

ORIGINAL ARTICLE

Airway Diseases

Coexistence of nasal reactivity to allergens with and without IgE sensitization in patients with allergic rhinitis

Ibon Eguiluz-Gracia¹  | Ruben Fernandez-Santamaria² | Almudena Testera-Montes^{1,3} |
 Adriana Ariza² | Paloma Campo¹ | Ana Prieto⁴ | Natalia Perez-Sanchez¹  |
 Maria Salas¹  | Cristobalina Mayorga²  | Maria Jose Torres^{1,3}  | Carmen Rondon¹ 

¹Allergy Unit, Hospital Regional Universitario de Malaga, Instituto de Investigación Biomédica de Málaga-IBIMA and ARADyAL, Málaga, Spain

²Allergy Research Group, Instituto de Investigación Biomédica de Málaga-IBIMA and ARADyAL, Málaga, Spain

³Universidad de Málaga-UMA, Málaga, Spain

⁴Pediatrics Unit, Hospital Regional Universitario de Malaga, Málaga, Spain

Correspondence

Ibon Eguiluz-Gracia, Laboratorio de Investigación, Hospital Civil, pabellón 5, sótano, Plaza del Hospital Civil, 29009 Malaga, Spain.
 Email: iboneguiluz@gmail.com

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Abstract

Background: Allergic rhinitis (AR) and local allergic rhinitis (LAR) are defined by nasal reactivity to aeroallergens with and without positive skin prick test (SPT), respectively. In this study, we aimed to investigate whether both types of allergen-specific reactivity can coexist in the same individual.

Methods: Forty-eight patients with perennial rhinitis symptoms and positive SPT with seasonal allergens only (discrepant group) were subjected to consecutive nasal allergen challenges (NAC) with seasonal (NAC-S) and perennial allergens (NAC-P). A nasal lavage was collected before and after the NACs to measure eosinophil cationic protein (ECP). A basophil activation test (BAT) with seasonal and/or perennial allergens was performed in ten patients from the discrepant group and in six seasonal allergic rhinitis (SAR), eight perennial local allergic rhinitis (LAR), six nonallergic rhinitis (NAR), and six healthy control (HC) individuals.

Results: All patients in the discrepant group tested positive in the NAC-S, and 41 of them (85.4%), also in the NAC-P (group A). Conversely, seven patients tested negative in the NAC-P (group B). ECP in the nasal lavage increased after the NAC-P in the group A ($P = .004$), but not in the group B. The BAT with seasonal allergens was positive in 100% of SAR and group A cases, whereas the BAT with perennial allergens was positive in 37.5% and 60% of LAR and group A cases, respectively. All NAR and HC subjects tested negative for the BAT.

Abbreviations: AA, *Alternaria alternata*; AR, allergic rhinitis; BAT, basophil activation test; DAR, dual allergic rhinitis; DP, *Dermatophagoides pteronyssinus*; ECP, eosinophil cationic protein; IDT, intradermal test; LAR, local allergic rhinitis; MR, mixed rhinitis; NAC, nasal allergen challenge; NAC-P, nasal allergen challenge with perennial allergens; NAC-S, nasal allergen challenge with seasonal allergens; NAR, nonallergic rhinitis; NHR, nasal hyperreactivity; SAR, seasonal allergic rhinitis; sIgE, allergen-specific IgE; SPT, skin prick test; VAS, visual analogue scale.

Ibon Eguiluz-Gracia, Ruben Fernandez-Santamaria and Almudena Testera-Montes contributed equally to this work.

Clinical Implications: Nasal reactivity to aeroallergens with and without systemic IgE sensitization can coexist in the same rhinitis patient.

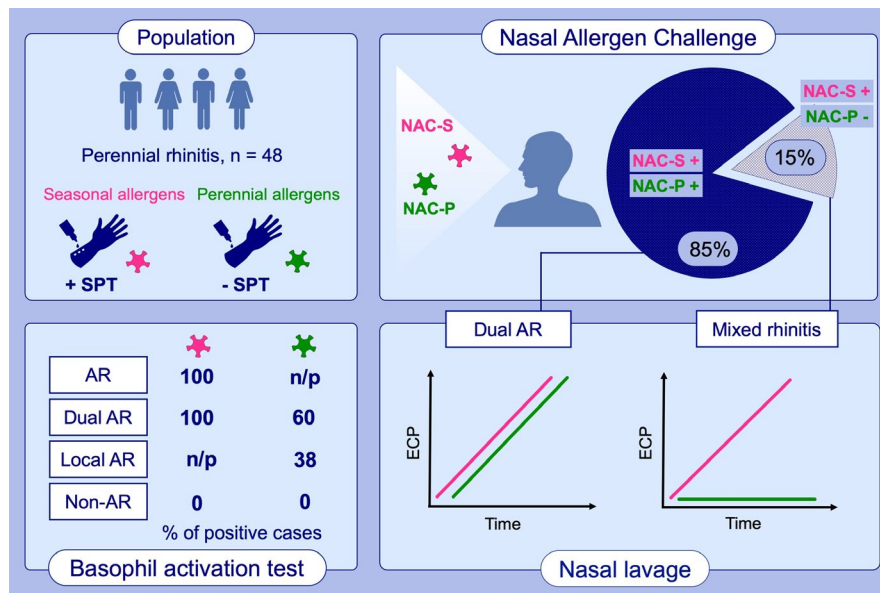
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Conclusion: This study shows that nasal reactivity to aeroallergens with and without positive SPT can coexist in the same patient. We propose the term dual allergic rhinitis for this rhinitis phenotype.

KEYWORDS

allergic rhinitis, basophil activation test, local allergic rhinitis, nasal allergen challenge



GRAPHICAL ABSTRACT

Forty-eight patients with perennial rhinitis symptoms and showing positive skin prick test with seasonal allergens only were recruited. All patients were subjected to consecutive nasal allergens challenges with seasonal (NAC-S) and perennial (NAC-P) allergens. All individuals tested positive in the NAC-S. Eighty-five percent of patients displayed positive NAC-P results (dual allergic rhinitis group), and 15% of subjects tested negative in the NAC-P (mixed allergic rhinitis group). The NAC-S induced an increase of nasal eosinophil cationic protein (ECP) in all patients, whereas the NAC-P induced an increase of nasal ECP only in dual allergic rhinitis patients. Additional patients with different rhinitis phenotypes were recruited for the peripheral immunophenotyping. The basophil activation test (BAT) with seasonal allergens was positive in all patients with seasonal allergic rhinitis and dual allergic rhinitis. The BAT with perennial allergens was positive in 60% and 38% of subjects with dual allergic rhinitis and perennial local allergic rhinitis, respectively. All individuals with nonallergic rhinitis tested negative in the BAT with both seasonal and perennial allergens.

1 | INTRODUCTION

Chronic noninfectious rhinitis is a nasal inflammatory disease affecting 30%-40% of the Western population.¹ The condition has been classically divided into the allergic rhinitis (AR) and the non-allergic rhinitis (NAR) phenotypes.^{2,3} Allergic rhinitis is a relatively homogeneous disorder arising from the IgE sensitization to aeroallergens.⁴ In AR patients, the exposure to the allergens induces rhinitis symptoms and eosinophilic nasal inflammation.⁵ By definition, AR patients test positive for the skin prick test (SPT) and/or serum allergen-specific IgE (sIgE) (atopy).¹ The NAR phenotype comprises a highly heterogeneous group of diseases including conditions mediated by immunological, hormonal, and/or neurogenic mechanisms.³ By definition, NAR patients are not atopic (they test negative for both SPT and serum sIgE).⁶ Nevertheless, in

recent years it has become apparent that a significant proportion of nonatopic rhinitis patients (25.7% according to a recent systematic review⁷) display nasal reactivity to aeroallergens.⁸ This disease phenotype has been termed local allergic rhinitis (LAR)⁹ and illustrates how the classical AR-NAR dichotomy does not reflect the complexity of the rhinitis syndrome.¹⁰ In this regard, an additional biomarker like the nasal allergen challenge (NAC) is crucial to identify LAR patients.¹¹ By definition, AR and LAR individuals test positive for the NAC, whereas NAR subjects display negative results.¹² Similar to AR, the allergen exposure induces nasal eosinophilic inflammation in LAR individuals.^{13,14} Some clinical features can help identify candidate subjects for a NAC among nonatopic rhinitis individuals. As compared to NAR patients, LAR subjects are more frequently younger nonsmoker women with earlier onset of rhinitis and a family history of atopy.⁸

Besides the NAC, the basophil activation test (BAT) has been proposed as a useful biomarker for LAR diagnosis.¹⁵ The BAT reproduces the allergen-triggered IgE-mediated activation of peripheral basophils in a controlled manner.¹⁶ Because the BAT is an *in vitro* test only requiring the collection of a blood sample, it might be more patient-friendly than a time-consuming *in vivo* procedure like the NAC.¹² The BAT displays a high specificity for LAR diagnosis (100%)¹⁷⁻¹⁹ and provides relevant information about disease mechanisms.¹⁶ On the other hand, its sensitivity for the diagnosis of LAR ranges from 50% to 66.6% depending on the allergen examined.¹⁷⁻¹⁹ Moreover, it is currently unknown how the NAC affects basophil reactivity.

In clinical practice, the results of the SPT and/or serum sIgE very often do not match the symptom pattern of the AR patients (eg, perennial symptoms with positive SPT to seasonal allergens only).¹ This phenomenon has been classically explained by the cooperation of allergic and nonallergic mechanisms in the same rhinitis patient.² In this regard, AR can coexist with different phenotypes of NAR in the same individual, and this clinical scenario has been called mixed rhinitis (MR).¹ However, the coexistence of AR and LAR in the same individual has not been reported to date. Moreover, nasal hyperreactivity (NHR) is a trait of nasal disease that can be present in both allergic and nonallergic rhinitis phenotypes.^{20,21} Nasal hyperreactivity is characterized by the onset of rhinitis symptoms upon unspecific stimuli such as rapid changes in temperature or irritant smells.²²

In this study, we aimed to progress in the phenotyping of rhinitis patients with discrepancies between the symptom pattern and the results of SPT, and to investigate whether nasal reactivity to allergens with and without systemic sensitization can occur in the same individual. To this end, we recruited rhinitis patients with perennial symptoms who only displayed positive SPT to seasonal allergens. All patients were subjected to consecutive NACs with seasonal and perennial allergens, and a nasal lavage was collected before and after each provocation to measure eosinophil cationic protein (ECP). Moreover, in a subgroup of rhinitis patients who tested positive in the NAC for both seasonal and perennial allergens, a BAT was performed before and after the provocations, and the results were compared to those obtained in seasonal AR (SAR), NAR, LAR, and healthy nonatopic control (HC) individuals.

2 | METHODS

2.1 | Study individuals

Individuals ≥ 14 years with the following features were prospectively recruited from the Allergy Department of Hospital Regional Universitario de Malaga from June 2017 to June 2018: perennial rhinitis with exacerbation during springtime, positive SPT to grass and/or olive pollen, and negative SPT to all other relevant aeroallergens (discrepant group). A detailed description of the other study groups (SAR, LAR, NAR, and HC) and the exclusion criteria can be found at the Appendix S1. The study was conducted according to the principles of the Declaration of Helsinki and approved by the local Ethics Committee.

2.2 | Demographic and clinical data

After inclusion, all subjects in the discrepant group filled a questionnaire including clinical and demographic data and triggers of rhinitis. A detailed description of the information requested can be found at the Appendix S1.

2.3 | Skin tests

The SPTs were performed with all relevant aeroallergens and according to the international recommendations.²³ A detailed description of all aeroallergens tested in SPT can be found at the Appendix S1. In addition, in patients of the discrepant group an intradermal skin test (IDT) was performed with freshly reconstituted freeze-dried allergen solutions of *Dermatophagoides pteronyssinus* (DP) (Der p 1 0.4 and 4 $\mu\text{g/mL}$) and *Alternaria alternata* (AA) (0.025 and 0.25 $\mu\text{g/mL}$) (Laboratorios Leti SL).

2.4 | Nasal allergen challenge

Patients in the discrepant and SAR groups underwent NACs with one allergen per session during the summer of 2018 and 2019. The minimum interval between consecutive NACs was 1 week. The allergen extracts administered were grass-mix pollen, *Olea europea*, DP, and AA (Laboratorios Leti SL). The NAC was monitored by both visual analogue scale (VAS) of nasal symptoms and acoustic rhinometry.^{11,24} The test was considered positive if an increase $\geq 30\%$ in the VAS and a bilateral decrease $\geq 30\%$ in the total sum of vol. 2-6 cm of the acoustic rhinometry (as compared to baseline measurement) occurred. Nasal hyperreactivity was excluded prior to allergen administration in every visit. A detailed description of the NAC procedure can be found at the Appendix S1.

2.5 | Eosinophil cationic protein in nasal lavage

In patients from the discrepant group, a bilateral nasal lavage with 8 mL of isotonic saline previously brought to room temperature was performed 24 hours before the NAC (baseline sample), and 15 and 60 minutes after the administration of the last allergen concentration or of that eliciting a positive NAC response. The lavage was used to measure ECP by commercial immunoassay (Thermo Fisher[®] Waltham). A detailed description of the lavage procedure can be found at the Appendix S1.

2.6 | Basophil activation test

Blood samples were collected immediately before and 24 hours after NAC from the different study groups to perform a BAT with grass-mix and olive pollen, DP, and AA extracts (Laboratorios Leti SL). Basophil activation was assessed by flow cytometry (FACSCalibur,

Becton-Dickinson Biosciences), and CD63 was used as activation marker.¹⁶ A detailed description of the BAT procedure can be found at the Appendix S1.

2.7 | Statistical analysis

SPSS version 15.0 was used for statistical analysis. Continuous data are expressed as mean and standard deviation or median and interquartile ranges, and categorical data as percentages. Comparisons were performed by chi-square analysis (categorical data), parametric test (Student's *t* test or Mann-Whitney *U* test for nonrelated samples), and nonparametric test (Student's *t* test or Wilcoxon test for related samples). All *P* values < .05 were considered statistically significant.

3 | RESULTS

3.1 | Clinical characterization

3.1.1 | Individuals included in the discrepant group

One hundred twenty-five patients with perennial rhinitis symptoms with seasonal exacerbation and positive SPT to olive/grass pollen only were identified in our clinic over a year. Sixty-three individuals accepted to participate in the study, signed the informed consent, and came to a second visit (selection visit) for nasal endoscopy, completion of the clinical history and collection of a blood sample (flowchart at Figure S1). After the visit, nine subjects were excluded for the following reasons:

1. Serum sIgE to perennial allergens >0.35 kUA/L: two patients (3.2%)
2. Clinical symptoms (nasal blockage, nasal discharge, decreased sense of smell, facial pain, etc) and/or endoscopic signs of chronic rhinosinusitis (mucopurulent discharge, edema in the middle meatus, nasal polyps, etc) (25): five patients (7.9%)
3. Nasal anatomical disorders: two patients (3.2%)

Twenty-four of the 54 patients recruited (44.4%) had positive SPT with olive pollen only (23 of them with serum sIgE >0.35 kUA/L), 17 patients (31.5%) with both grass and olive pollens (15 of them with detectable serum sIgE to both allergens), and 13 subjects (24.1%) with grass pollen only (all of them with detectable serum sIgE). Two patients did not have detectable serum sIgE to any seasonal allergen, and one subject had detectable serum sIgE to olive pollen only, despite displaying positive SPT to both grass and olive pollens. The IDTs with DP and AA were negative in all patients.

3.1.2 | NAC with seasonal allergens

Six patients of the discrepant group (11.1%) had three consecutive positive nasal control challenges, and thus, they were excluded from further analysis (see NAC procedure at the Appendix S1).^{11,24} In the

TABLE 1 Demographic and clinical characteristics of the patients from the discrepant group

	Study subjects N = 48
Female gender, N (%)	32 (66)
Age (y), mean (SD)	31.30 (10.9)
FHA, N (%)	19 (39.6)
Urban dwelling, N (%)	33 (68.8)
Urban employment, N (%)	38 (79.2)
Smoking habit, N (%)	
Nonsmoker	40 (83.3)
Ex-smoker	4 (8.5)
Current smoker	4 (8.5)
Time of evolution (y), mean (SD)	8 (8.2)
Severity of seasonal symptoms, N (%)	
Mild	4 (8.3)
Moderate	32 (66.7)
Severe	12 (25)
Severity of perennial symptoms, N (%)	
Mild	16 (33.3)
Moderate	28 (58.3)
Severe	4 (8.3)
Rhinitis evolution, N (%)	
From seasonal to perennial	25 (52.1)
Perennial with seasonal exacerbation since rhinitis onset	23 (47.9)
Co-morbidities, N (%)	
Conjunctivitis symptoms	31 (64.6)
Asthma symptoms	18 (37.5)
Triggering factors, N (%)	
Vegetation	34 (70.8)
House dust	26 (54.2)
Temperature changes	8 (16.7)
Irritant smells	15 (31.3)
Dampness	9 (18.8)

Abbreviations: FHA, family history of atopy; N, number; SD, standard deviation.

remaining 48 patients, provocation with seasonal allergens (NAC-S) was performed with grass and olive pollen, obtaining a perfect concordance between the NAC-S and the corresponding SPT results (*kappa* index = 1), confirming the causative role of pollens in the seasonal exacerbation of rhinitis. Three of them (6.3%) had a positive control challenge in a previous visit, but tested negative to saline in subsequent visits. The demographic and clinical characteristics of the 48 subjects included are shown in Table 1.

3.1.3 | NAC with perennial allergens

Patients testing positive for the NAC with perennial allergens

Forty-one of 48 individuals of the discrepant group (85.4%) tested positive for at least one perennial allergen (group A). Four of them (9.8%) had had a positive saline challenge in a previous visit (one

FIGURE 1 Sensitization to seasonal and perennial allergens in patients from the group A. sIgE, allergen-specific IgE; SPT, skin prick test; NAC, nasal allergen challenge; IDT, intradermal test; AA, *Alternaria alternata*; DP, *Dermatophagoides pteronyssinus*

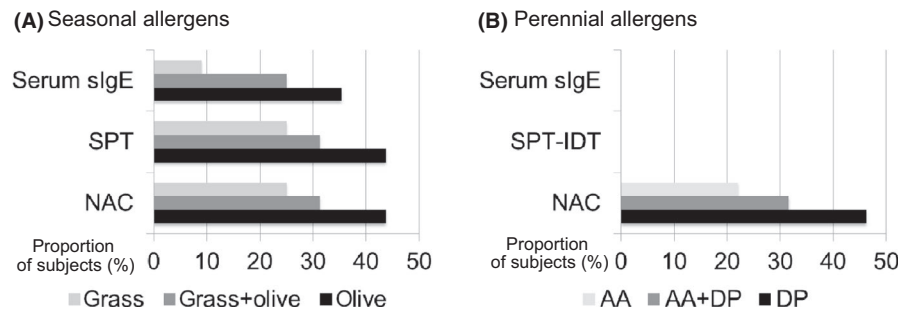
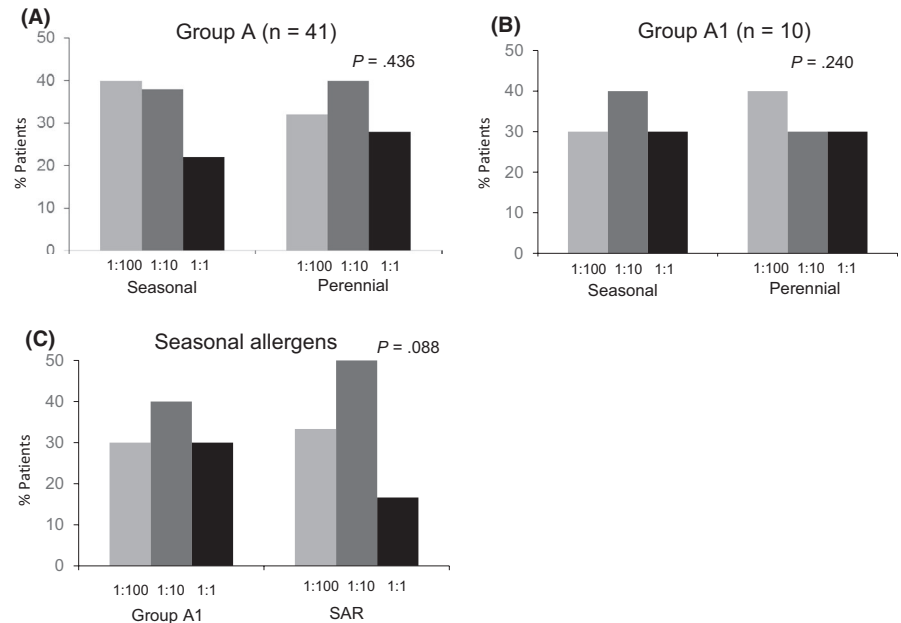


FIGURE 2 Proportion of patients reacting to the different concentrations of allergens during the NAC: comparison between seasonal and perennial allergens in group A (A) and group A1 (B, concentrations relative to the second NACs with seasonal and perennial allergens), and comparison between group A1 and SAR for the seasonal allergens (C, for A1 patients concentration relative to the second NAC)



of them also displayed a positive saline challenge before the NAC-S). Nineteen patients from group A (46.3%) reacted to DP only, 13 subjects (31.7%) to both AA and DP, and 9 individuals (22%) to AA only (Figure 1). No significant differences were observed between seasonal and perennial allergens in terms of concentration eliciting a nasal response ($P = .436$), with % of the subjects from group A reacting to sub-maximal concentrations of both allergens (Figure 2A). There was a predominance of nonsmoker women (mean age 32.2 years). The exposure to vegetation was the main trigger eliciting rhinitis (73.2%), followed by the exposure to indoor dust (58.5%). Most patients (58.5%) had seasonal rhinitis at disease onset, with subsequent progression to perennial symptoms (Table 2).

Patients testing negative for the NAC with perennial allergens

Seven patients of the discrepant group displayed negative NAC-P responses to both DP and AA (group B). Three individuals (42.9%) had experienced a positive saline challenge in a previous visit (two of them had had also a positive saline challenge before the NAC-S). Among group B patients, there was a predominance of nonsmoker men (median age: 49 years). Interestingly, subjects from group B were significantly older than individuals from group A ($P = .023$). Only one patient from group B (14.3%) reported the progression from seasonal to perennial symptoms during disease evolution, which significantly differed from group A ($P = .044$). The main triggers eliciting rhinitis

in group B were rapid changes in temperature (71.4%) and irritant smells (71.4%). Of note, the ability of these triggers to induce rhinitis was significantly higher in group B as compared to group A ($P = .001$ and $P = .024$, respectively) (Table 2).

3.2 | Immunological characterization

3.2.1 | Local level

The concentration of ECP in the nasal lavage was significantly higher 60 minutes after the administration of the seasonal allergen in both group A ($P < .001$) and group B ($P = .017$). Conversely, only group A showed a significant increase after the administration of the perennial allergen ($P < .001$). Despite displaying positive SPT with seasonal allergens only, individuals in group A experienced comparable increases in ECP concentrations in the nasal lavage after the NAC-S and the NAC-P (Figure 3).

3.2.2 | Peripheral level

We performed additional NAC-S and NAC-P in ten patients from group A (with positivity to only one perennial allergen) (group A1)

TABLE 2 Comparative features of individuals of groups A and B from the discrepant group

	Group A (N = 41)	Group B (N = 7)	P value
Female gender, N (%)	29 (70.7)	3 (42.9)	.201
Age (y),			
Mean (SD)	32.2 (12.0)	47.71 (16.8)	
Median (range)	33 (14-55)	49 (26-68)	.023
FHA, N (%)	16 (39)	3 (42.9)	1.000
Urban dwelling, N (%)	30 (73.2)	3 (42.9)	.183
Urban employment, N (%)	30 (73.2)	3 (42.9)	.183
Smoking habit, N (%)			
Nonsmoker	33 (80.5)	7 (100)	1.000
Ex-smoker	4 (9.8)	0 (0)	
Current smoker	4 (9.8)	0 (0)	
Time of evolution (y),			
Mean (SD)	8.8 (8.9)	5.3 (3.2)	
Median (range)	5.5 (1-40)	5 (2-12)	.549
Severity of seasonal symptoms, N (%)			.686
Mild	4 (9.8%)	0 (0)	
Moderate	27 (65.9)	5 (71.2)	
Severe	10 (24.4)	2 (28.6)	
Severity of perennial symptoms, N (%)			
Mild	14 (34.1)	2 (28.6)	.897
Moderate	23 (56.1)	5 (71.4)	
Severe	4 (9.8)	0 (0)	
Rhinitis evolution, N (%)			
From seasonal to perennial	24 (58.5)	1 (14.3)	.044
Perennial with seasonal exacerbation since rhinitis onset	17 (41.5)	6 (85.7)	
Co-morbidities, N (%)			
Conjunctivitis	28 (68.3)	3 (42.9)	.226
Asthma symptoms	16 (39)	2 (28.6)	.696
Triggering factors, N (%)			
Vegetation	30 (73.2)	4 (57.1)	.400
House dust	24 (58.5)	2 (28.6)	.223
Temperature changes ^a	3 (7.3)	5 (71.4)	.001
Irritant smells ^a	10 (24.4)	5 (71.4)	.024
Dampness	8 (19.5)	1 (14.3)	1.000

Bolditalics identifies comparisons where statistical significance was found.

Abbreviations: FHA, family history of atopy; N, number; NAC-P, nasal allergen challenge with perennial allergen; NAC-S, nasal allergen challenge with seasonal allergen; SD, standard deviation.

^aDuring all the year.

and NAC-S in six SAR subjects. In these individuals, we compared the ability of the allergens to activate peripheral basophils and the levels of serum sIgE before and 24 hours after the NACs. We selected this time point because previous studies from our group demonstrated that nasal sIgE progressively increases during the 24 hours following a positive NAC.^{13,26} All SAR subjects displayed positive NAC-S responses, and all patients from group A1 tested positive in both NAC-S and NAC-P (perfect concordance with previous NAC).²⁷ The BAT with seasonal allergens was positive in 100% of SAR individuals and patients from group A1, whereas the BAT with perennial allergens was positive in 60% of subjects from group A1. The surface expression of CD63 induced by seasonal allergens in peripheral basophils (Figure 4A) and the concentrations eliciting a positive NAC-S response (Figure 2C) were comparable between SAR subjects and patients from group A1 ($P = .51$ and $P = .088$, respectively). Conversely, serum sIgE for seasonal allergens was significantly lower in group A1 than in SAR subjects ($P = .040$) (Figure 4E), and no correlation was observed between serum sIgE and CD63 expression for seasonal allergens in patients from group A1 ($R^2 = .06$, $P = .53$). On the other hand, the basophils of patients from group A1 displayed significantly higher CD63 surface expression after stimulation with seasonal allergens as compared to perennial allergens ($P = .03$) (Figure 4C), yet no difference was observed in the allergen concentration eliciting a positive NAC response ($P = .240$) (Figure 2B). Interestingly, neither the NAC-S nor the NAC-P induced significant changes in basophil reactivity in SAR patients or individuals from group A1 (Figure S2). To investigate the correlation between nasal allergen-specific reactivity and basophil activation, we performed additional BATs in eight LAR (all of them with perennial symptoms and a previous positive nasal challenge with DP), six NAR (all of them with perennial symptoms and previous negative nasal challenges with all relevant aeroallergens), and six HC (no symptoms) individuals. Because NAC-P did not increase basophil reactivity in DAR patients, we did not repeat the nasal challenge with DP in LAR individuals. The BAT with perennial allergens was positive in 37.5% of LAR patients, whereas no NAR and HC individuals displayed positive results for either perennial or seasonal allergens (Figure 4D). Despite differences in BAT positivity between LAR subjects and patients in group A1 (37.5% and 60%, respectively), the surface expression of CD63 in peripheral basophils after stimulation with perennial allergens was comparable between the two groups ($P = .36$) (Figure 4B).

4 | DISCUSSION

In this study, we show that a significant proportion of rhinitis patients showing SPT positivity to seasonal allergens only, but suffering from perennial symptoms, display nasal allergen-specific reactivity to both perennial and seasonal allergens. Moreover, both perennial and seasonal allergens induce comparable increases in nasal ECP upon nasal challenge, and the BAT confirmed the IgE sensitization to perennial allergens in 60% of the individuals with this phenotype. Overall, these data indicate that clinically relevant

FIGURE 3 Concentration of eosinophil cationic protein (ECP) in the nasal lavage after nasal challenge with seasonal allergens (NAC-S) and nasal challenge with perennial allergens (NAC-P) in individuals from groups A (A) and B (B)

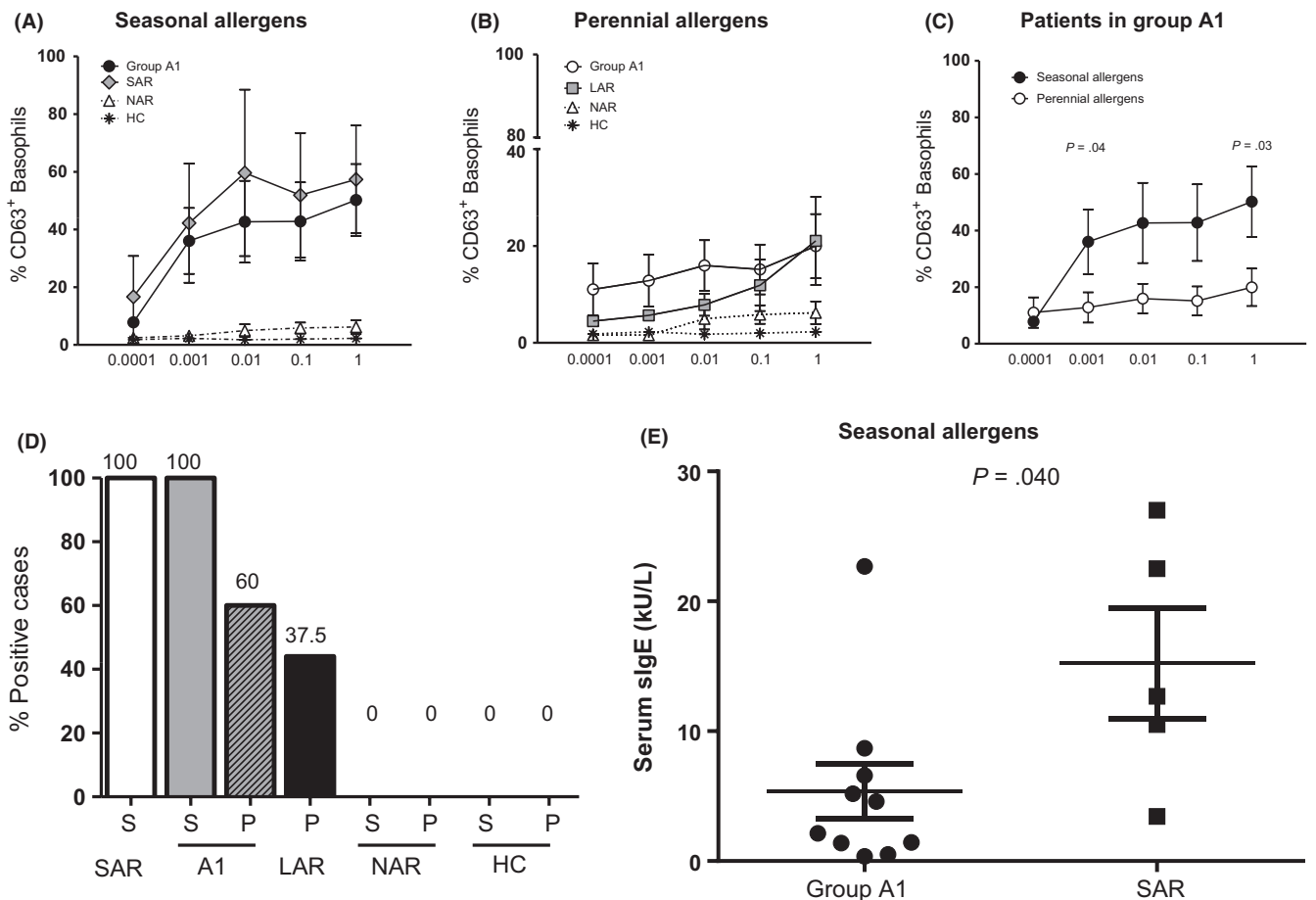
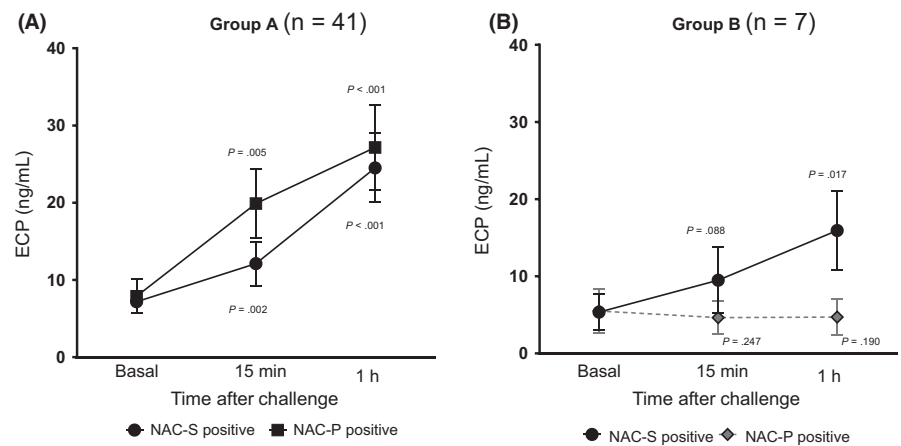


FIGURE 4 Peripheral immune characterization of patients with different rhinitis phenotypes. A: Basophil activation test (BAT) with seasonal allergens (olive or grass pollens) in group A1, seasonal allergic rhinitis (SAR), nonallergic rhinitis (NAR), and healthy nonatopic control (HC) subjects; B: BAT with perennial allergens (*Dermatophagoides pteronyssinus* or *Alternaria alternata*) in group A1, perennial local allergic rhinitis (LAR), NAR, and HC subjects. C: BAT with seasonal and perennial allergens in individuals from group A1. D: Proportion of individuals with positive BAT among different rhinitis phenotypes. A Stimulation index ≥ 2 was required for a BAT to be considered positive (see Appendix S1). S, seasonal allergen; P, perennial allergen. E, serum allergen-specific IgE in patients from group A1 and SAR subjects

allergen sensitization with and without SPT positivity can coexist in the same individual, thus identifying a new rhinitis phenotype for which we propose the name dual allergic rhinitis (DAR). We believe that the term “dual” properly reflects the coexistence of two forms of allergen sensitization within the context of an allergic phenotype

of rhinitis, in contrast to the term “mixed,” which implies the cooperation of allergic and nonallergic mechanisms to drive rhinitis in the same individual.¹

In the clinical practice is very common to observe patients with perennial symptoms who only display SPT positivity to seasonal

allergens. One potential explanation for this phenomenon is the presence of MR and/or NHR.²² Nevertheless, the objective identification of NHR is challenging, as its intensity varies along disease evolution.²⁰ On the other hand, there is a good correlation between a clinical suspicion of NHR and a positive diagnostic test (eg, cold dry air challenge).²¹ In our study, individuals from group B identified temperature changes and irritant smells as triggers of rhinitis significantly more often than patients from group A (DAR group). These observations suggest that the coexistence of AR, NAR, and NHR accounted for most rhinitis cases in group B (MR group). Moreover, MR individuals were significantly older and had had perennial symptoms more often since disease onset. Conversely, DAR patients were significantly younger and their nasal symptoms started more frequently with a seasonal pattern. These differences might be useful to identify candidate patients for a NAC, among those with discrepancies between the SPT results and the symptom pattern in clinical practice.

In this study, the administration of both perennial and seasonal allergens induced a similar increase in nasal ECP in DAR individuals, unlike MR patients who only experienced increases upon the provocation with seasonal allergens. Therefore, the diagnosis of DAR in individuals from group A was supported by the allergen-induced increases in nasal ECP, regardless of the atopic status for the corresponding allergen. This finding is consistent with previous works demonstrating that allergen-specific nasal reactivity is associated with the increase in eosinophilic inflammation in both SPT+^{5,28,29} and SPT-^{13,14,30} rhinitis patients.

Despite showing a high specificity, the measurement of sIgE in the nasal secretions has a low diagnostic sensitivity in LAR.^{13,14,26,30} On the other hand, the BAT displays a higher sensitivity for LAR diagnosis and permits the investigation of both the presence and the functionality of sIgE.¹⁷⁻¹⁹ Therefore, in this study, we investigated the involvement of sIgE in DAR pathophysiology by means of a BAT (seasonal and perennial allergens), in addition to an immunoassay in serum (seasonal allergens). All SAR and DAR patients displayed positive BAT responses with seasonal allergens. This finding is consistent with previous studies reporting an almost perfect concordance between NAC and BAT in SPT+ rhinitis individuals.³¹⁻³³ Importantly, SAR and DAR patients displayed no difference in the proportion of peripheral basophils expressing CD63 after stimulation with seasonal allergens. On the other hand, serum sIgE against seasonal allergens was significantly lower in DAR as compared to SAR individuals, and serum sIgE did not correlate with CD63 expression in basophils. This observation contrasts with previous works reporting a positive correlation between basophil sensitivity and serum sIgE in AR patients.^{31,33} Nevertheless, the former studies included only patients with perfect concordance between SPT positivity and symptom pattern, and thus, a distinct basophil sensitivity for the DAR phenotype cannot be excluded. On the other hand, 37.5% of LAR individuals (all of them with positive NAC with DP) and 60% of DAR patients had positive BAT responses with perennial allergens, in contrast to none of the NAR and HC subjects. The positivity of BAT with DP in

50%-55% of LAR subjects had been previously reported^{17,19} and, importantly, wortmannin experiments confirmed that the basophil activation in these patients is mediated by sIgE.¹⁷ In our study, there were no differences in the proportion of CD63 expression after basophil stimulation with perennial allergens between DAR and LAR patients. Interestingly, the NAC did not induce any significant change in basophil reactivity with either perennial or seasonal allergens. This finding facilitates the implementation of the BAT as a tool for LAR/DAR diagnosis in the daily practice, as it does not require a previous NAC to increase its sensitivity.

The investigation of the mechanisms explaining why DAR and LAR patients display sIgE against perennial allergens attached to the membrane of peripheral basophils is beyond the scope of this study. Nevertheless, we hypothesize that similar to AR,^{34,35} in DAR and LAR individuals, sIgE against aeroallergens is produced at the mucosal level, following sequential class switch recombination of IgG₁+ B cells.³⁶ After sensitizing nasal resident effector cells, sIgE would traffic to the bloodstream through the lymphoid vessels to sensitize circulating basophils in first place (elaborated in³⁷). The extremely high affinity of IgE for FcεRI ($K_d: 10^{-10}$)³⁸ would explain why LAR and DAR patients can display membrane-bound sIgE in peripheral basophils without having free sIgE detectable in serum.

The relatively small number of subjects included in the BAT analysis constitutes a limitation of our study. Moreover, in this work we only evaluated patients sensitized to seasonal allergens who reported perennial symptoms with seasonal exacerbation. We decided to focus on this phenotype because it is the more frequent scenario among patients with discrepancies between IgE-sensitization tests and symptoms pattern (personal observation). Moreover, we restricted the NAC-P to AA and DP because in our population those two allergens account for the vast majority of perennial cases of LAR.³⁹ Nevertheless, other discrepant scenarios (eg, positive SPT to perennial allergens only with seasonal symptoms, or with perennial symptoms and seasonal exacerbation) can also occur. Therefore, the proportion of DAR cases found in this work cannot be directly extrapolated to the global population. Moreover, the design of this study did not permit to investigate the natural evolution of DAR. Although recent data suggest that most LAR patients do not develop systemic atopy over disease evolution,⁴⁰ we cannot exclude that DAR individuals evolve to other rhinitis phenotypes over time. Nevertheless, our results indicate that DAR is a relevant differential diagnosis in AR patients with suboptimal response to allergen immunotherapy (AIT) or with discrepancies between symptoms and IgE-sensitization tests. Besides the well-established long-term beneficial effect of AIT in AR,⁴¹ recent evidence indicates that AIT is able to control LAR symptoms.^{42,43} Thus, it is tempting to speculate that AIT would be an effective treatment in DAR individuals. Moreover, similar to other rhinitis phenotypes with eosinophilia,⁴⁴ DAR patients can be expected to obtain a benefit from oral antihistamines and nasal steroids.

In summary, this work identifies DAR as a new allergic phenotype of rhinitis with clinical and immunological features intermediate between AR and LAR. Our work expands the complexity of the rhinitis syndrome and supports the existence of intermediate phenotypes

other than MR. These findings emphasize the need to count with appropriate diagnostic tools in the clinical practice, importantly the NAC. Further studies are warranted to elucidate the prevalence in the general population, the mechanisms, the natural evolution, and the therapeutic options of DAR.

CONFLICT OF INTEREST

The authors have no conflict of interest to disclose.

AUTHOR CONTRIBUTIONS

CR, CM, and MJT designed the study and coordinated the work of the rest of the authors. IEG, PC, ATM, NPS, MS, and CR recruited the study individuals. IEG, PC, and AP conducted the clinical procedures. RFS and AA performed the in vitro studies. IEG, CM, MJT, and CR wrote the manuscript, which was reviewed by the rest of the authors.

ORCID

Ibon Eguiluz-Gracia  <https://orcid.org/0000-0002-3774-931X>
 Natalia Perez-Sanchez  <https://orcid.org/0000-0001-9399-8188>
 Maria Salas  <https://orcid.org/0000-0002-0583-9492>
 Cristobalina Mayorga  <https://orcid.org/0000-0001-8852-8077>
 Maria Jose Torres  <https://orcid.org/0000-0001-5228-471X>
 Carmen Rondon  <https://orcid.org/0000-0003-0976-3402>

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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1 **Supplementary material**

2 **Title:** Coexistence of Nasal Reactivity to Allergens with and without IgE-sensitization in
3 Allergic Rhinitis patients

4 **Author list:** Ibon Eguiluz-Gracia*¹, Ruben Fernandez-Santamaria*², Almudena Testera-
5 Montes*^{1,3}, Adriana Ariza², Paloma Campo¹, Ana Prieto⁴, Natalia Perez-Sanchez¹, Maria
6 Salas¹, Cristobalina Mayorga², Maria Jose Torres^{1,3}, Carmen Rondon¹.

7 These authors contributed equally to this work.

8 **Affiliations:**

9 1. Allergy Unit, Hospital Regional Universitario de Malaga. Instituto de Investigación
10 Biomédica de Málaga-IBIMA and ARADyAL. Málaga, Spain.

11 2. Allergy Research Group, Instituto de Investigación Biomédica de Málaga-IBIMA
12 and ARADyAL. Málaga, Spain.

13 3. Universidad de Málaga-UMA. Málaga, Spain.

14 4. Pediatrics Unit, Hospital Regional Universitario de Malaga. Málaga, Spain.

15

16 **Corresponding author:**

17 Ibon Eguiluz-Gracia

18 Laboratorio de Investigación

19 Hospital Civil, pabellón 5, sótano

20 Plaza del Hospital Civil

21 29009 Malaga, Spain

22 Tel: +34 951290313; Fax: +34 951290302.

23 e-mail: iboneguiluz@gmail.com

24 Methods

25 *Study individuals:* In addition to the subjects of the discrepant group, we screened the
26 Rhinitis Database of our Department for patients ≥ 14 years with the following features:

- 27 1. Seasonal symptoms only, positive skin prick test (SPT) to grass and/or olive pollen
28 and negative SPT to all other relevant aeroallergens in our area: seasonal allergic
29 rhinitis (SAR) group.
- 30 2. Perennial symptoms without exacerbation at any month, negative SPT and serum
31 allergen-specific (sIgE) for every relevant aeroallergen in our area, positive nasal
32 allergen challenge (NAC) with *Dermatophagoides pteronyssinus* (DP), and negative
33 NAC with *Alternaria alternata* (AA) and *Parietaria judaica*: local allergic rhinitis
34 (LAR) group
- 35 3. Perennial symptoms without exacerbation at any month, negative SPT and serum
36 sIgE for every relevant aeroallergen in our area, and negative NAC with grass-mix,
37 *Olea europea*, DP, AA, and *Parietaria judaica*: non-allergic rhinitis (NAR) group.

38 Healthy non-atopic individuals were recruited from the adult companions of the patients
39 referred to our Allergy department (healthy control group). For all study groups, perennial
40 symptoms were defined as the presence of two or more nasal symptoms (nasal itching,
41 sneezing, rhinorrhea or nasal congestion) all year round, whereas seasonal
42 rhinitis/exacerbation was defined as the presence/aggravation of symptoms from March to
43 June. The exclusion criteria were: rhinitis of < 2 years of evolution, long-lasting use of nasal
44 vasoconstrictors, pregnancy or breastfeeding, systemic uncontrolled disorders (immune,
45 oncologic, endocrine or cardiovascular), previous diagnosis of chronic rhinosinusitis or
46 nasal anatomical abnormalities, and FEV1 persistently $< 80\%$. No procedure was performed

during the month following a respiratory infection, and treatment with corticosteroids, antihistamines or anti-leukotrienes was stopped 10 days before every visit.

Clinical and demographical questionnaire: after inclusion, all subjects in the discrepant group filled a questionnaire including data about family history of atopy, profession, smoking habits, structural features of the household, pet keeping and other environmental exposures, triggers of rhinitis, natural history of their nasal symptoms, frequency and severity of rhinitis symptoms, and occurrence of conjunctivitis and symptoms suggestive of asthma (wheezing, shortness of breath, cough or chest tightness) (1). Severity of rhinitis was classified as mild, moderate or severe according to the modified ARIA classification (2). Patients were asked to indicate their triggers of rhinitis among the following: exposure to vegetation (visits to urban green areas or trips to the countryside including mountains and forests around Malaga and olive tree plantations), indoor dust, rapid temperature changes, irritant smells and dampness. Patients were allowed to choose more than one option.

Skin prick test: the skin prick test (SPT) were performed with all relevant aeroallergens in our area: house dust mites (*Dermatophagoides pteronyssinus* (DP), *Dermatophagoides farinae*, *Lepidoglyphus destructor*) pollens (*Phleum pratense*, *Lolium perenne*, *Cupressus arizonica*, *Platanus acerifolia*, *Olea europaea*, *Chenopodium spp*, *Plantago lanceolata*, *Artemisia artemisifolia*, *Parietaria judaica*, *Salsola kali*), molds (*Alternaria alternata* (AA), *Aspergillus*, *Cladosporium*, and *Penicillium*) and animal epithelia (dog, cat and hamster) (ALK-Abelló, Madrid, Spain).

Lung function tests: flow-volume spirometry was performed in patients of the discrepant group with a Spirobank spirometer (Medical International Research, Rome, Italy) and according to the recommendations of the American Thoracic Society (3).

Nasal allergen challenge: patients in the discrepant and SAR groups underwent NACs with one allergen per session during the summer of 2018 and 2019. The minimum interval between consecutive NAC was one week. The allergen extracts and concentrations administered were grass-mix pollen (0.001–0.01–0.1 µg/mL), *Olea europea* (0.006–0.06–0.6 µg/mL), DP (0.04–0.4–4 µg/mL) and AA (0.0025–0.025–0.25 µg/mL) (Laboratorios Leti S.L., Spain). The NAC was performed as described elsewhere (4, 5). In brief, the subjects spent 30 minutes acclimating to the room conditions. The room was maintained at a constant temperature and free of irritant smells. Thereafter, baseline measurements of visual analogue scale (VAS) of nasal symptoms and acoustic rhinometry were carried out. If the patient scored higher than 30 points in the VAS, the procedure was stopped, and the NAC was rescheduled for a later appointment with a minimal interval of 1 week. In case the VAS was lower than 30 points, the control challenge was performed by the application of 100 µL of saline serum to each nostril. If the control challenge was positive (see NAC monitoring), the procedure was stopped and a new appointment was scheduled (minimal interval of 1 week). The patient was excluded from further studies after three consecutive baseline VAS scores higher than 30 points or after three consecutive positive control challenges. If the control challenge was negative, 100 µL of three progressively increasing concentrations of a single freshly reconstituted allergen solution were administered to each nostril with a 15-minute interval and using a micropipette. The response to the NAC was monitored at baseline, 15 minutes after the application of every concentration and 1 hour after the last allergen dose, by both VAS of nasal symptoms and acoustic rhinometry. The NAC was considered positive if an increase $\geq 30\%$ in the total VAS and a bilateral decrease $\geq 30\%$ in the total sum of vol 2-6cm of the acoustic rhinometry, as compared to reference measurement, occurred. The NAC was stopped before the last allergen dose in case of a

positive response.

Eosinophil cationic protein in nasal lavage: bilateral nasal lavage with 8 mL of isotonic saline previously brought to room temperature was performed 24 hours before the NAC (baseline sample), and 15 and 60 minutes after the administration of the allergen concentration eliciting a positive NAC response. The lavage was always collected after the corresponding monitoring measurement. The procedure was carried out as described elsewhere (6, 7). The subjects remained seated with their necks extended 30° from the vertical axis. After 10 seconds, the subjects flexed back the necks and the sample was expelled into a container. Samples were centrifuged at 1069 g and 4° C for 7 minutes and the supernatant was stored at – 20° C. Eosinophil cationic protein (ECP) was measured by immunoassay according to manufacturer's instructions (UniCAP, Phadia). The detection threshold of the immunoassays was 2 ng/mL.

Serum specific IgE: serum sIgE against the same allergens included in the SPT panel were determined by fluoroenzyme immunosorbent assay (UNICAP; Pharmacia Diagnostics, Uppsala, Sweden). The detection threshold for sIgE was 0.35 kU/mL.

Basophil activation test: as previously described, 100 µL of heparinized whole blood per test were incubated with 20 µL of stimulation buffer for 10 min at 37°C (8). Thereafter, 100 µL of the allergen extracts (grass and olive pollen, DP and AA, Laboratorios Leti S.L. Spain) were added for final concentrations of 0.001, 0.01, 0.1, 1 and 10 ng/mL. These concentrations were based on a preexisting dose-response curve (data not shown). The negative control tube was added 100 µL of washing solution, and the positive control tube 100 µL of rabbit anti-human IgE (BD Pharmingen San Diego, CA, USA). All samples were incubated for 30 min at 37°C in a water bath in agitation. The samples were put on ice for 5

min to stop the degranulation and subsequently stained for 20 minutes with 3 μ L of mouse anti-human CD63-FITC, CD203c-PE, and CD193-APC (Caltag Laboratories, Burlingame, CA, USA). After staining, 2 mL of prewarmed lysis solution was added to each tube before centrifugation for 5 min at 4°C. Thereupon the cells were analysed by flow cytometry (FACSCalibur, Becton-DickinsonBioscience, San Jose, CA, USA) and 500–1000 basophils were acquired per sample. The results were expressed as stimulation index (SI), which was calculated as the ratio between the proportion of CD63 expression in the allergen-stimulated basophils and in the non-stimulated basophils (negative control tube). The test was considered positive if a $SI \geq 2$ was found for one or more of the allergen concentrations provided a proportion of activated basophils $\geq 5\%$ (9).

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144 **References**

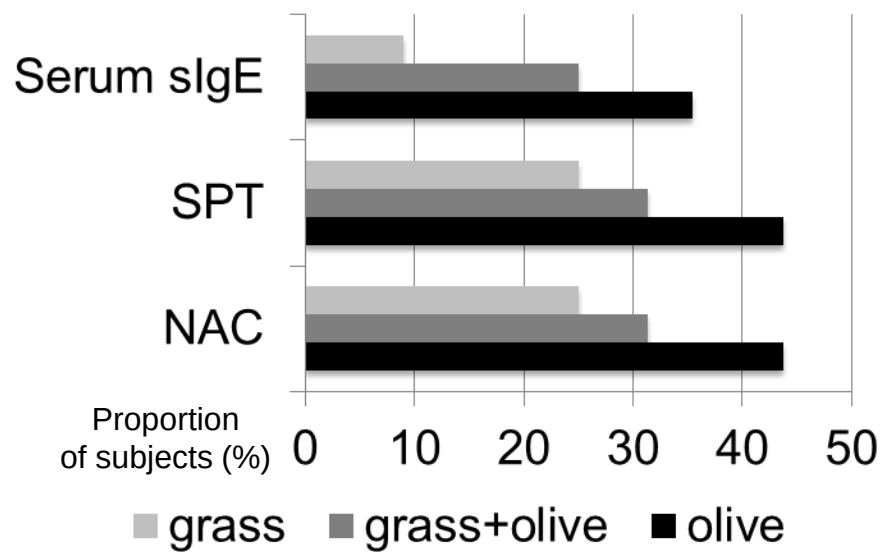
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Figure 1

A) Seasonal allergens



B) Perennial allergens

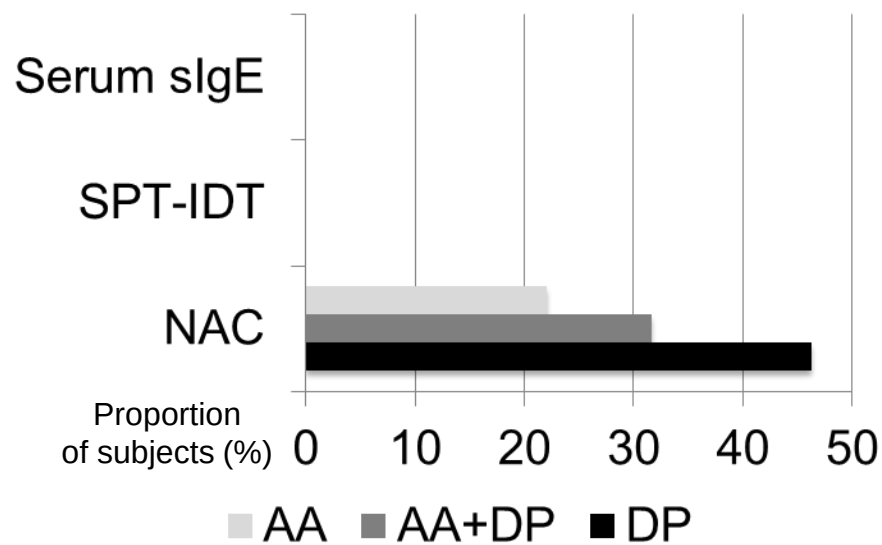


Figure 2

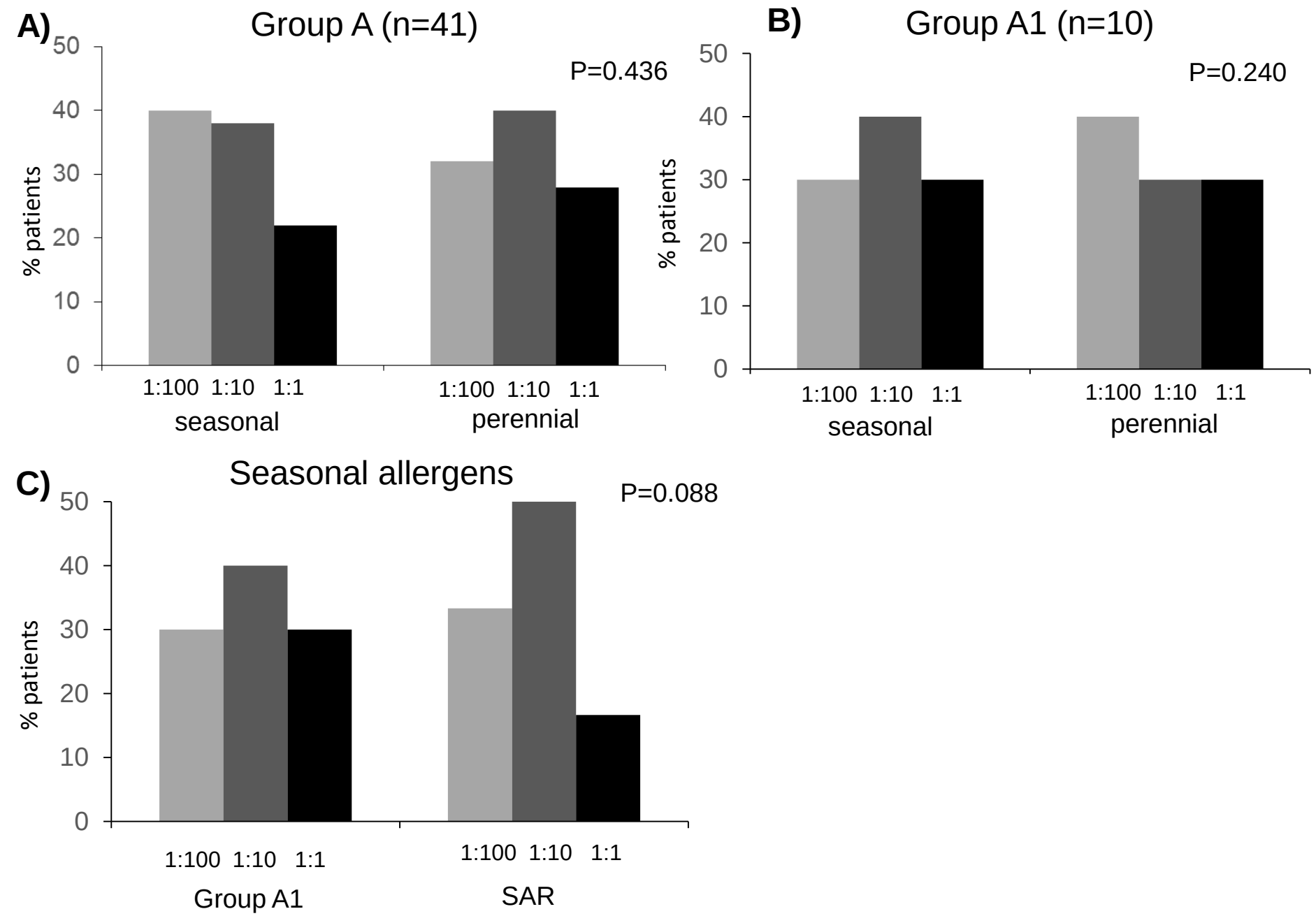


Figure 3

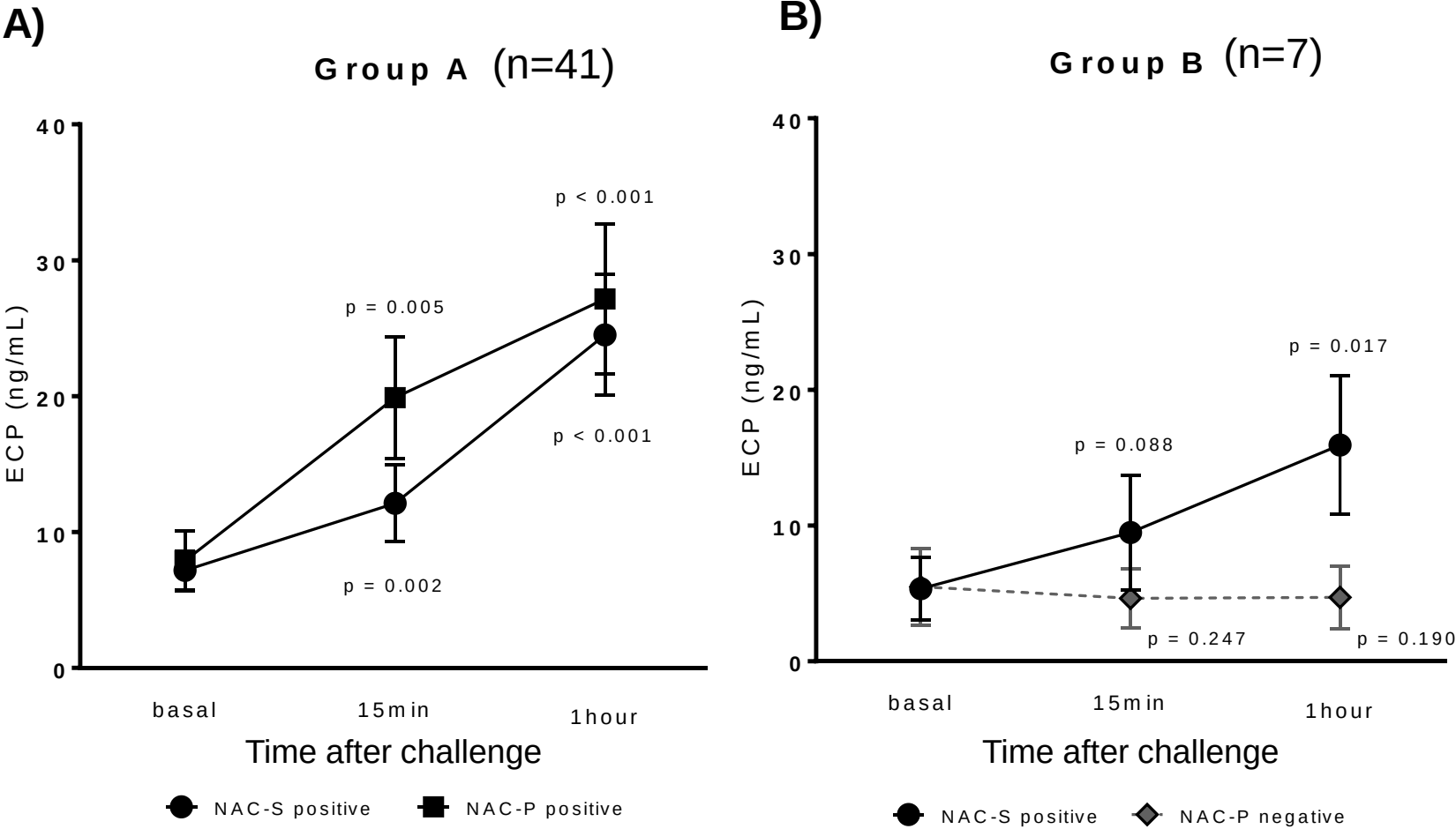
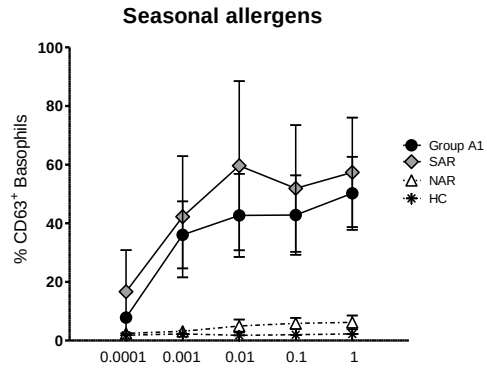
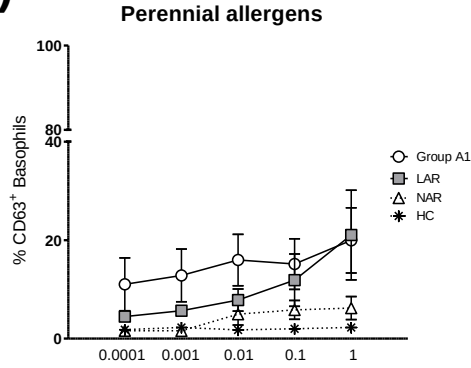


Figure 4

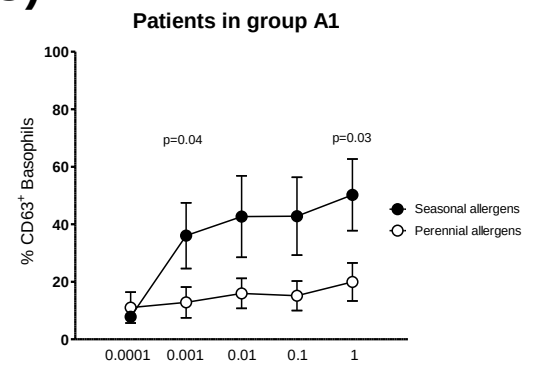
A)



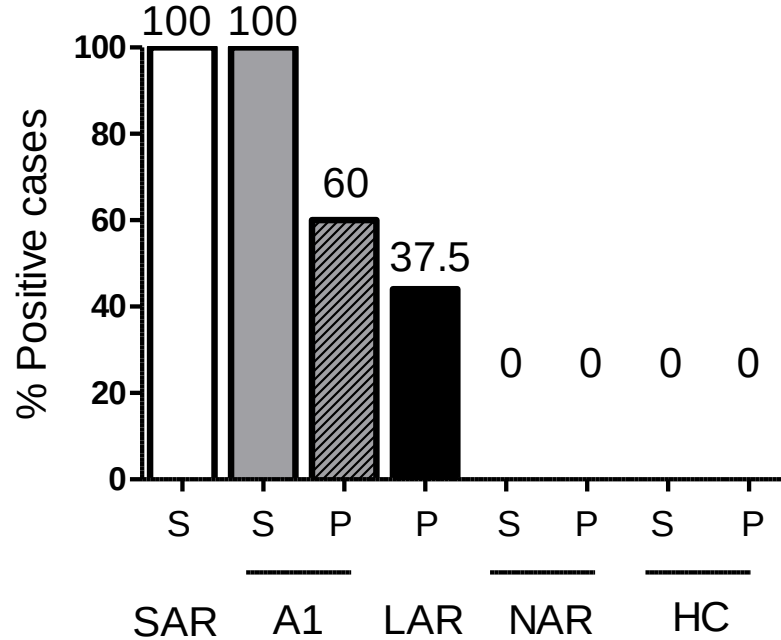
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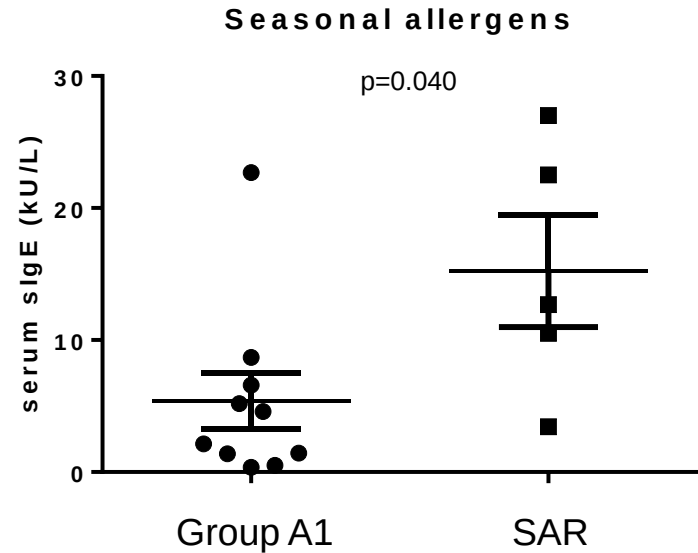
C)



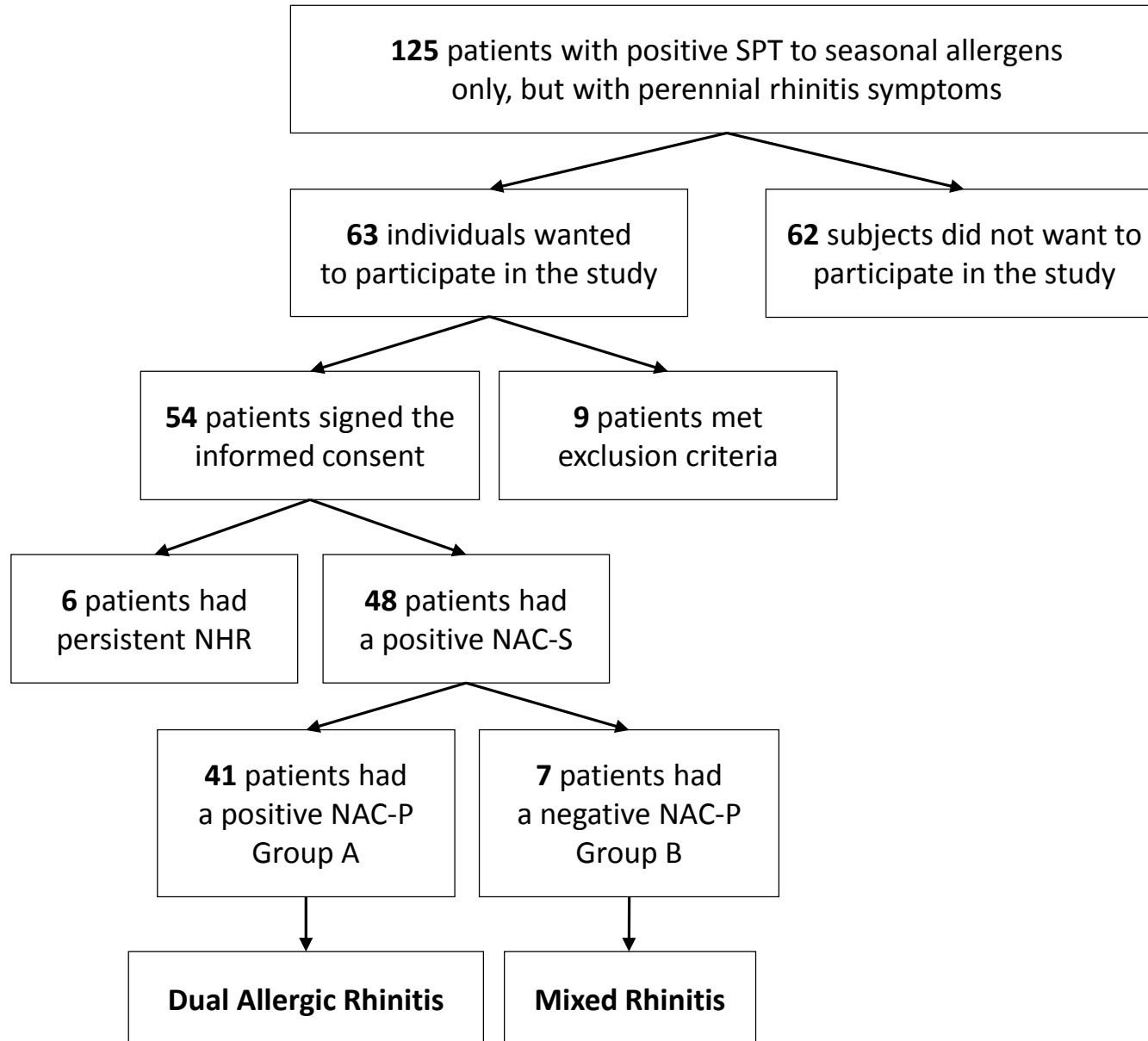
D)



E)



Supplementary figure 1



Supplementary figure 2

