

Immunologic responses to the major allergen of *Olea europaea* in local and systemic allergic rhinitis subjects

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Summary

Objective To evaluate the *in vivo* and *in vitro* responses to nOle e 1 in allergic rhinitis (AR) and local allergic rhinitis (LAR) patients sensitized to olive tree pollen (OL) confirmed by nasal allergen provocation test (NAPT).

Methods Twelve subjects with AR, 12 with LAR and 12 subjects as control group (CG) were selected. Skin testing and NAPT with nOle e 1 were performed. Eosinophilic cationic protein (ECP) and tryptase were measured in nasal lavages before and after NAPT. Serum IgE to OL allergens was measured by ELISA. Basophil activation tests (BAT) with OL and nOle e 1 and dendritic cell maturation/proliferation studies were carried out.

Results All AR (12/12) and 10/12 (83%) of LAR had a +NAPT to nOle e 1. ECP levels in nasal lavages were significantly increased after NAPT in both AR and LAR compared with CG at 15 min ($P < 0.05$). Serum IgE was positive only in AR. All AR had +BAT responses to OL and 10/12 to nOle e 1 (83%); 8/12 LAR (66.6%) had a +BAT to OL and 4/12 (33%) to nOle e 1, with only one subject of the CG with a +BAT to both OL and nOle e 1 (8%). Dendritic cell proliferation to nOle e 1 was increased in AR compared to LAR and CG ($P = 0.019$ and $P = 0.001$, respectively).

Conclusion Both AR and LAR had a similar *in vivo* response to nOle e 1 with release of inflammatory mediators. Specific basophil activation with OL and nOle e 1 was observed in LAR confirming previous data obtained with dust mites.

Keywords basophil activation test, local allergic rhinitis, nasal allergen provocation test, natural Ole e 1, olive tree pollen, specific IgE

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Introduction

Allergic rhinitis (AR) is a prevalent disease that affects 20–40% of population, including both children and adults [1]. Allergic rhinitis constitutes a burden from the health and economic point of view, with important associated comorbidities such as asthma. Local allergic rhinitis (LAR) is a phenotype of AR characterized by the presence of a positive response to nasal allergen provocation test (NAPT) and local production of specific IgE (sIgE) antibodies [2, 3]. In LAR subjects, specific nasal responses to allergens have been demonstrated with house dust mites [4], alternaria [5] and pollens such as grass and olive [6]. Inflammatory mediators' release and a cellular Th2 pattern in nasal lavages have

also been detected after the challenge with all these allergens [7, 8].

Olive tree (*Olea europaea*) pollen allergy represents one of the most important causes of respiratory allergy in Mediterranean regions and some areas of North America [9–11]. Olive tree is closely related to other plants from the Oleaceae family such as the ash tree (*Fraxinus excelsior*), which is widely distributed in the temperate zones of Europe and America, and also with jasmine (*Jasminum*), lilac (*Syringa*), privet (*Ligustrum*) and forsythia (*Forsythia*) [12]. Ole e 1, the major olive allergen, has an IgE-binding frequency of almost 80% among patients with olive pollinosis [13–15]. The structure and characteristics of Ole e 1 have been thoroughly studied and constitute an interesting model of purified allergen [15–17]. Ole e 1 exhibits 88% sequence identity to Fra e 1 from ash pollen, 89%

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identity to Syr v 1 from lilac and 87% identity to Lig v 1 from privet [12].

A recent study has demonstrated that LAR is a disease that shows many similarities with AR in terms of clinical presentation, association with other comorbidities and inflammatory patterns [18]. However, LAR seems to constitute a unique entity that does not evolve to AR [19]. In LAR subjects, NAPT have been performed in all cases with whole commercial extracts, which contain a known amount of major allergens reported by the company. There are some studies evaluating nasal responses after challenge with purified allergens performed in classic AR patients [20, 21], but up to date, there are not equivalent studies performed in LAR subjects. Also, each allergen may produce a different response in the nasal mucosa according to their properties [22]. Whether the response in the target organ is the same in AR and LAR subjects still remains unknown.

Moreover, a recent study by Gómez et al. [23] demonstrated a positive basophil response in 50% of subjects with LAR to *D. pteronyssinus* and that the basophil stimulation was IgE specific as demonstrated by wortmannin pretreatment of the assay. As these studies had been performed only with dust mites, it seemed interesting to confirm these results with other allergens such as pollens.

The aim of this study was to thoroughly evaluate the *in vivo* and *in vitro* responses to natural Ole e 1 purified from olive pollen (nOle e 1) as a model of purified allergen from a relevant pollen with high exposure in many areas around the world. The study was performed in a well-phenotyped group of AR and LAR patients with known sensitization to OL demonstrated by NAPT. The AR group represents the classic model of rhinitis, and the LAR group represents a novel phenotype whose responses to purified allergens have not been investigated yet. The *in vivo* responses to nOle e 1 were evaluated by means of skin testing and NAPT, with measurement of inflammatory mediators before and after challenge. The *in vitro* responses were evaluated by measuring sIgE to nOle e 1 and other OL allergens in serum, basophil activation test (BAT) with both whole extract and nOle e 1, along with dendritic cell maturation and T cell proliferation studies in response to nOle e 1 in all subjects.

Methods

Study subjects

The study included subjects who were classified in three groups: subjects with AR, subjects with LAR and healthy subjects as control group (CG). Subjects were recruited in the Allergy Department at the Regional

Hospital of Málaga. The study was performed outside the pollen season when patients were symptom free and no treatment was required. A general scheme of patient's selection and study performance is shown in Fig. 1.

AR inclusion criteria. History of seasonal rhinitis symptoms due to exposure to olive tree pollen (OL) ≥ 2 years, positive NAPT with olive tree pollen (NAPT-OL), positive skin prick test (SPT) and serum-specific IgE (sIgE) to OL.

LAR inclusion criteria. History of seasonal rhinitis symptoms due to exposure to olive tree pollen (OL) ≥ 2 years, positive NAPT-OL, negative skin testing to OL (intradermal (ID)/SPT), and negative sIgE to OL and a battery of common aeroallergens.

CG inclusion criteria. The control subjects had no allergic or nasal diseases and negative NAPT-OL. They also had negative SPT/ID and serum sIgE to aeroallergens.

Exclusion criteria. Subjects with chronic rhinosinusitis evaluated by CT scan; massive polyposis, septal perforation, total nasal blockage, upper respiratory infection or any other condition that does not allow to perform the nasal challenge; pregnant or breastfeeding patients; patients who had autoimmune and/or any other severe disease that contraindicated NAPT; and subjects with psychosomatic disorders or unable to follow the instructions.

The study was conducted according to the principles of the Declaration of Helsinki, and the whole study protocol was approved by the Ethic Committee of Málaga. All participants and parents of patients below 16 years of age were informed and signed the corresponding informed consent. Blood sample was processed following standard procedures and, where appropriate, frozen immediately after their reception by the Málaga Hospital-IBIMA Biobank, Andalusian Public Health System Biobank.

Purification and characterization of nOle e 1

nOle e 1 allergen was purified from ethyl-ether-defatted olive tree (*Olea europaea*) pollen (Allergon AB) extracted in 50 mM ammonium bicarbonate pH 8.0, containing 1 mM PMSF (1 g pollen/30 mL) for 1 h as described [14]. After centrifugation at 20 000 g for 30 min at 4 °C, the supernatant was collected. The procedure was repeated three times, and supernatants were pooled together and stored at -20 °C. Three chromatographic steps, two gel permeation and a reverse-phase HPLC, were necessary for the purification of nOle e 1. First, olive pollen extract was loaded into a Sephadex

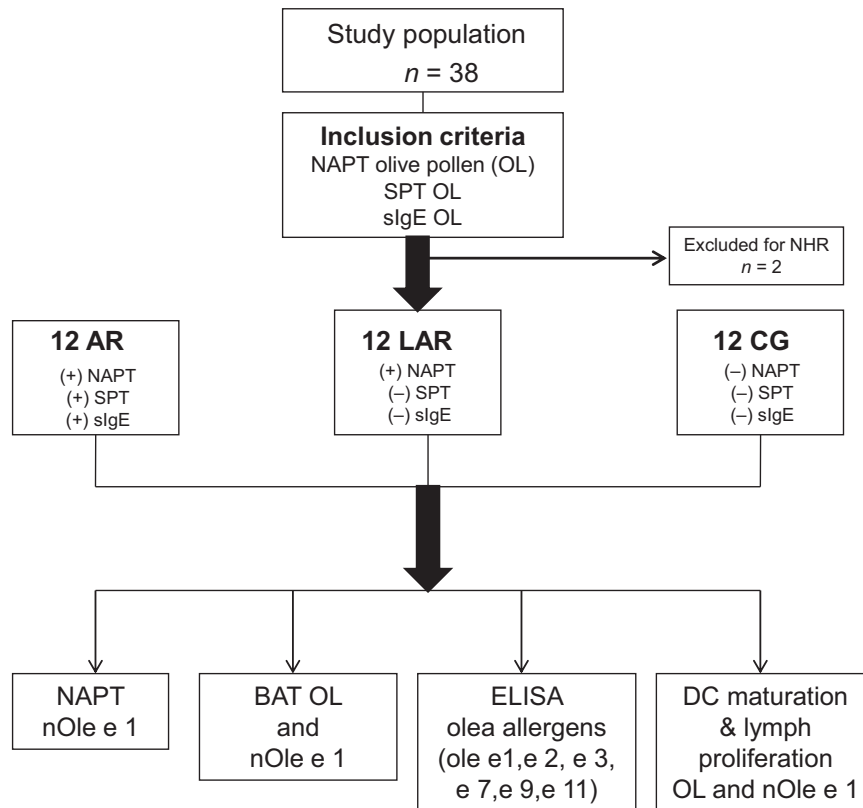


Fig. 1. Patients' selection and study flow chart. NHR, nasal hyperresponsiveness; OL, olive tree pollen; NAPT, nasal allergen provocation test; SPT, skin prick test; AR, allergic rhinitis; LAR, local allergic rhinitis; CG, control group.

G-75 medium column and afterwards in a Sephadex G-75 superfine, both in 0.2 M ammonium bicarbonate, pH 8.0. Fractions containing nOle e 1 were finally loaded onto a Nucleosil C₁₈ column with an acetonitrile gradient (0–60%) in 0.1% trifluoroacetic acid. The elution profile was continuously monitored at 214 and 280 nm.

Skin testing

Skin prick tests were performed with a wide panel of prevalent aeroallergenic sources in the area: *D. pteronyssinus*, *D. farinae*, *Phleum pratense*, *Lolium perenne*, *Cupressus arizonica*, *Platanus acerifolia*, *Olea europaea*, *Chenopodium album*, *Artemisia vulgaris*, *Parietaria judaica*, *Salsola kali*, *Alternaria alternata*, *Aspergillus fumigatus*, *Cladosporium herbarum*, *Penicillium*, and dog and cat epithelia (ALK-Abelló, Madrid, Spain) following current guidelines [24]. Also, SPTs with nOle e 1 at 0.5, 1, 5 and 10 µg/mL were performed in all subjects. ID skin test was performed with freshly reconstituted freeze-dried OL extract (0.6 µg/mL) (ALK-Abelló) in all LAR and CG subjects, but was not performed with nOle e 1 because there is not a standardized technique for purified allergens [24].

Nasal provocation test with whole olive extract and nOle e 1

Nasal allergen provocation test with OL extract and nOle e 1 was performed in all patients according to published methods [6, 7]. For the challenge with OL (inclusion criteria), two puffs (100 µL) of freshly reconstituted extract (6 µg/mL Ole e 1, ALK-Abelló, Denmark) were applied in both nostrils using a metered pump. A nasal challenge with 0.9% saline was performed prior to the application of the allergen to rule out nasal hyperresponsiveness. The nasal response to allergen was monitored by symptoms' score and acoustic rhinometry as described [25]. Briefly, nasal symptoms (rhinorrhea, itching, nasal obstruction and sneezing) were scored placing a vertical mark on a horizontal visual analogue scale (VAS) of 100 mm at baseline and 15, 60 and 120 min after the challenge. The volume of the nasal cavity that corresponds to the lower turbinate (vol 2–6 cm) in each nostril was measured after challenge at different time-points (baseline, 15, 60 and 120 min) by acoustic rhinometry (SRE 2000 Rhinometer; Rhinometrics, Lyngø, Denmark). A nasal lavage was performed at baseline, 15 and 60 min following the Naclerio method [26]. A positive NAPT was considered to be an increase of ≥ 30% in the total nasal symptoms and a decrease of

$\geq 30\%$ in the total vol 2–6 cm of both nasal cavities compared with the baseline test.

At least two weeks later, the challenge with nOle e 1 was performed, applying 100 μL per nostril of nOle e 1 at serial dilutions of 0.5, 1, 5 and 10 $\mu\text{g}/\text{mL}$ using a micropipette because the amount of available allergen was much less compared to the commercial extract. All the challenges were performed with the same pipette and the same nurse. After 15 min of each allergen dose, an evaluation of the response was performed as explained, and if negative response, the next dilution was applied.

ECP and tryptase measurement in nasal lavages

Measurement of tryptase and eosinophilic cationic protein (ECP) was made by UNICAP method (Thermo Fisher Scientific, Uppsala, Sweden). All *in vitro* analyses were performed in a blinded fashion.

Total and specific IgE measurement in serum

Serum total and sIgE to the same aeroallergens of the SPT panel including OL were measured by fluoroenzyme immunosorbent assay (UNICAP; Thermofisher). Also, specific IgE antibodies against nOle e 1, rOle e 2, rOle e 3, nOle e 7, rOle e 9 and rOle e 11 were measured by ELISA in all participants. ELISA was performed in microtitre plates coated with 100 $\mu\text{g}/\text{well}$ of each purified allergen. Plates were incubated with sera (diluted 1 : 10). The binding of human IgE was detected by mouse anti-human IgE antibodies (diluted 1 : 5000) donated by ALK-Abelló (Madrid, Spain) followed by horseradish peroxidase-labelled goat anti-mouse IgG (diluted 1 : 5000; Pierce Chemical Co, Rockford, IL, USA). The reaction was developed with o-phenylenediamine and the optical density (OD) measured at 492 nm. Each value was calculated as mean of two determinations [16].

Basophil activation test with whole olive extract and nOle e 1

A hundred microlitres of heparinized whole blood per test was aliquoted, and 20 μL of stimulation buffer was added and incubated for 10 min with agitation at 37 °C in a water bath as described [23]. After this, 100 μL of washing solution was added to the negative control tube, and 100 μL of anti-human IgE antibodies (BD Pharmingen San Diego, CA, USA) to the positive control tubes and 100 μL of the allergen (OL extract, ALK-Abello, Denmark and purified nOle e 1) were added at final concentrations of 5, 1, 0.5 and 0.1 $\mu\text{g}/\text{mL}$ based on a dose–response curve (data not shown). The samples were incubated for 30 min at 37 °C in a water bath in

agitation. The degranulation was stopped by incubating the samples on ice for 5 min, and then, 3 μL of monoclonal antibodies, anti-CD63-FITC, CD203c-PE, and CD193-APC (Caltag Laboratories, Burlingame, CA, USA), was added to each tube. After 20 min at 4 °C in dark, 2 mL of prewarmed lysis solution was added and centrifuged 5 min at 4 °C. Cells were washed and analysed in a FACSCalibur flow cytometer (Becton-Dickinson Bioscience, San Jose, CA, USA) by acquiring at least 500–1000 basophils per sample. Results were expressed as stimulation index (SI), calculated as the ratio between the percentage of CD63⁺ in the stimulated basophils and in the non-stimulated basophils (negative control). Basophil activation test was considered positive when $\text{SI} \geq 2$ in at least one of the allergen concentrations. When the percentage of spontaneously activated basophils was lower than 2.5%, an additional condition was required, that is that the percentage of basophils activated after contact with the antigen should be ≥ 5 .

Generation of monocyte-derived DC

From each subject, 40 mL of peripheral blood was obtained. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Paque density gradient (GE Healthcare UK Ltd, Buckinghamshire, UK). Monocytes were purified from PBMC by positive selection using CD14 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) following the manufacturer's protocol. A purity of 90–95% was assessed by flow cytometry. Immature dendritic cells (imDC) were derived from monocytes by culturing the CD14⁺ fraction in complete medium R10 [RPMI 1640, 10% foetal bovine serum (BioWhittaker, Pittsburgh, PA, USA), 2 mM L-glutamine (BioWhittaker), and 5 mg/mL gentamicin (Normon, Madrid, Spain)] with 200 ng/mL rhGM-CSF and 100 ng/mL rhIL-4 (both from R&D Systems Inc, Minneapolis, MN, USA), for 5–6 days at 5% CO₂ and 37 °C. The CD14[−] fraction was frozen in a culture medium containing 10% dimethyl sulphoxide (DMSO) (Sigma, St Louis, MO, USA) for further experiments of lymphocyte transformation test (LTT) [27].

DC maturation

imDC were incubated in complete medium at 5×10^5 cells/mL in 48-well plates (Nunc AS, Roskilde, Denmark) with Ole e 1 at 10, 1 and 0.1 $\mu\text{g}/\text{mL}$, including LPS at 1 mg/mL (Sigma) and TNF- α (R&D Systems) at 10 ng/mL as positive controls. After 72 h of stimulation at 37 °C in 5% CO₂, treated or untreated imDC were harvested and the maturation state was assessed by up-regulation of CD80, CD86 and CD83 costimulatory molecules (all three from Immunotech, Marseilles, France) and HLA-DR (BD Pharmingen, San Diego, CA,

USA), in a FACSCanto II Cytometer (BD Biosciences, Milpitas, CA, USA). Data were processed with FACSDiva (BD Biosciences). Results were expressed both as a percentage of positive cells compare to non-stimulated cells and as maturation index (MI), calculated as the ratio between stimulated DC and non-stimulated cells, considered positive when > 2 .

Lymphocyte transformation tests (LTT) by CFSE dilution proliferation assay

The LTT were performed using imDC as antigen presentation cells, the CD14⁺ fraction [that included autologous lymphocytes (lymphs)] and nOle e 1 at different concentrations (10, 1 and 0.1 µg/mL). Autologous lymphs at $0.5-1 \times 10^7$ /mL were labelled with CFSE (5 (6)-carboxyfluorescein diacetate N-succinimidyl ester, Molecular probes) following the manufacturer's instructions. One hundred microlitres of CFSE-labelled lymphs at 1.5×10^6 cells/mL was cultured with imDC at 1.5×10^5 cells/mL (ratio 10 : 1) at a final volume of 250 µL of complete medium, in 96-well plates in triplicate, with or without allergen, for 7 days at 37 °C and 5% CO₂. Tetanus toxoid (TT) at 5 µg/mL (Calbiochem, San Diego, CA, USA) and phytohaemagglutinin (PHA) at 10 µg/mL (Sigma) were used as positive proliferative controls. The proliferation of different lymphs subsets, either T cells, NK cells, B cells or T regulatory cells, was assessed by flow cytometry, analysing the percentage of CD3⁺, CD56⁺, CD19⁺ or CD4⁺CD25^{high}CD127⁺ cells that expressed CFSE^{low}, respectively. The results were considered positive when the proliferation index (PI), calculated for each subset as the ratio [%CFSE^{low} stimulated-(Lymphs + DC)]-%CFSE^{low} unstimulated-(Lymphs + DC)] / %CFSE^{low} (Lymphs), was > 3 .

Statistical analysis

Data were expressed as median and range. The clinical and demographic data were compared between groups by chi-square analysis and the Mann-Whitney *U*-test. Friedman's test was used to examine the overall differences. If significant differences occurred, the Wilcoxon's signed-rank test was used to identify them within groups. For cellular studies, comparisons of quantitative variables were carried out by nonparametric Kruskal-Wallis test and Mann-Whitney *U*-test. All *P*-values of < 0.05 were considered statistically significant.

Results

Clinical characteristics of participants

The study included a total of 36 subjects divided in three groups (two patients were excluded for nasal

hyperresponsiveness, Fig. 1). In all groups, subjects were mostly non-smoker young women [AR mean age: 33.1 year (15–64), LAR: 38.2 year (27–52), CG: 37.6 year (27–58)], although there were more active smokers in both LAR (17%) and CG (17%) ($P < 0.05$) compared to AR (0%). The mean time of rhinitis symptoms was 8.5 years (range 2–30) for AR subjects and 9.7 years (3–20) for LAR. Sixty-six percent of AR subject had also asthma symptoms compared to 58% of LAR patients.

In vivo responses to OL and nOle e 1

Skin testing. Skin prick tests with nOle e 1 at 0.5, 1, 5 and 10 µg/mL were performed in all subjects. All AR subjects recognized nOle e 1 in SPT and none of the LAR or CG subjects.

NAPT responses to nOle e 1 and mediators' release in nasal lavages. All AR and LAR subjects had a positive NAPT to whole OL commercial extract (containing 6 µg/mL of Ole e 1) as an inclusion criterion. Also, all AR subjects (12/12) had a positive response to nOle e 1, and 10/12 (83%) of LAR subjects had a positive NAPT. Healthy controls did not react to nOle e 1 at the highest concentration. As shown in Table 1, 58.3% of AR vs. 41.6% of LAR patients reacted to 0.5 µg/mL, whereas 58.3% of LAR required 1 µg/mL or higher nOle e 1 concentration to obtain a positive NAPT response compared to 41.6% of AR subjects. Chi-square analysis showed no differences in the nOle e 1 concentration to obtain a positive NAPT between AR and LAR patients.

The median vol 2–6 cm decreased significantly from 100% (value obtained after saline lavage) to 50% at 15 min in AR subjects and to 55% in LAR after NAPT with nOle e 1 (Fig. 2a). The maximum reduction was observed at 15 min in both groups. Patients from AR and LAR groups also showed significant increases in total VAS scores compared to controls (Fig. 2b).

Eosinophilic cationic protein levels (Fig. 3a) in nasal lavages were significantly increased at baseline, 15 and 60 min after NAPT in AR subjects compared with CG ($P < 0.05$). Comparisons for related samples showed a significant increase of ECP at 15 min after challenge in LAR subjects ($P < 0.05$). Trypsin levels in nasal lavages

Table 1. Number and percentage of positive nasal allergen provocation test (NAPT) responses based on the dose of nOle e 1 required in allergic rhinitis (AR) and local allergic rhinitis (LAR) patients

	nOle e 1 dose (µg/mL) required to obtain + NAPT			
	0.1	1	5	10
AR [n, (%)]	7 (58.3)	3 (25)	1 (8.3)	1 (8.3)
LAR [n, (%)]	5 (41.6)	3 (25)	1 (8.3)	3 (25)

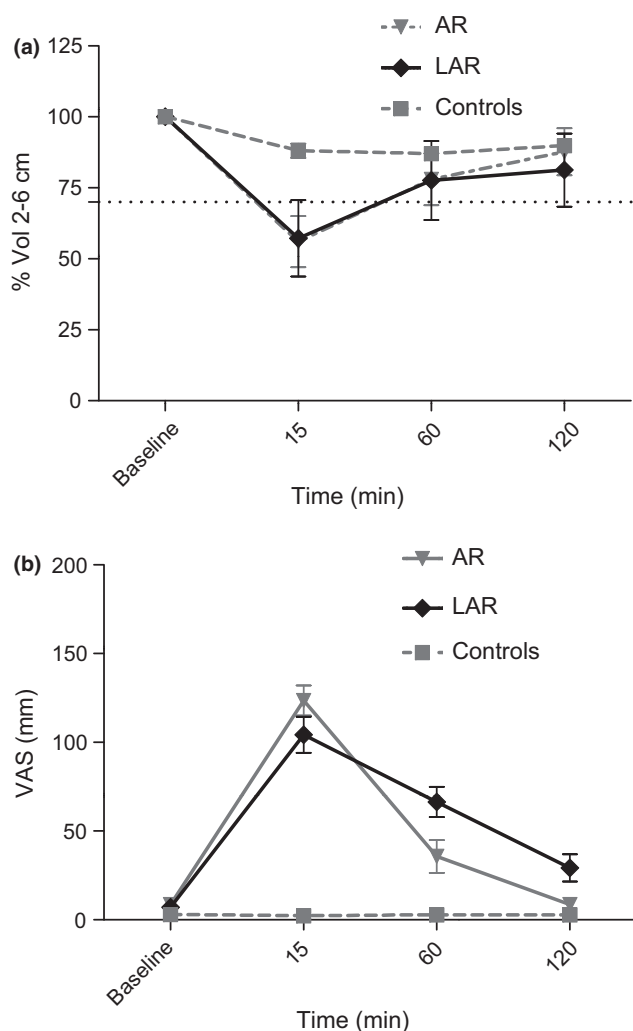


Fig. 2. (a) Nasal allergen provocation test (NAPT) with nOle e 1 in allergic rhinitis (AR), local allergic rhinitis (LAR) and control subjects (CG). Figure shows decrease of vol 2–6 cm values at different time-points. The solid horizontal bar represents the cut-off point for a positive NAPT response (decrease of 30% of vol 2–6 cm). (b) Mean visual analogue scale (VAS) values after nOle e 1 NAPT in allergic rhinitis (AR), local allergic rhinitis (LAR) and control subjects (CG). Values are represented at different time-points after NAPT.

were increased at 15 and 60 min after NAPT in AR patients ($P = \text{n.s.}$, Fig. 3b), and no increase was observed in LAR or CG subjects.

In vitro responses to nOle e 1

Serum sIgE to nOle e 1 and other OL allergens. In AR subjects, total IgE was significantly higher compared to those determined in LAR and CG ($P < 0.05$). Specific IgE to OL was also significantly higher in AR group as expected ($P < 0.05$) (Table 2). Serum-specific IgE antibodies against nOle e 1, rOle e 2, rOle e 3, nOle e 7, rOle e 9 and rOle e 11 were also measured by ELISA in

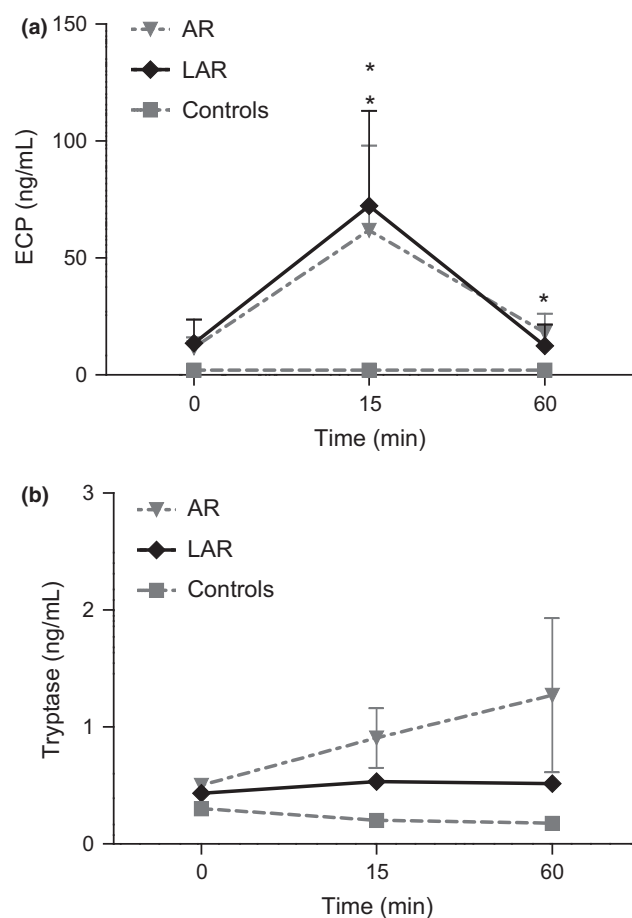


Fig. 3. (a) Levels of eosinophilic cationic protein in nasal lavages at 0, 15 and 60 min after nOle e 1 NAPT in AR, LAR and CG. * $P < 0.05$. (b) Levels of tryptase in nasal lavages at 0, 15 and 60 min after nOle e 1 NAPT in AR, LAR and CG.

all subjects. Both LAR and CG subjects had undetectable serum levels of sIgE to all allergens tested. AR subjects showed positive values to allergens, being nOle e 1 and rOle e 2 the most commonly detected (60%), followed by nOle e 7, rOle e 9 and rOle e 11 (20%) and rOle e 3 (10%).

Basophil activation test (BAT) with OL and nOle e 1. Basophil activation test was performed with OL whole commercial extract and nOle e 1 in all subjects at final concentrations of 0.1, 0.5, 1 and 5 $\mu\text{g/mL}$. In these groups, all patients were responders to BAT and showed a positive response to the anti-human IgE. Fig. 4a–c represents the SI of all subjects after basophil stimulation with both OL and nOle e 1. All AR subjects had positive responses to OL stimulation, and 10/12 AR subjects (83.3%) had a positive response to nOle e 1. In the LAR group, 8/12 subjects (66.6%) had a positive BAT with OL and 4/12 with nOle e 1 (33%). In the CG, only one subject (8.3%) had a positive BAT with both

Table 2. Serum total IgE and specific IgE determination to olive tree pollen (OL) and purified OL allergens nOle e 1, rOle e 2, rOle e 3, nOle e 7, rOle e 9 and rOle e 11 IU/mL: international units per millilitre kU/L: kilounits per litre

	Total IgE OL (IU/mL) Median (range)	sIgE OL (kU/L) Median (range)	Serum sIgE nOle e 1 (% positive)	Serum sIgE rOle e 2 (% positive)	Serum sIgE rOle e 3 (% positive)	Serum sIgE nOle e 7 (% positive)	Serum sIgE rOle e 9 (% positive)	Serum sIgE rOle e 11 (% positive)
AR <i>n</i> = 12	322.89 (12–909)*	12.9 (0.7–53.7)*	60%*	60%*	10%*	20%*	20%*	20%*
LAR <i>n</i> = 12	26.17 (9–68)	< 0.35	0%	0%	0%	0%	0%	0%
CG <i>n</i> = 12	13 (8–18)	< 0.35	0%	0%	0%	0%	0%	0%

**P* < 0.05.

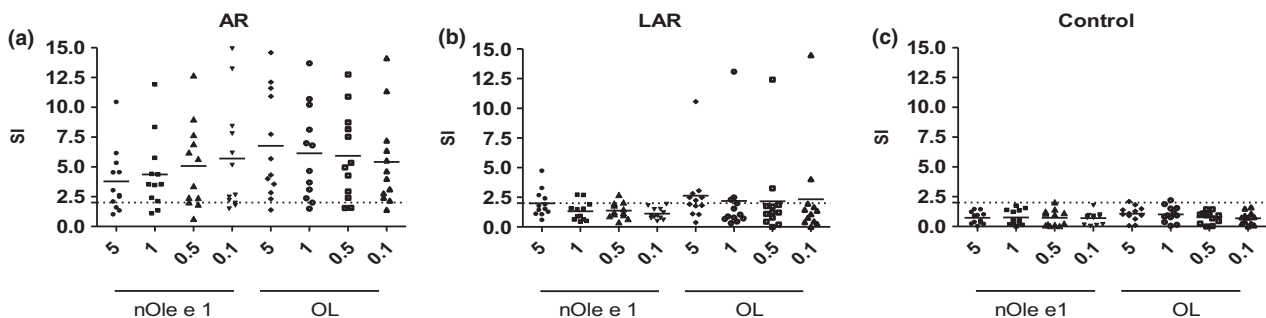


Fig. 4. Basophil activation test results after adding OL and nOle e 1 at different concentrations. The horizontal line indicates the cut-off point of the assay. (a) Allergic rhinitis (AR) subjects (b) Local allergic rhinitis (LAR) subjects (c) control subjects (CG) SI: stimulation index; OL: olive tree pollen extract; IgE C+: positive IgE control.

OL and nOle e 1 stimulation. Specificity of the test was 91.7% for OL and nOle e 1 in both groups.

Dendritic cell maturation and T cell proliferation studies. Changes in DC maturation after stimulation with nOle e 1 were assessed (Fig. 5). The percentage of cases with positive DC maturation was 66.6% for AR, 57.4% for LAR and 40% for CG. There were no significant differences in the percentage of positive maturation among the groups for any of the markers evaluated. The analysis of the proliferative response of T lymphocytes after stimulation with nOle e 1 was also assessed by flow cytometry. The percentage of cases with positive proliferative responses was again higher in AR (90%) than in LAR (37.5%) and CG (11.1%). Chi-square analysis demonstrated that AR showed a significant increase in the percentage of proliferation compared to LAR and CG (*P* = 0.019 and *P* = 0.001, respectively). Although results were higher in LAR compared to CG, the differences were not significant.

Discussion

Olive tree pollen (OL) is one of the most important causes of allergy in the Mediterranean countries [11, 13, 28].

Several allergens have been characterized, many of which show sequence similarity to proteins from different vegetable tissues [9, 12, 28]. Ole e 1 is the most

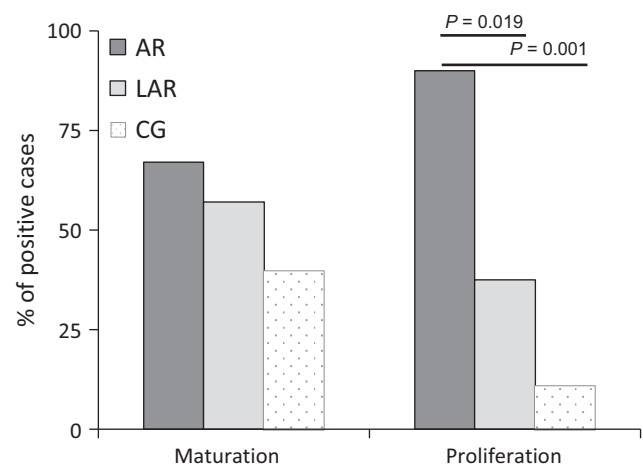


Fig. 5. Percentage of positive responses in terms of maturation and proliferation of dendritic cells after OL and nOle e 1 incubation in allergic rhinitis (AR), local allergic rhinitis (LAR) and control subjects (CG).

abundant protein of olive pollen extracts, being up to 20% of the total protein content. It is a polymorphic protein of 145 amino acids with a glycosylated (80–85% of the total allergen) and a non-glycosylated forms [14]. Its structure has been thoroughly investigated and for that reason constitutes a very interesting model of purified allergen [29, 30].

Several studies have been conducted to test its reactivity *in vivo* and *in vitro*, (measurement of sIgE [31],

SPT [32], genetic association studies [9–11] using natural and recombinant forms of Ole e 1. However, to the best of our knowledge, there are no studies assessing the response after nasal challenge with nOle e 1 in AR patients, because the reports published till now were performed with whole olive extract mostly for diagnostic purposes [33–36]. Moreover, the cellular responses (basophil and DCs) after nOle e 1 stimulation have not been studied in depth. A recent study evaluated the Th1, Th2 and T regulatory responses in nasal biopsies of subjects with AR due to OL sensitization [37], using an extract containing a mix of OL allergens, and there is only one study performing histamine release in 5 patients after incubation with Ole e 1 [38].

Our aim was to investigate the *in vivo* and *in vitro* responses to nOle e 1 as a model of purified allergen in a group of AR and LAR patients sensitized to OL where the phenotype had been confirmed by NAPT. The AR patients represented the classic model of rhinitis, whereas LAR group was a more novel phenotype where responses to purified allergens have not been investigated yet. Moreover, the basophil response of LAR subjects to pollens was also unknown, because the prior study performed in this kind of patients was only performed with dust mites [23].

In this study, all AR subjects and almost all LAR subjects (10/12, 83.3%) reacted to nOle e 1 after NAPT. This is the first time where *in vivo* positive responses to a purified structure of known allergenicity are demonstrated in LAR subjects. Also, in both AR and LAR subjects, a significant decrease in vol 2–6 measured by acoustic rhinometry and an increase in VAS scores were detected, whereas none of the healthy controls reacted to either OL or nOle e 1. These results correlated with ECP release that increased at the same time-points in nasal lavages in both AR and LAR groups. Tryptase levels were elevated only in AR subjects with no increase in LAR or the controls, in contrast to what has been observed in other studies where allergic patients were challenged with purified allergens [20]. These data could indicate that mast cells may not play a major role in these responses in LAR. This is the first study performing nasal provocation test with a natural purified allergen in LAR subjects, so more studies are necessary to better understand the mechanism of the response.

In general, LAR subjects required higher doses of nOle e 1 to obtain a positive response in the nasal mucosa, but still under the maximum dose tolerated by controls that did not elicit nasal obstructions, symptoms or release of mediators. As expected, serum sIgE to OL and to nOle e 1, rOle e 2, rOle e 3, nOle e 7, rOle e 9 and rOle e 11 was only found in AR subjects, with nOle e 1 and rOle e 2 the most commonly detected, followed by nOle e 7, 9 and 11 (20%). nOle e 3 was only detected in 10% of AR patients.

Regarding the cellular response, BAT was positive to OL stimulation in 100% of AR subjects and in 10/12 with nOle e 1 (83.3%). In the LAR group, 8/12 subjects (66.6%) had a positive BAT with OL. The BAT responses with purified nOle e 1 were lower in this group, with 4/12 (33%) allergen stimulation. In the CG, only one subject (8%) had a positive BAT with both OL and nOle e 1 stimulation indicating a good specificity (91.7%) for both OL and nOle e 1. These results are in agreement with the ones obtained in a previous study [23], where a group of subjects with LAR to DP showed a 50% of positive results in the BAT with commercial whole extract of DP. In that study, 7% of controls had a positive response in BAT, similar to the figures reported here for OL and nOle e 1. This study confirms the presence of allergen-specific IgE in the surface of basophils, in both local and systemic allergic rhinitis subjects, because this cell may be the target of the specific IgE leaked from the nose or other organs [39, 40]. It has been demonstrated that basophils circulate to allergic inflamed sites in allergic individuals [41] and have also been found in nasal secretions after allergen challenge [42]. The demonstration of the reactivity of basophils with no presence of specific IgE antibodies could be explained by the fact that basophils are highly responsive to IgE-mediated activation, being twice more sensitive than mast cells [43]. However, the exact mechanism that may cause this phenomenon and how the exchange of IgE antibodies between the nasal mucosa and the general circulation is produced still remains unclear [40].

Also, this work represents the first study that evaluates the cellular responses to purified allergens in LAR patients. These results demonstrated that although nOle e 1 induced DC maturation in a similar way in all groups analysed, both AR and LAR showed a higher specific T cell proliferation compared to CG, although with a very low sensitivity in the latter. The significant differences obtained in AR are in agreement with those obtained previously with other purified allergens as Pru p 3, Bet v 1 and Phl p 5 [27, 44, 45]. These results demonstrated for the first time that, although weaker than in AR subjects, there is a systemic cellular response (DC and basophils) in subjects with LAR.

In conclusion, this study has described the *in vivo* and *in vitro* responses to a purified allergen, nOle e 1, in 3 different groups of subjects, demonstrating *in vivo* a nasal response with obstruction, mediators' release by classic AR patients and a different form of AR known as LAR or 'entropy' that had not been yet demonstrated. The *in vitro* studies confirmed the absence of serum-specific IgE to OL and OL allergens in both LAR and controls. The BAT was positive in AR and LAR subjects with both OL and nOle e 1. This study confirmed the presence of positive BAT with pollens in LAR subjects at a similar percentage described with dust mites before, and also responses to

nOle 1 from the basophils of these patients but in a lower percentage. The cellular studies revealed that nOle e 1 can induce mild maturation and proliferation of dendritic cells derived of both classes of patients with no significant differences, but further studies will be able to clarify this event. Further studies with higher number of subjects and other whole and purified allergens will be performed in the near future to gain insights into the pathogenesis of this novel form of AR.

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Conflict of interests

The authors declare no conflict of interest.

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