28.27±22.08	1.74±1.40	4.32±3.48	33.99±19.04	0.55±0.86
		V3	26.60±16.38	1.59±2.12	4.91±3.35	35.63±15.99	0.54±0.48
		p	0.755	0.761	0.595	0.805	0.956
AR n=18	BPT(+) n=15 (AA)	V1	39.01±23.66	5.46±8.58	6.28±5.39	26.18±11.70	0.28±0.19
		V3	38.90±22.74	7.41±7.54	5.61±2.71	38.56±14.91	0.46±0.29
		p	0.939	0.219	0.666	0.064	0.070
	BPT(-) n=3	V1	23.45±8.56	2.30±2.98	2.49±0.28	41.25±6.72	0.58±0.11
		V3	33.00±28.99	0.62±0.73	3.28±0.58	41.65±22.70	0.39±0.34
		p	n/a	n/a	n/a	n/a	n/a
NAR n=19	BPT(-) n=19	V1	26.40±30.21	2.67±5.61	2.87±2.59	37.04±20.58	0.34±0.32
		V3	32.76±27.50	2.92±2.79	3.46±2.65	33.54±19.87	0.29±0.23
		p	0.535	0.879	0.388	0.550	0.552
HC n=8	BPT(-) n=8	V1	32.42±21.23	2.38±1.85	5.74±3.75	35.58±19.72	0.50±0.24
		V3	27.48±17.00	2.08±1.78	6.05±1.27	34.56±8.89	0.30±0.17
		p	0.725	0.699	0.847	0.917	0.076

Table E1

Group	BPT	Visit	N% (median, range)	Eo% (median, range)	Mo% (median, range)	T-Iy% (median, range)	%NK (median, range)
LAR n=28	BPT(+) n=8 (LAA)	V1	29.00 (55.20)	3.44 (8.05)	1.25 (3.34)	1.18 (2.87)	0.19 (1.69)
		V3	50.90 (39.00)	6.55 (8.24)	3.14 (5.47)	2.06 (5.63)	0.13 (1.35)
		p	0.735	0.018	0.028	0.310	0.933
	BPT(-) n=20	V1	43.95 (86.85)	2.41 (34.45)	0.89 (3.12)	1.89 (4.35)	0.17 (0.07-0.30)
		V3	42.30 (69.33)	2.99 (8.72)	0.60 (5.84)	2.02 (4.89)	0.21 (0.01-0.43)
		p	0.587	0.281	0.698	0.938	0.170
AR n=18	BPT(+) n=15 (AA)	V1	18.20 (53.48)	1.26 (7.47)	0.89 (5.10)	1.91 (10.02)	0.14 (0.61)
		V3	31.75 (53.20)	3.75 (14.70)	4.32 (12.55)	3.23 (11.47)	0.25 (0.55)
		p	0.139	0.028	0.038	0.959	0.477
	BPT(-) n=3	V1	46.70 (26.20)	5.14 (0.24)	2.70 (0.52)	2.89 (4.05)	0.12 (0.07)
		V3	41.60 (9.00)	2.06 (1.83)	1.98 (1.22)	1.33 (0.76)	0.22 (0.34)
		p	0.655	0.180	0.655	0.655	0.655
NAR n=19	BPT(-) n=19	V1	33.30 (66.40)	3.35 (43.89)	1.07 (7.55)	1.84 (50.50)	0.05 (0.57)
		V3	25.15 (88.91)	2.03 (30.67)	1.05 (24.9)	3.49 (13.00)	0.05 (0.40)
		p	0.427	0.013	0.055	0.394	0.254
HC n=8	BPT(-) n=8	V1	59.35 (66.90)	5.12 (10.48)	0.93 (2.17)	0.63 (3.38)	0.04 (0.24)
		V3	36.15 (40.00)	3.49 (64.80)	0.49 (12.20)	0.47 (29.60)	0.02 (0.15)
		p	0.123	0.889	0.575	0.674	0.116

Table E2

			SERUM		SPUTUM	
Group	BPT	Visit	ECP ng/ml (mean, SD)	Tryptase ng/ml (mean, SD)	ECP ng/ml (median, range)	Tryptase ng/ml (median,range)
LAR n=28	BPT(+) n=8 (LAA)	V1	13.97 (12.02)	5.03 (2.61)	11.64 (2.63-27.88)	1.00 (1.00-1.00)
		V3	24.82 (12.04)	5.24 (2.68)	16.48 (2.05-40.18)	1.00 (1.00-1.19)
		p	0.073	0.373	0.031	0.5
	BPT(-) n=20	V1	14.06 (7.65)	5.60 (2.40)	22.40 (4.09-200.00)	1.00 (1.00-1.00)
		V3	19.76 (14.93)	5.41 (1.90)	35.30 (21.80-195.00)	1.00 (1.00-1.22)
		p	0.067	0.610	0.500	1.000
AR n=18	BPT(+) n=15 (AA)	V1	20.45 (12.68)	5.10 (1.50)	41.30 (6.40-156.50)	1.00 (1.00-1.00)
		V3	24.81 (14.83)	5.18 (1.71)	123.00 (10.22-200.00)	1.00 (1.00-1.34)
		p	0.086	0.823	0.027	0.250
	BPT(-) n=3	V1	28.77 (31.81)	4.13 (0.68)	23.10 (23.10-23.10)	1.00 (1.00-1.00)
		V3	29.19 (30.99)	5.16 (0)	29.20 (29.20-29.20)	1.00 (1.00-1.00)
		p	NA	NA	NA	1.000
NAR n=19	BPT(-) n=19	V1	16.30 (9.68)	5.12 (1.20)	8.80 (6.97-20.55)	1.00 (1.00-1.00)
		V3	16.24 (10.58)	5.14 (2.03)	14.10 (8.50-35.60)	1.00 (1.00-1.00)
		p	0.845	0.828	0.469	1.000
HC n=8	BPT(-) n=8	V1	15.15 (7.40)	3.57 (1.31)	2.00 (2.00-3.25)	1.00 (1.00-1.00)
		V3	11.63 (5.06)	3.86 (1.38)	2.00 (2.00-2.59)	1.00 (1.00-1.00)
		p	0.228	0.925	0.750	1.000

Table E3

1 **ONLINE REPOSITORY**

2 **Title:**

3 Bronchial asthma triggered by house dust mites in patients with local allergic rhinitis

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18 None of the authors has any conflict of interest for the present study

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27 **Key words:** allergic, bronchial, asthma, IgE, local, rhinitis

28

29 **Abbreviations**

30 AA: allergic asthma

31 AHR: bronchial hyperresponsiveness

32 AIT: allergen immunotherapy

33 AR: allergic rhinitis

34 BAPT: bronchial allergen provocation test

35 BPT: bronchial provocation test

36 DP: *Dermatophagoides pteronyssinus*

37 ECP: eosinophil cationic protein

38 FEV1: forced expiratory volume in the 1st second

39 ID: intradermal test

40 sIgE: specific IgE

41 HC: healthy controls

42 HDM: house dust mites

43 LAR: local allergic rhinitis

44 LAA: local allergic asthma

45 MT: metacholine test

46 NAR: non-allergic rhinitis

47 NAPT: nasal allergen provocation test

48 PC₂₀: FEV1 by 20% at baseline

49 SPT: skin prick test

50 V: visit

51

Methods

Exclusion criteria

The exclusion criteria for all groups were:

Evidence of immunological disease, respiratory infection during the previous 4 weeks, severe asthma or FEV1<60%, uncontrolled asthma, pregnancy/breastfeeding, anti-IgE treatment or allergen immunotherapy during the past 10.

Skin Prick and Intradermal Test

Skin prick test (SPT) was performed with a complete panel of the most prevalent aeroallergens in our area, including house dust mites (HDM) (*Dermatophagoides pteronyssinus* (DP), *Dermatophagoides farinae*, *Lepidoglyphus Destructor* and *Blomia Tropicalis*), pollens (grass pollen, *Parietaria Judaica*, *Cupressus* species, *Platanus* species, *Olea Europaea*, *Chenopodium* species, *Plantago* species, *Artemisia* species and *Salsola Kali*), molds (*Alternaria Alternata*, *Aspergillus Fumigatus*), dog and cat dander (ALK-Abelló, Denmark) following the current guidelines (E1). Intra-dermal test was performed with freshly reconstituted solution (ALK-Abelló, Der p1 concentration 0.4 µg/ml).

Nasal Allergen Provocation Test (NAPT)

NAPT was performed according to previously described methods (E2). Subjects had to stop treatment with leukotriene antagonists, antihistamines and nasal corticosteroids (1 week prior), and systemic corticosteroids 4 weeks prior to the study. Nasal hyperresponsiveness was ruled out by performing a nasal challenge with 0.9% saline before applying the allergen in the nose. Later on, two puffs (100 µL total) of freshly reconstituted allergen extract were applied on each nostril with a metered pump (*Alternaria alternata* 0.25 µg/mL and *Dermatophagoides pteronyssinus* 4 µg/mL from ALK-Abelló (Madrid, Spain) and *Parietaria judaica*, cat and dog epithelia 30 HEP/mL from Leti S.L. (Tres Cantos, Madrid, Spain)). Responses were monitored by means of acoustic rhinometry (SRE 2000 rhinometer, Rhinometrics, Lynge, Denmark) and symptoms' score. Acoustic rhinometry was performed following the guidelines of the Standardization Committee on Acoustic Rhinometry (E3), measuring the volume of the nasal cavity that corresponds to the lower turbinate (vol 2–6 cm) in each nostril. Nasal obstruction, rhinorrhea, itching, sneezing, and ocular symptoms were recorded by using a visual analog

scale (VAS) of 100 mm. The NAPT was performed in symptom-free patients (total VAS, <60 mm), and with 1-week interval between different allergens. A positive NAPT was considered to be an increase $\geq 30\%$ in the total VAS and a decrease $\geq 30\%$ in the total vol 2–6 cm of both nasal cavities compared with the baseline test.

Methacholine Test (MT)

Treatment with asthma medication (short/long acting beta-agonists, inhaled corticosteroids, anti-leukotriens) was discontinued for a minimum of 48 hours before the challenge as described (E4,E5). Antihistamines had to be stopped 10 days prior to the test. All participants were stable, with no evidence of respiratory infection within the past 6 weeks or treatment with oral corticosteroids. Methacholine (Provocholine®, Diater Laboratories, Madrid, Spain) was administrated in doubling doses from 0.03 mg/ml to 16 mg/ml using the tidal breath method following current guidelines (E4, E5). The test was interrupted when a fall $\geq 20\%$ was observed and provocative concentration causing a 20% drop in FEV₁ (PC₂₀) was calculated. MT was considered positive (MT (+)) when PC₂₀ < 4 mg/ml (E4, E5).

Bronchial Provocation Test with D. Pteronyssinus (BPT-DP)

BPT-DP was performed with a commercial whole DP extract for bronchial challenge containing 4 µg of Der p 1/ml (ALK-Abelló, Denmark) one week after the baseline MT (V1). Current treatment for asthma was interrupted as described in the MT section. The challenge was performed using a DeVilbiss 646 nebulizer, inhaling at tidal breath for 2 minutes on each dilution (E6, E7). First, patients inhaled 0.9% CINA in order to rule out bronchial hyperreactivity; if FEV₁ dropped <10% from baseline after saline inhalation, then DP extract was administrated by increasing concentrations. The starting dilution of allergen was a 1:1000 dilution of the DP extract (0.004 µg/ml of Der p 1/ml), and a new dilution was administered if no immediate asthmatic response occurred (1:100, 0.04 µg/ml Der p1), 1:10 (0.4 µg/ml Der p1) and 1:1 (4 µg/ml Der p1). Symptoms and FEV₁ were assessed at 0, 5, 10 and 15 minutes after the allergen exposition. The challenge was interrupted if a drop of $\geq 20\%$ of FEV₁ was detected or until the maximal dose was administrated. If the test was positive, patients were treated with 500 µg Terbutaline, and lung function was monitored during two hours to assess total recovery.

Afterwards, if stable, patients were discharged and measured peak expiratory flow values at home each hour to evaluate late responses.

Induced Sputum Sampling and Processing

Serial induced sputum samples were obtained prior to and 24 hours after BPT-DP and processed within two hours, following the method of Pizzichini (E8). FEV₁ was measured before sputum induction and participants were administrated 500 µg of terbutaline. Three different concentrations of hypertonic saline (3%, 4% and 5% NaCl) were nebulized through an ultrasonic device (Omron® Ultra A-I-R NE U-17, Japan) for 7 minutes each. In case of baseline FEV₁ below 70%, a 0.9% solution was used. After each nebulization period, patient had to cough and to spit sputum in a sterile container. In that moment, FEV₁ was also monitored, and the procedure was stopped as soon as a good quality sputum sample was obtained.

Sputum was processed according to published methods (E9, E10). Sputum plugs were selected in a Petri dish, weighted and suspended in 4 times the volume in PBSx1 supplemented with 2% of a protease inhibitor cocktail (Sigma-Aldrich, ST Louis, MO), and centrifuged 10 minutes 1500xg. The pellet was then diluted in a solution of dithiothreitol (DTT) 10⁻³M (Sigma-Aldrich), supplemented with protease inhibitor cocktail 4:1 vol/w, incubated for 15 minutes and centrifuged at 389 xg 15 minutes 4°C. Supernatants were stored at -80°C for further analysis and pellet resuspended in PBS. Viability was determined by trypan blue exclusion staining. Cells were phenotyped by flow cytometry (see *Cell population analysis* section).

In vitro measurements

The cellular populations and the levels of inflammatory mediators (ECP and tryptase), total IgE and sIgE to DP were analysed in blood and induced sputum samples at V1 (baseline) and V3 (24 hours post BPT-DP):

Cell population analysis

Cell populations (eosinophils, monocytes, T-lymphocytes and NK cells) in both blood and sputum were analysed by flow cytometry. Processed sputum and blood samples were incubated for 15 minutes in the darkness with a series of monoclonal antibodies: CD45-APC,

CD16-FITC, CD33-PE, CD3-PerCP, (Becton-Dickinson, San Jose, CA), following previously published methods (E11). Six-parameter analysis was performed on a FACSCalibur flow cytometer and analyzed with FlowJo® software (Tree Star, Inc., Ashland, OR, USA). Results were expressed as percentage of each population from CD45⁺ cells (Figure E1).

Mediators' measurement

ECP and tryptase expressed in ng/mL, were measured in serum and sputum supernatant by CAP method (Thermo Fisher, Waltham, Mass). Cut-off values were 2ng/mL for ECP and 1 ng/mL for tryptase. ***Total IgE and specific IgE measurement***

Total IgE and sIgE to DP were measured in peripheral blood by CAP method (Thermo Fisher). The detection limit was 4 UI/ml for total IgE and 0.35 kU/L for sIgE according to manufacturer's instructions. Moreover, sIgE to DP was measured in sputum supernatants by two different methods: immunoCAP (Thermo Fisher) and radioimmunoassay (RIA) as previously described (E12). Briefly, cyanogen bromide-activated cellulose discs were used as solid phase, and conjugated to DP extract at 50µg/mL (Leti Laboratories) as previously described (E13). The RIA was performed by incubating 30 µl of supernatant with the discs for 3 hours. After washing, 25 µL of anti-IgE labelled with I¹²⁵ (ALK-Abelló, Spain) was added and incubated for 16 hours. The radioactivity was then measured in a gamma counter (Packard BioScience Company, Frankfurt, Germany) as count per minute (cpm). Results were calculated as a percentage of the maximum of cpm and considered positive if they were higher than 2.5% of label uptake, which was the mean ±2 SD of the negative control group.

Statistical analysis

Data are expressed as means ± standard deviation, median values with interquartile ranges, or number and percentage, as appropriate. Comparisons between groups were performed by Student's T-test for normal distribution and Mann-Whitney U-test for non-normal distribution respectively. The Pearson chi-square or the Fisher-exact tests were used comparisons between categorical variables, and Bonferroni method for multiple comparisons. Differences between before vs. after bronchial challenges interventions (PC₂₀ and BPT-DP) were analysed by Paired sample-t test or Wilcoxon's signed-ranks test for normal and non-normal distribution

respectively. Concordance analysis was used to assess the relationship between MT and BPT-
DP results. Statistical analyses were performed using SPSS 15.0 (SPSS Inc., Chicago, IL,
USA). A two-sided p value of < 0.05 was considered significant.

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FIGURE AND TABLE LEGENDS

FIGURE E1.

Gating strategy for cellular analysis by flow cytometry in sputum and peripheral blood of patients included in the study

TABLE E1.

Results of flow cytometry analysis in peripheral blood of all participants comparing data from baseline (V1) with after bronchial provocation test (BPT) with D. pteronyssinus (DP) (V3).

N: neutrophils; Eo: eosinophils; Mo: monocytes; T-Ly: T-lymphocytes; NK: natural killer

AA: allergic asthma; AR: allergic rhinitis; HC: healthy controls; LAA: local allergic asthma; LAR: local allergic rhinitis; NAR: non-allergic rhinitis; SD: standard deviation;

TABLE E2.

Results of flow cytometry analysis in sputum of all participants comparing data from baseline (V1) with after bronchial provocation test (BPT) with D. pteronyssinus (DP) (V3).

N: neutrophils; Eo: eosinophils; Mo: monocytes; T-Ly: T-lymphocytes; NK: natural killer

AA: allergic asthma; AR: allergic rhinitis; HC: healthy controls; LAA: local allergic asthma; LAR: local allergic rhinitis; NAR: non-allergic rhinitis; SD: standard deviation;

TABLE E3.

Levels of mediators (ECP and tryptase) in serum and sputum of all participants comparing data from baseline (V1) with after bronchial provocation test (BPT) with D. pteronyssinus (DP) (V3).

AA: allergic asthma; AR: allergic rhinitis; HC: healthy controls; LAA: local allergic asthma; LAR: local allergic rhinitis; NAR: non-allergic rhinitis; SD: standard deviation;