

Are Basophil Activation and Sulphidoleukotriene Determination Useful Tests for Monitoring Patients With Peach Allergy Receiving Sublingual Immunotherapy With a Pru p 3-Enriched Peach Extract?

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■ Abstract

Introduction: Treatment of food allergy essentially consists of food avoidance, but immunotherapy with food is emerging as a new therapeutic option.

Objective: To evaluate clinical improvement and immunological changes in patients with peach allergy following sublingual immunotherapy (SLIT) with a Pru p 3 quantified peach extract.

Methods: A randomized, double-blind, placebo-controlled clinical trial with peach SLIT was conducted. We assessed clinical efficacy after 6 months of treatment by means of double-blind, placebo-controlled oral challenges with peach and also evaluated immunological changes (basophil activation test [BAT] and determination of sulphidoleukotriene production) following stimulation with peach peel and pulp, rPru p 3, rMal d 1, and rMal d 4 stimulation. We also measured specific IgE and IgG₄ to Pru p 3.

Results: After 6 months of SLIT (T6), the active group showed a 3-fold improvement in tolerance to Pru p 3 and a significant increase in IgE to rPru p 3 and in sLT production following stimulation with peach peel and rPru p 3. There was also a significant increase in BAT results after stimulation with rPru p 3 at 1 month of SLIT (T1). Statistically significant between-group differences were only observed for BAT with peach peel and pulp at T1 and T6 and for BAT with rPru p 3 at T6. No changes were observed in BAT with rMal d 1 or rMal d 4 or in IgG₄ levels to nPru p 3.

Conclusions: SLIT with a Pru p 3 quantified peach extract is clinically effective and leads to an increase in basophil activation and sulphidoleukotriene production following stimulation with rPru p 3 and peach peel in the first months of treatment.

Key words: Basophil. CAST. Food immunotherapy. Lipid transfer protein. Sublingual immunotherapy. Peach immunotherapy. LTP.

■ Resumen

Introducción: El tratamiento de la alergia alimentaria se basa en la evitación del alimento. La inmunoterapia con alimentos está emergiendo como una nueva opción terapéutica. Evaluar la mejoría clínica y los cambios inmunológicos de una inmunoterapia sublingual (ITSL) de melocotón (cuantificada en Pru p 3) en pacientes con alergia a melocotón.

Métodos: Ensayo clínico doble-ciego controlado con placebo con una SLIT de melocotón. Valoramos la eficacia clínica a los 6 meses del tratamiento mediante provocaciones orales doble-ciego controladas con placebo (PODCCP) y los cambios inmunológicos (test de activación de basófilos -BAT- y liberación de sulfidoleucotrienos -sLT-) tras estimulación celular con piel y pulpa de melocotón, rPru p 3, rMal d 1 y rMal d 4, IgE e IgG₄ a Pru p 3.

Resultados: A los 6 meses del tratamiento (T6), la tolerancia a Pru p 3 mediante PODCCP en el grupo activo fue 3 veces superior a la basal (T0), se observó un incremento significativo en la IgE específica a rPru p 3 y en la liberación de sLT tras estimulación con piel de melocotón y rPru p 3, así como en el TAB tras estimulación con rPru p 3 al mes del tratamiento (T1).

Se observaron diferencias intergrupo (activo-placebo) en T1 y T6 para piel y pulpa de melocotón y en T6 para rPrup3 mediante TAB. No se observaron modificaciones en rMald1 y rMald4 o en los niveles de IgG₄ a nPrup3.

Conclusiones: La ITSL con un extracto de melocotón cuantificado en Prup3, es clínicamente efectiva y provoca un incremento en la activación basófila y en la liberación de sLT tras estimulación celular con rPrup3 y piel de melocotón en los primeros meses de tratamiento.

Palabras clave: Basófilos. CAST. Inmunoterapia alimentaria. Proteínas transportadoras de lípidos. Inmunoterapia sublingual. Inmunoterapia con melocotón. PTL.

Introduction

Sublingual immunotherapy (SLIT) is gaining importance in food allergy treatment in the light of studies published on kiwi [1], milk [2], hazelnut [3], and peanut [4], with encouraging efficacy and tolerability results.

It has been shown that subcutaneous immunotherapy (SCIT) is able to increase the allergen activation threshold of mast cells and basophils, resulting in a decrease in the release of histamine and other mediators such as sulphidoleukotrienes (sLTs) [5-7]. However, few studies have monitored the sensitivity of basophils and their mediators during food SLIT [2,4,8-10].

We previously conducted a double-blind, placebo-controlled, 2-center trial to assess the effect of SLIT with peach extract quantified in micrograms of Prup3 in a group of 49 patients with peach allergy and observed clinical (double-blind, placebo-controlled food challenge [DBPCFC]) and immunological (specific IgE and IgG₄ to Prup3) efficacy after 6 months [11].

Assuming that the antigen-specific activation of effector cells could be a useful biomarker for monitoring clinically effective SLIT, 27 patients at 1 of the participating sites were subjected to further cell-stimulation techniques (basophil activation test [BAT] and determination of sLT), with monitoring of progress during the course of treatment.

The aim of this study was to assess changes in basophil activation and sLT production during SLIT with Prup3 quantified peach extract.

Methods

Patients

Thirty-one patients were selected according to the following inclusion criteria: age 18 to 65 years, history of peach allergy with positive skin tests (wheal diameter ≥ 3 mm) to peach extract (ALK-Abelló, S.A.) and/or positive specific IgE to peach by CAP (≥ 0.70 kU/L) (Phadia) and a positive DBPCFC with peach. Exclusion criteria were a positive DBPCFC with placebo, history of an anaphylactic reaction to any food with hypotension, history of allergy to coconut (contained in the challenge vehicle), pollen immunotherapy in the previous 2 years, and any contraindication for immunotherapy [12]. All patients provided written informed consent.

Study Design

Enrolled patients were randomized to receive active treatment or placebo at a ratio of 2:1. Randomization was stratified by a history of systemic symptoms to peach. The outcomes recorded were BAT response and sLT release to peach peel, peach pulp, rPrup3, rMald1, and rMald4 evaluated before treatment (T0), after 1 month of treatment (T1), and at the end of treatment (6 months, T6).

Extracts and Purified Allergens

The peach extract for immunotherapy and DBPCFC was obtained from fresh peelings and quantified in micrograms of the major allergen Prup3, as described by Duffort et al [13]. The SLIT extract was prepared as a glycerinated, phenolated saline solution of peach extract. The placebo was identical to the active preparation, but without the allergen content. The immunotherapy regimen was as described previously [11].

The BAT and CAST (cellular antigen stimulation test) were carried out with peach-peel and peach-pulp extracts (Bial-Aristegui), rMald1, rMald4, and rPrup3 (supplied by E.T.S. Ingenieros Agrónomos, Universidad Politécnica de Madrid and ALK-Abelló, S.A) and produced as described previously [14].

DBPCFC With Peach

Peach SLIT efficacy was analyzed by means of DBPCFC at T0 and T6. Up to 7 doses of peach extract, each increased by a factor of 3 (3-2167 μ g), were administered as previously described [11].

Specific IgE and IgG₄ to Prup3

Serum samples to determine specific IgE to rPrup3 were analyzed at T0, T1, and T6 using the ADVIA Centaur platform (Bayer Health-Care Diagnostics Division) [15,16]. IgG₄ to nPrup3 was determined by means of ELISA [11].

Basophil Activation Test

The BAT was performed as described previously [17,18]. Two concentrations of each allergen studied were established according to the preliminary results [16,19]: 2 and 0.5 mg/mL for peach peel, 1.2 and 0.3 mg/mL for peach pulp, and 0.33 and 0.165 μ g/mL for the purified allergens rPrup3, rMald1, and rMald4. For the analysis, we selected the maximum activation achieved by either of the 2 concentrations tested

for each allergen. Results were considered positive if the basophil activation rate was over 20% with a stimulation index (SI, test value/background value) of greater than 2. The optimal cutoff point was calculated by receiver operating characteristic (ROC) curves, giving preference to specificity over sensitivity [16,19].

Antigen-Specific sLT Determination

sLT release was measured using the CAST-ELISA technique (Bühlmann Laboratories) [20]. The final concentrations of the study allergens were the same as those used for the BAT and the maximum sLT release achieved by either of the 2 concentrations tested for each allergen was selected. sLT production greater than 300 pg/mL with an SI (antigen response/baseline response) equal to or greater than 3 was considered positive. The cutoff points were established using ROC curves [19], selecting optimal specificity values in preference to sensitivity.

Statistical Analysis

The data were analyzed with the statistical program SPSS 15.0. Descriptive statistics included median and interquartile range [IQR] for nonnormally distributed quantitative variables, except for specific IgE for which the mean and IQR were calculated. We compared quantitative variables with the Mann-Whitney U test for 2 independent groups and the Friedman test for k related samples, followed by the Wilcoxon matched pair test when results were significant. *P* values of less than .05 were considered statistically significant.

The DBPCFC results were studied by survival analysis (log-rank test), where time was the challenge dose and event was a positive reaction. This test was applied to study within-group differences for T0 vs T6 and between-group differences at T6. In addition, within-group differences were analyzed by the Wilcoxon test, and between-group differences by discrete-time survival analysis in which the study-end score was the outcome variable and the baseline score and therapy were the regressors [21].

Results

Patients

Of the 37 prescreened patients, 6 were excluded by protocol (3 had a negative DBPCFC and 3 had a placebo-positive DBPCFC). Of the remaining 31 patients, 21 were randomized to active treatment and 10 to placebo. Two patients in each group dropped out for personal reasons at T0 without having received any SLIT doses. The study was completed by 27 patients: 19 in the active group and 8 in the placebo group (Figure 1). All the patients reached the SLIT maintenance dose (30 µg of Pru p 3 per week). The patient and clinical data and sensitization profiles of the evaluated patients are shown in the Table.

DBPCFC With Peach

In the survival analysis, significant changes were found in the active group (log-rank test, *P*=.002). Median (IQR) and

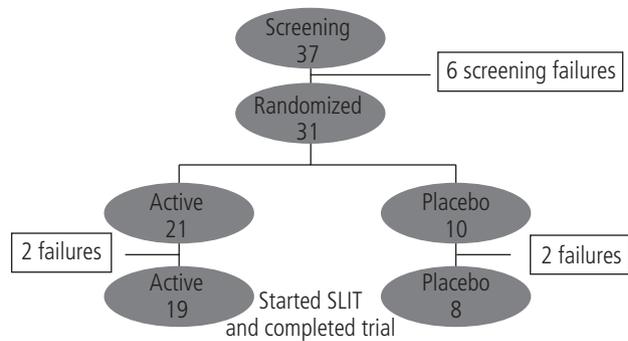


Figure 1. Flow diagram of patients.

Table. Patient and Clinical Characteristics

	Patients (n=19)	Controls (n=8)
Mean age, y	32.8	34.6
Sex (F/M)	8 (42.1)/11 (57.9)	5 (62.5)/3 (37.5)
Mean peach allergy duration, y	15.1	14.4
Peach symptoms		
Local only (OAS)	12 (63.1)	4 (50)
Systemic only	3 (15.8)	3 (37.5)
Local and systemic	4 (21.1)	1 (12.5)
Associated pollen allergy	11 (57.9)	3 (37.5)
Pollen allergy symptoms		
Rhinoconjunctivitis	9 (47.4)	3 (37.5)
Rhinoconjunctivitis and asthma	2 (10.5)	0 (0)
Other food allergies	19 (100)	8 (100)
IgE to rPru p 3	19 (100)	8 (100)
IgE to rMal d 1	4 (21)	1 (12.5)
IgE to rMal d 4	6 (31.6)	1 (12.5)

Abbreviations: F, female; M, male; OAS, oral allergy syndrome.
^aData presented as number (%) unless otherwise specified.

Wilcoxon *P* values for dose changes resulting in a reaction outcome were 1 (0–2) (*P*=.065). This means that after 6 months of SLIT in the active treatment group, the dose of Pru p 3 needed to induce local and systemic reactions was 3 (3¹) times higher (Figure 2A).

No significant differences were observed in the placebo group (log-rank test, *P*>.05; Wilcoxon paired test, *P*=.197) (Figure 2A).

Between-group differences observed at T6 for local or systemic symptoms were not significant (log-rank test, *P*=.933). Also, the discrete-time survival analysis performed to analyze differences between active and placebo groups adjusted for the baseline score did not produce significant results (Figure 2B).

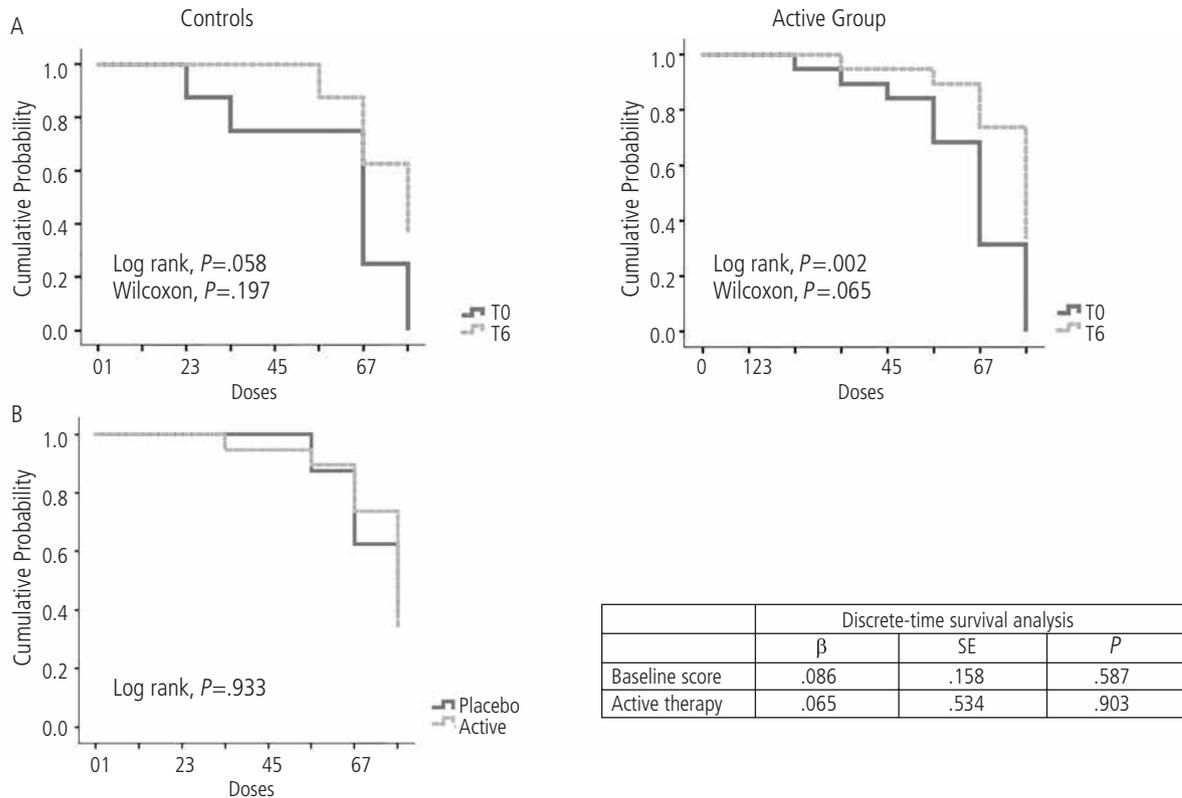


Figure 2. Results of double-blind placebo-controlled food challenge with peach before (T0) and after 6 months (T6) of sublingual immunotherapy (SLIT).

Specific IgE and IgG₄ to Pru p 3

Specific IgE to rPru p 3 showed a significant increase in the active group from T0 (mean, 9.08 kU/L; IQR, 1.48-9.43) to T1 (14.64 kU/L, 1.94-30.8) ($P=.037$) and T6 (12.83 kU/L, 2.7-16.7) ($P=.009$). No significant differences were observed in the placebo group (mean; IQR) (T0: 6.44 kU/L; 1.86-12.74 kU/L; T1: 8.98 kU/L; 1.79-19.56 kU/L; T6: 9.95 kU/L; 1.82-16.84 kU/L) ($P=.565$) and no between-group differences were observed at the different study time points (T0: $P=.696$; T1: $P=.735$, and T6: $P=.449$) (Figure 3).

Values for IgG₄ to nPru p 3 did not change in the active group (median; IQR) (T0: 0.15 kU/L; 0.07-0.32 kU/L; T1: 0.15 kU/L; 0.08-0.28 kU/L; T6: 0.22 kU/L; 0.07-0.38 kU/L) or in the placebo group (T0: 0.17 kU/L; 0.08-0.28 kU/L; T1: 0.18 kU/L; 0.08-0.26 kU/L; T6: 0.16 kU/L; 0.08-0.21 kU/L). No within-group differences were detected either (T0: $P=.938$; T1: $P=.815$; T6: $P=.481$).

Biological Cellular Antigen-Specific Stimulation Tests

Basophil Activation Test

During the study, no significant changes were detected in the BAT with peach peel in either the active or control group. However, in the within-group comparison, starting with similar values at T0, the percentage of basophil activation was higher in the active group at T1 ($P=.029$) and T6 ($P=.049$). Statistical significance was not reached either in the within-group analysis of the results from the BAT with peach pulp in the active or

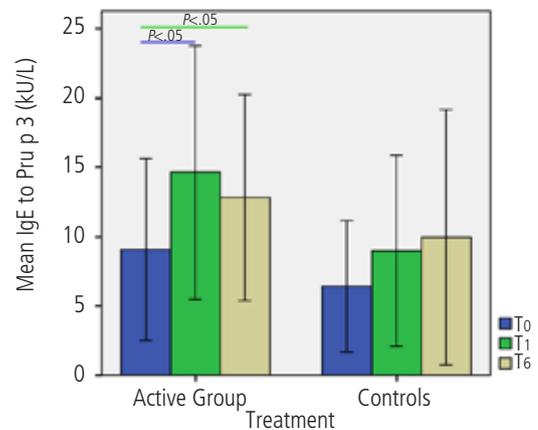


Figure 3. Results of specific IgE to rPru p 3 (mean values).

placebo groups. As with the results after peach peel stimulation, the BAT results with peach pulp in the active group were also significantly higher than those obtained in the placebo group at T1 ($P=.007$) and T6 ($P=.022$). The differences were not observed at T0.

In the active group, the BAT values with rPru p 3 increased significantly from T0 to T1 ($P=.014$). Changes at T6 did not attain statistical significance ($P=.169$).

In the active group, the BAT with rPru p 3 was only higher than the BAT in the placebo group at T6 ($P=.034$). No differences were observed between the 2 study arms at T0 ($P=.360$) or T1 ($P=.095$). There were also no differences in

BAT values with rPru p 3 at the different study time points in the placebo group ($P=.882$). Finally, no differences were observed after antigenic stimulation with rMal d 1 and rMal d 4 in the within- or between-group analyses (Figure 4).

with T1 were not significant. No changes were observed in the placebo group ($P=.882$). Significant changes were not observed either in the CAST with peach pulp in the active group ($P=.241$) or the control group, or between the 2 study arms. In the active group, a significant increase was observed in the CAST with rPru p 3 from T0 to T6 ($P=.029$). Variations compared with T1 were not significant. No changes were observed in CAST with rMal d 1 and rMal d 4 in the within- or between-group analyses (Figure 5).

Cellular Antigen Stimulation Test

In the active group, an increase was observed in the quantity of sLT released after peach peel stimulation at 6 months of SLIT with peach ($P=.045$). Variations compared

	Active (n=19)			Placebo (n=8)		
	T0	T1	T6	T0	T1	T6
Peach peel	78.8 (51.0-86.3)	84.6 (48.0-89.7)§	78.2 (55.4-88.3)§	71.9 (22.3-83.2)	62.8 (12.5-79.3)§	45.9 (3.97-73.9)§
Peach pulp	80.3 (64.5-92.2)	87.0 (79.4-92.2)§	84.9 (65.0-91.4)§	62.5 (26.5-81.8)	73.2 (27.4-80.1)§	52.1 (14.6-74.9)§
rPru p 3	68.1 (44.4-85.0)*	85.7 (61.5-89.6)*	80.5 (53.3-90.1)§	58.8 (12.9-78.2)	66.4 (13.8-81.9)	49.6 (15.1-73.2)§
rMal d 1	6.80 (2.60-63.9)	5.02 (1.30-16.9)	5.70 (2.60-19.5)	11.7 (2.00-20.4)	5.20 (1.31-41.6)	12.6 (2.40-57.4)
rMal d 4	17.8 (2.40-63.2)	32.6 (1.90-73.0)	7.70 (3.60-79.9)	3.70 (1.20-15.8)	16.3 (2.00-52.0)	2.90 (1.12-31.1)

* Within-group differences § Between-group differences

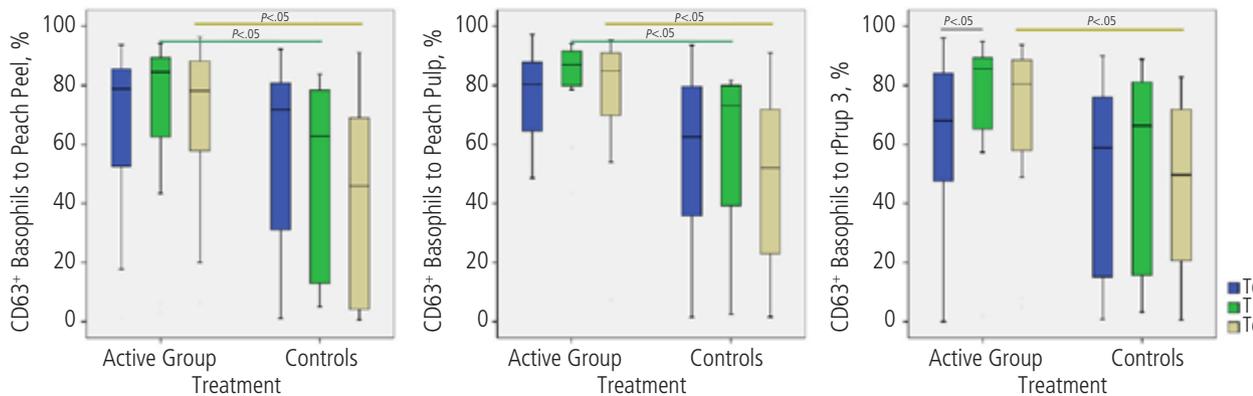


Figure 4. Results of basophil activation test (%) to peel and pulp peach, rPru p 3, rMal d 1, and rMal d 4 (medians and interquartile range).

	Active (n=19)			Placebo (n=8)		
	T0	T1	T6	T0	T1	T6
Peach peel	1318 (807-1936)*	2151 (521-3980)	2520 (1277-3140)*	927 (547-1985)	611 (150-2111)	1204 (136-2189)
Peach pulp	1523 (821-2303)	1993 (1295-5629)	2385 (1471-3240)	743 (242-1204)	1273 (202-3193)	1527 (129-3001)
rPru p 3	1128 (591-1837)*	2268 (610-4540)	2249 (1189-3240)*	756 (89-997)	951 (115-4192)	1426 (477-2367)
rMal d 1	161 (91-857)	138 (106-430)	447 (143-1655)	134 (40-464)	217 (158-517)	554 (211-1302)
rMal d 4	255 (135-1115)	589 (130-1234)	1096 (174-1852)	113 (15-179)	205 (155-830)	221 (62-2654)

* Within-group differences

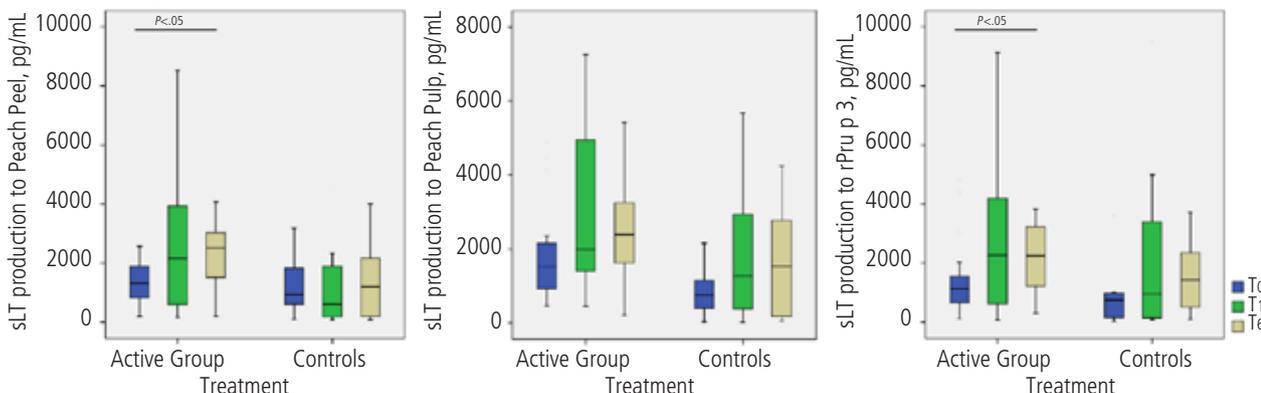


Figure 5. Results of CAST to peel and pulp peach, rPru p 3, rMal d 1, and rMal d 4 (medians and interquartile range). sLT indicates sulphidoleukotriene.

Discussion

As in the case of the 2-center clinical trial [11], in this group of patients, we have also demonstrated the clinical efficacy of SLIT with peach extract through DBPCFC and an increase in specific IgE to rPru p 3 levels. We re-analyzed the results of clinical and immunological efficacy in view of the interest of assessing these in parallel with cell-activation tests.

SCIT has been shown to cause a decrease in allergen-specific basophil activation and in histamine and sLT release from the very first doses of treatment [5,6]. However, at the time of writing, few studies had evaluated how SLIT with inhalants [8-10] or food [2,4] acts on basophils and/or their mediators. Furthermore, such studies are hard to compare because of their heterogeneity. In one study on SLIT with latex [8] (maintenance dose of 40 µg/d), a decrease in the percentage of activated basophils was observed in the active group (n=11) after 2 years of treatment, but not after 1 year. However, in an analysis of a grass-pollen SLIT tablet (20 µg/d), no changes were observed in basophil activation at 4 months of treatment, leading to the conclusion that basophil activation is not a good biomarker of clinical improvement [9,10].

In the 2 SLIT studies that had been performed with food at the time of writing—one with milk [2] (open-label-study, 10 patients, maintenance dose of 7000 µg/d) and the other with peanut [4] (DBPCS, 6 patients, maintenance dose of 2000 µg/d)—a decrease was observed in basophil activation at 3 and 15 months in the first case and at 12 months in the second case. Only 1 out of 10 patients receiving milk SLIT tolerated the challenge dose (10-fold) at the end of treatment. The peanut SLIT group ingested 20 times more peanut protein than the placebo group.

Our study design was double-blind and placebo-controlled in order to evaluate clinical and immunological parameters and thus identify immunological changes due to spontaneous evolution in molecular sensitizations and/or DBPCFC exposure from those induced by immunotherapy. In our study, (maintenance dose, 30 µg Pru p 3/wk), there was a higher percentage of activated basophils after stimulation with peach peel and pulp at 1 and 6 months and also at 6 months after stimulation with rPru p 3 in the active treatment group. The active group had a higher percentage of activated basophils after stimulation with rPru p 3 at 1 month of treatment. In parallel, after 6 months of SLIT with peach, there was an increase in sLT release after antigenic stimulation of basophils with rPru p 3 and peach peel.

It is well known that allergen-specific IgE concentrations increase in the early stages of specific immunotherapy [22]. In our study, the association observed between the increase in specific IgE to rPru p 3 and BAT results with peach and Pru p 3 may have a reciprocal explanation. On the one hand, the increase in the serum concentration of allergen-specific IgE would sensitize the basophils, resulting in the high basophil stimulation rate found in our study. And on the other hand, it was recently discovered that basophils are able to stimulate antigen-specific CD4⁺ T-cell proliferation [23] and act as antigen-presenting cells to naïve T lymphocytes [24,25], which, in turn, are needed for the immune system to produce IL-4 and IgE. Basophils are thus becoming important therapeutic targets in IgE-mediated reactions. The above could

explain the parallelism we found in BAT and CAST results, with increased specific IgE to rPru p 3 during the first 6 months of treatment. Thus, basophils stimulated with rPru p 3 would appear to initiate and amplify the allergen-specific IgE response in the early stages of treatment.

No changes were observed in IgG₄ levels or skin test results (data not shown), compared with the same 2-center trial results, probably due to the smaller sample size.

Finally, SLIT with peach did not modify basophil response or sLT release after stimulation with rMald 1 or rMald 4; nor did it modify levels of IgE to these allergens (data not shown). We used Mald 1 and Mald 4 as homologs of Pru p 1 and Pru p 4 (with a sequence identity of 86% and >90%, respectively [26,27]) due to the absence of commercial extracts for *in vitro* assays.

Our findings coincide with those of studies that did not find any significant changes on analyzing Mald 1 behavior after the administration of birch pollen SLIT [28,29]. The authors of those studies noted that the T-cell response was dependent on the antigen and that the underlying immune mechanism in SLIT was restricted to the allergen administered (Bet v 1 in their case).

It is noteworthy that in our study SLIT was capable of inducing systemic immunologic changes (increased sLT release and basophil stimulation), specifically in response to peach peel and Pru p 3 but not to other peach components (pulp, Pru p 1 and Pru p 4 homologs). However, in light of our current results, BAT and CAST should be carefully considered for monitoring clinically effective SLIT, because a decrease would be expected in basophil activation and sLT release.

In this respect, we believe that the limitations of the 6-month study duration, the low concentrations of Pru p 3 administered during the maintenance phase, and the reduced sample size influenced our results and did not allow us to evaluate whether the increase in specific IgE and basophil sensitivity is a transitory phenomenon in SLIT with food allergens as it is in SCIT.

In conclusion, SLIT with a Pru p 3 quantified peach extract is clinically effective and leads to an increase in basophil activation and sLT production following stimulation with rPru p 3 and peach peel in the first months of treatment. However, more studies with a larger number of cases are needed to determine the kinetics of these parameters in the longer term and also to investigate whether there is a correlation with the clinical improvement seen with immunotherapy.

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Conflicts of Interest

The authors declare that they have no conflicts of interest.

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