

Unraveling Kiwifruit Allergy Diagnosis: Usefulness of the Current Diagnostic Tests

Short title: Understanding the Gap in Diagnosing Kiwifruit Allergy

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ABSTRACT

Objectives: To determine the usefulness of the *in vitro* and *in vivo* methods used in the diagnosis of kiwifruit allergy, focusing on the impact of the seed proteins on their sensitivity.

Methods: Skin prick tests (SPTs) using different commercial extracts, homemade pulp and seed extracts, and prick-prick test with kiwifruit were performed on 36 allergic patients. The presence of specific IgE (sIgE) was assessed using ImmunoCAP (kiwifruit extract), ELISA (Act d 1, Act d 2), ISAC and FABER assays. Immunoblotting of seed extract was carried out, and a single blinded oral food challenge with whole seeds was performed in seed-sensitized subjects.

Results: The prick-prick test with kiwifruit demonstrated the highest diagnostic capacity (81.8% sensitivity and 94.1% specificity) among the *in vivo* tests. The sIgE levels measured by ImmunoCAP-kiwifruit extract showed a similar sensitivity to that of global ISAC and FABER (63.9%, 59.5% and 58.3%, respectively). Act d 1 was the major allergen, and sensitization to it was associated with positive sIgE to whole kiwifruit extract detected by ImmunoCAP ($p < 0.000$). A positive SPT with kiwifruit seeds was associated with severe symptoms with kiwifruit ($p = 0.019$) as a marker of an advanced disease, but not with clinically relevant sensitization. The challenge to kiwifruit seeds performed on eight seed-sensitized patients resulted negative.

Conclusions: Sensitization to Act d 1 is related to a positive result in conventional diagnostic techniques, whereas kiwifruit seed sensitization does not increase the sensitivity of the evaluated diagnostic techniques.

Key words: Allergy, Component-resolved diagnosis, Kiwifruit, Skin test, Storage protein

RESUMEN

Objetivos: determinar la rentabilidad diagnóstica de las técnicas *in vitro* e *in vivo* utilizadas en el diagnóstico de alergia al kiwi y estudiar la influencia de las proteínas alergénicas de las semillas en su sensibilidad.

Métodos: Se seleccionaron 36 pacientes alérgicos a kiwi. Se les realizó prick test con cuatro extractos comerciales diferentes y prick-prick con kiwi. Se determinó IgE específica mediante ImmunoCAP (extracto de kiwi), ELISA (Act d 1, Act d 2), las micromatrices ISAC y FABER e Immunoblotting de extracto de semilla de kiwi. Se realizó exposición oral simple ciego frente a semilla de kiwi en pacientes sensibilizados a la semilla.

Resultados: El prick-prick de kiwi fue la prueba *in vivo* con mayor rendimiento (Sensibilidad 81.8%, Especificidad 94.1%). El ImmunoCAP de extracto de kiwi mostró una sensibilidad similar a la global del ISAC y del FABER (63.9%, 59.5% y 58.3%, respectivamente). Act d 1 fue el alérgeno mayoritario. Se encontró asociación entre los niveles de IgE específica frente a Act d 1 (ISAC) y el extracto de kiwi mediante ImmunoCAP ($p < 0.000$). La prueba cutánea positiva con semilla se asoció con mayor gravedad de síntomas frente a kiwi ($p = 0.019$), como marcador de enfermedad avanzada, pero no como sensibilización clínicamente relevante. La prueba de provocación con semillas fue negativa en los ocho pacientes provocados.

Conclusiones: La sensibilización a Act d 1 se asocia con resultados positivos con las técnicas diagnósticas convencionales. La sensibilización frente a semillas no mejora el rendimiento de las técnicas evaluadas.

Palabras clave: Alergia, Diagnóstico por componentes. Kiwi, Prueba cutánea, Proteína de almacenamiento.

INTRODUCTION

Kiwifruit (*Actinidia deliciosa*) has been one of the most important causes of food allergies in Western countries ever since it was first described in 1981 [1-3]. Although kiwifruit allergy has been associated with polysensitization in relation to the latex-fruit syndrome,[4] as well as birch and grass pollinosis [5,6], monosensitization is strongly related to Act d 1, a cysteine protease of 30 kDa and its major allergen, thus suggesting a primary sensitization to kiwifruit [3,7].The clinical manifestations of this allergy range from local oral symptoms to severe systemic reactions, including anaphylaxis [1,3]. Fourteen allergens have been described in green kiwifruit, with marked geographical differences having been detected in the molecular profile of sensitized patients [1,8].

A poor sensitivity (Se) of commercial extracts used in skin prick tests (SPTs) has been observed in the diagnostic approach followed for patients with kiwifruit allergy, with a Se of 17-61%[1,3,7,9], and improvable results for the ImmunoCAP (Phadia, Uppsala, Sweden) assay using kiwifruit extract (Se: 17-55%) [1,3,7,9]. The determination of sIgE by component-resolved diagnosis (CRD) using ImmunoCAP testing for pulp allergens has been reported to increase this sensitivity to 65-77% [1,3], whereas the ISAC microarray raises it to 66% [7]. Based on these low values, Sirvent *et al.* suggested that the inclusion of kiwifruit seed allergens Act d 12 (11S globulin) and Act d 13 (2S albumin) in the CRD of kiwifruit allergy might significantly reduce the number of misdiagnosed patients [10,11]. No studies have been conducted thus far to demonstrate this hypothesis, and the clinical relevance of kiwifruit seed allergens in kiwifruit-allergic patients has yet to be established.

The aim of this study was to determine whether sensitization to kiwifruit seed storage proteins Act d 12 and Act d 13 explains the low sensitivity of the currently available techniques and to assess the clinical relevance of the kiwifruit seed allergens. We also aimed

to analyze the diagnostic performance of the commercial *in vivo* and *in vitro* techniques that are currently available to evaluate sensitization for the diagnosis of kiwifruit allergy.

MATERIALS AND METHOD

Study Population

A sample of 36 kiwifruit-allergic patients (>6 years old) was prospectively recruited from the Allergology Service of three hospitals of Northern Spain: *Clínica Universidad de Navarra* (Pamplona, Spain), *Complejo Hospitalario de Navarra* (Pamplona, Spain) and *Hospital Universitario Central de Asturias* (Oviedo, Spain) throughout 2017. Allergy was demonstrated by an open oral food challenge (OFC) with kiwifruit (see Supplementary files). Patients with previous history of anaphylaxis or recent clear kiwifruit ingestion-related systemic symptoms (generalized acute urticaria or gastrointestinal symptoms or respiratory symptoms) and positive tests (SPT or specific immunoglobulin E [sIgE] against kiwifruit as determined by an ImmunoCAP assay) were excluded from OFC. Anaphylaxis was defined following the 2014 European Academy of Allergy and Clinical Immunology (EAACI) anaphylaxis guideline [12]. The exclusion criteria are defined in the Supplement. All patients completed the study questionnaire regarding clinical data and symptoms experienced following kiwifruit intake, and tolerance to other foods. Symptom severity was measured according to a scale of symptoms (contact rash, oral allergy syndrome [OAS], systemic symptoms, and anaphylaxis).

Additionally, 31 atopic, adult controls sensitized to pollen or plant food allergens were prospectively recruited for the analysis of the SPT's specificity (Sp). An OFC was performed in this control group following a six-month period during which the fruit had not been eaten. All participants signed the informed consent that had previously been approved by the Research Ethics Committee of the University of Navarra (2016.052) and supported by the

ethics committees of all participating hospitals. The demographic and clinical data of these patients and controls are summarized in Table I.

In addition, to analyze the Sp of the *in vitro* techniques, sera from 35 controls (14 atopic controls sensitized to dust mite and 21 non-atopic controls) from a previous multicenter study (FIS PI 11/01634) were also used.

Skin Tests

All prospectively recruited cases and controls underwent a prick-prick (p-p) test with kiwifruit, SPTs with four different commercial kiwifruit extracts (ALK-Abelló [Hørsholm, Denmark], Bial [Madrid, Spain], Diater [Madrid, Spain] and Leti [Tres Cantos, Spain]), homemade kiwifruit seed and pulp extract (preparation described in the Supplement). Peach lipid transfer protein (LTP) extract (0.1 mg/ml) (Bial), profilin extract (ALK-Abelló), *Betula verrucosa* extract (ALK-Abelló) and mustard extract (Leti) were tested to evaluate possible cross reactivity with other foods. The wheal and flare size were measured after 15 minutes, and wheals with a diameter ≥ 3 mm were considered to be positive [13].

Specific Immunoglobulin E Determination

The presence of sIgE against Act d 1, Act d 2, Act d 5, and Act d 8 was determined in all 36 patients of our study using microarray ImmunoCAP ISAC CRD112 (ThermoFisher, Uppsala, Sweden), with sIgE levels ≥ 0.3 ISU being considered positive. sIgE against Act d 1, Act d 2, Act d 5, and Act d 10 were determined using nanobead-based microarray FABER (CAAM, Italy), with sIgE levels ≥ 0.3 FUI/ml being considered positive. The levels of sIgE against kiwifruit extract (f84) and Act d 8 were measured by fluorescence enzyme immunoassay (FEIA) ImmunoCAP (Thermo Fisher Scientific), with sIgE levels ≥ 0.35 kUA/L being considered positive. In addition, the presence of sIgE against Act d 1, Act d 2, and Act d 5 was repeatedly determined by enzyme-linked immunosorbent assay

(ELISA), in which the positive optical density values for each allergen were as follows: Act d 1 ≥ 0.235 units, Act d 2 ≥ 0.142 units, and Act d 5 ≥ 0.162 units (see more details in the Supplement).

The ISAC, FABER, ImmunoCAP (kiwifruit extract), and ELISA (Act d 1) assays were also used to determine the presence of sIgE in sera obtained from the controls of said retrospective sample ($n = 35$). Some of the analyses were limited due to the reduced availability of sera samples from certain prospectively and retrospectively recruited patients.

Immunoblotting with kiwifruit seed extract ($20 \mu\text{g}/\text{strip}$) was performed with sera from the 36 patients comprising our study population. In addition, immunoblotting with kiwifruit seed extract at $40 \mu\text{g}/\text{strip}$ was performed with the sera from the 17 patients who had a positive SPT against seed extract (see the methodological details in the Supplement).

Specific IgE inhibition studies with purified Act d 1 were performed to evaluate the presence of Act d 1 in the ImmunoCAP assay (see details in the Supplement).

Single Blind Oral Food Challenge with Kiwifruit Seeds

To assess the clinical relevance of kiwifruit seed allergens' sensitization, a single blind oral food challenge (SBOFC) with kiwifruit seeds was performed in eight patients sensitized to kiwifruit seeds. Two types of smoothies (active smoothie [AS] containing all seeds of one kiwifruit and inactive smoothie [IS] as a placebo) were prepared to perform the SBOFC. Food products used for the smoothie's recipe were evaluated with SPTs (see details in the Supplement). Doses of the smoothies were administered progressively, at 30-minute intervals, starting with the IS and continuing with the AS (0.5 gr, 3.5 gr, 7 gr, 15 gr, 30 gr and 70 gr of AS), until all seeds present in the kiwifruit had been consumed. Whenever patients reported subjective symptoms after taking the AS, they were administered the same dose of the IS. The challenge was interrupted in the presence of objective or subjective

symptoms on two occasions after taking the AS and graded with at least two more points in the visual analog scale (VAS) compared with the equivalent dose of the IS. The clinical and serological data of these patients are described in Table II.

Statistical Analysis

The data were analyzed with statistical software Stata/IC 12.0. The sample size was calculated for 60 patients (30 cases and 30 controls) to detect a difference of 30 % between two diagnostic tests (sensitivities of 50% and 80%, respectively) with a power of 70%, assuming a two-tailed alpha value of 5% and a proportion of discordant pairs of 40%.

Quantitative variables were described as medians and interquartile ranges (IQRs) (25-75 percentile) and qualitative variables were described as frequencies (percentages) and compared using the Chi-square or Fisher's exact test. Ordinal and quantitative variables were analyzed using the Mann-Whitney U test. The positive concordance between the evaluated skin tests was calculated considering the sum of positive and negative results, respectively. The correlation between the presence of sIgE against Act d 1 using the ISAC array and against kiwifruit extract using the CAP was evaluated based on Spearman's rho. A concordance analysis between the *in vitro* techniques was performed using McNemar's test. A *p* value of less than 0.05 was considered to be statistically significant.

RESULTS

Study Population

Thirty-six kiwifruit-allergic patients (12 male [33.3%]; mean age of 27 years [6-62]) were recruited. The thirty-one controls (6 male [19.4%]; mean age of 33 years [18-59]) were significantly older than the cases ($p = 0.043$) because only adults were included in this group. Most of the patients, 47.2% (17/36), suffered anaphylaxis after ingesting kiwifruit, 30.6%

(11/36) experienced non-anaphylactic systemic symptoms, 19.4% (7/36) developed OAS, and one patient (2.8%) had a contact rash with kiwifruit. Twenty five percent (9/36) of the patients showed sensitization to profilin, 41.7% (15/36) to *Betula verrucosa* and 19.4% (7/36) to LTP in SPT.

Prick-prick Test with Kiwifruit is the Most Sensitive *In Vivo* Test

The sensitivity of the p-p test using kiwifruit was 81.8% ([18/22]). The sensitivity of the test using the four kiwifruit commercial extracts studied was 52.8% (19/36) with the ALK-Abelló extract, 61.1% (22/36) with the Leti extract, 63.9% (23/36) with the Bial extract, and 66.7% (24/36) with the Diater extract. When comparing the kiwifruit extracts used in the prick tests by pairs, a concordance of 80.6% to 88.9% was found between the commercial extracts. The sensitivity of the pulp and seed extracts was 61.1% (22/36) and 47.2% (17/36), respectively and, interestingly, the concordance of positive SPT between pulp and seed extracts was 80.6% (29/36). The specificity of the *in vivo* tests is summarized in Table III.

The ImmunoCAP Assay with Complete Kiwifruit Extract Showed a Similar Diagnostic Capacity to ISAC and FABER Microarrays

The sensitivity of ImmunoCAP using kiwifruit extract was 63.9% (23/36) (median of 1.15 kUA/L [IQR 0.19-3.37]). The pooled results of the two platforms analyzed considering all kiwifruit allergens present in the microarrays yielded a similar sensitivity to that of the ImmunoCAP assay with kiwifruit extract (FABER Se: 55.9% [19/34]; ISAC Se: 58.3% [21/36]). Regarding the component sensitization profile among the patients included in the study, the rate of positives obtained with the ELISA, ISAC, and FABER assays in the detection of major allergen Act d 1 was 100% (36/36) (median of 0.394 units [0.355-0.454]), 58.3% (21/36) (median of 0.54 ISU [0-1.78]), and only 11.8% (4/34) (median of 0 FIU/ml [0-0]), respectively. The frequency of sensitization to Act d 2 detected by the ELISA, ISAC,

and FABER assays was 41.7% (15/36) (median of 0.144 units [0.113-0.262]), 2.8% (1/36) (median of 0 ISU [0-0]), and 0%, respectively. The frequency of sensitization to Act d 5 detected by the ELISA, ISAC, and FABER assays was 0% (0/36), 2.8% (1/36), and 2.9% (1/34), respectively. Finally, the frequency of sensitization to Act d 8 detected by the ISAC and CAP assays was 8.3% (3/36) in both cases, and the percentage of sensitization to Act d 10 detected by the FABER assay was 5.9% (2/34).

The specificity of the ImmunoCAP and FABER tests in the detection of sIgE against kiwifruit extract was 87.5% [14/16] (FABER test was only performed in 16 subjects) and 97.1% (34/35), respectively. The negative rate obtained in the detection of Act d 1 by the ELISA, ISAC, and FABER tests was 100% (16/16), 97.1% (34/35), and 100% (35/35), respectively. The global specificity of the ISAC and FABER assays was 97.1% (34/35) and 100% (35/35), respectively.

To analyze the lack of correlation between the detection of sIgE against Act d 1 measured by the ELISA and ISAC assays, we performed a SPT with purified Act d 1 on 10 patients (14), detecting sIgE against Act d 1 in all patients with the ELISA, but only three positive cases of sIgE against Act d 1 with the ISAC assay (patients 6, 7, and 24). These three patients had a positive SPT against purified Act d 1, but the other seven ones (patients 5, 9, 17, 21, 22, 23, and 25) had a negative result for this allergen. The results obtained with the ELISA were finally not considered in the analysis due to its possible overestimation (Supplement Table I). The analysis of the concordance between the available *in vitro* techniques is shown in the Supplement.

The kiwifruit extract used in the ImmunoCAP assay contains a sufficient amount of major allergen Act d 1.

ImmunoCAP kiwifruit extract inhibition was performed using purified Act d 1 at five different concentrations in sera of four patients who showed a positive ImmunoCAP against kiwifruit extract. Two of them had a positive ISAC against Act d 1 (patient 6: kiwifruit CAP 10.6 kUA/L and Act d 1 ISAC 11.62 ISU; patient 29: kiwifruit CAP 40.1 kUA/L and Act d 1 ISAC 44.6 ISU) and two had a negative ISAC against Act d 1 (patient 22: kiwifruit CAP 2.53 kUA/L and Act d 1 ISAC 0 ISU; patient 25: kiwifruit CAP 2.21 kUA/L and Act d 1 ISAC 0 ISU). Patients 6 and 29 showed an inhibition of 86%, and patients 22 and 25 showed an inhibition of 19% and 43%, respectively. Based on the results of our study, the major allergen Act d 1(15) is sufficiently represented in the whole kiwifruit extract (Supplement Figure 1). Interestingly, both a good correlation and a significant association was observed between the ISAC against Act d 1 and ImmunoCAP against kiwifruit extract (Spearman's $\rho = 0.8896$; $p < 0.000$).

ISAC Act d 1 sensitization was also associated with a higher frequency of positive SPTs using commercial extracts (Bial: $p < 0.000$; Leti: $p = 0.001$; ALK-Abelló: $p = 0.002$; Diater: $p = 0.01$) and pulp extract ($p = 0.014$), but not with the kiwifruit p-p ($p = 0.117$).

The Role of Sensitization to Kiwifruit Seeds.

Immunoblotting (IB) with kiwifruit seed extract at 20 $\mu\text{g}/\text{strip}$ was performed for all patients ($n = 36$) (Figure 1). Bands with a molecular mass of 51 kDa (expected for Act d 12) and 12 kDa (expected for Act d 13) were detected in 19.4% (7/36; patients 9, 18, 20, 22, 23, 31 and 32) and 11.1 % (4/36; patients 11, 24, 29 and 30) of the studied subjects, respectively. In no patient were both bands detected simultaneously. None of the seven patients who detected

bands at 51kDa presented positive SPT with seed extract and 3 patients out of the 4 who detected bands at 12 kDa, presented positive SPT with the seed extract.

In order to evaluate the role of the seeds in the diagnosis of kiwifruit allergy the participants were divided into two groups: sensitized and not sensitized to kiwifruit seeds according to the results of the SPTs. Seventeen out of 36 (47.2%) patients had a positive SPT against kiwifruit seed extract. Severe symptoms following kiwifruit ingestion (anaphylaxis) were significantly more frequent among patients who showed sensitization to the seeds ($p = 0.019$). Seed sensitization was associated with a higher frequency of positive SPTs against commercial extracts (Bial: $p < 0.000$; Leti: $p = 0.002$; ALK: $p < 0.000$; Diater: $p = 0.014$) and pulp extract ($p < 0.014$), but not in the kiwifruit p-p test ($p = 0.293$). Seed sensitization was also associated with higher levels of sIgE against Act d 1 (ISAC; $p = 0.004$) and complete kiwifruit extract (ImmunoCAP; $p = 0.029$). The clinical data of these patients are summarized in Table IV.

The addition of the SPT with seed extract did not sufficiently improve the diagnostic performance of the available techniques, as the Se of the p-p test only increased from 81.8% (18/22) to 86.4% (19/22), Se of the commercial extract (Diater) increased from 66.7% (24/36) to 72.2% (26/36), Se of ImmunoCAP increased from 63.9% (23/36) to 69.4% (25/36), Se of ISAC increased from 58.3% (21/36) to 66.7% (24/36), and Se of FABER increased from 55.9% (19/34) to 70.6% (24/34).

Eight patients with a positive SPT against kiwifruit seeds and/or bands at 51 kDa (1/8) and/or 12 kDa (1/8) in the IB underwent a kiwifruit seed SBOFC, with all of these provocation tests resulting negative.

Given the low amount of kiwifruit seed proteins found in the IB of patients with a positive SPT against seed extract, a new IB was performed using a greater concentration of seed extract (40 $\mu\text{g}/\text{strip}$) for these patients ($n = 17$). The IB of ten out of 17 (58.8%) patients

showed a 25 kDa protein that, when identified by mass spectrometry, corresponded to a seed-specific thaumatin-like protein that was different from the Act d 2 of the pulp (see more details in the Supplement).

DISCUSSION

In our study, we have evaluated different *in vivo* and *in vitro* tests used to diagnose kiwifruit allergy. According to our results, the best *in vivo* test is the p-p test using fresh kiwifruit, as it yielded the highest sensitivity (81.8%) coinciding with the results reported in other studies [7,16]. As for the SPT, our results showed a low sensitivity of this test using commercial kiwifruit extracts (52.8-66.7%). Allergen Act d 1 (actinidin cysteine protease) might comprise about 50% of kiwifruit proteins and degrade other allergen proteins present in the pulp [1,17]. This can lead to a lower presence of other proteins in the commercial extracts and, hence, to a lower capacity to detect patients allergic to other proteins. In fact, 60% (9/15) of the patients with a negative response against Act d 1 in the ISAC assay were misdiagnosed by the SPT using commercial extracts. Moreover, most of the patients not sensitized to Act d 1 in the ISAC assay were also misdiagnosed by other *in vitro* tests, including the ImmunoCAP (13/15; 86.6%), the FABER (11/15; 73.3%), and the ISAC assays (15/15; 100%). In contrast, fresh kiwifruit seems to preserve all of its proteins, thus offering a good diagnostic capacity.

Sensitization to Act d 1 (58.3%, measured by ISAC) in our results was more frequent than in previous studies [1,3]. The ImmunoCAP- kiwifruit extract assay also yielded higher sensitivity than reported in previous studies [1,3,7] and similar to that of global ISAC and FABER, probably because of the higher prevalence of sensitization to Act d 1 among our population. In addition, we found that sera from Act d 1-sensitized patients were highly

inhibited by purified Act d 1 in terms of their capacity to bind to the kiwifruit extract CAP, which demonstrates a high representation of this allergen in this whole extract ImmunoCAP. There is also a good correlation between the ISAC against Act d 1 and ImmunoCAP against kiwifruit extract (Spearman's $\rho = 0.8896$; $p < 0.000$), that corroborates this fact.

Based on these results, the ImmunoCAP-kiwifruit extract was similar to CRD in the diagnosis of kiwifruit allergy and with good ability to detect Act d 1, the major allergen. Thus, ImmunoCAP-kiwifruit extract is good, although improvable in diagnosis of kiwifruit allergy. The addition of a SPT using seed extract did not sufficiently improve the diagnostic performance of the available techniques. Therefore, in our opinion, the absence of seed allergens for use in the currently available diagnostic *in vitro* and *in vivo* tests is not the cause of their low capacity to diagnose kiwifruit allergy. Interestingly, we found that patients sensitized to kiwifruit seeds in the SPT had higher levels of sIgE against the whole kiwifruit extract and Act d 1, and also experienced more severe allergic symptoms after ingesting kiwifruit compared with patients not sensitized to kiwifruit seeds. Therefore, our results suggest that sensitization to kiwifruit seeds could be considered a marker of and advanced disease and more severe kiwifruit allergy.

In addition, we were unable to demonstrate the clinical relevance of sensitization to kiwifruit seeds, as none of the eight subjects who were sensitized to kiwifruit seeds showed symptoms after undergoing an oral challenge with the seeds. A possible limitation of this challenge is the fact that the subjects swallowed the seeds by taking the smoothie, rather than chewing them, which could have resulted in a decreased availability of the seed allergens. However, it has been reported that seed allergens Act d 12 and Act d 13 can be released from intact kiwifruit seeds after one hour of exposure to simulated gastric and intestinal fluids [11], therefore, the allergen release should have not been negatively influenced. Our approach tried

to mimic real-life conditions in which subjects swallow the seeds along with the kiwifruit pulp.

In conclusion, the *in vivo* diagnostic test with the highest capacity is the p-p test with kiwifruit. In our study, the ImmunoCAP assay using kiwifruit extract showed a similar sensitivity to CRD techniques. The addition of a SPT using seed extract did not sufficiently improve the diagnostic performance of the available techniques. However, the presence of sensitization to the seed allergens seems to be a marker of advanced disease and a more severe kiwifruit allergy. Sensitization to kiwifruit seeds is not clinically relevant in our population.

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Conflicts of interest

The authors have no conflicts of interest to declare.

REFERENCES

1. Le TM, Bublin M, Breiteneder H, Fernández-Rivas M, Asero R, Ballmer-Weber B, et al. Kiwifruit allergy across Europe: Clinical manifestation and IgE recognition patterns to kiwifruit allergens. *J Allergy Clin Immunol*. 2013 Jan 1;131(1):164–71.
2. Fine AJ. Hypersensitivity reaction to kiwi fruit (Chinese gooseberry, *Actinidia chinensis*). *J Allergy Clin Immunol* [Internet]. 1981 [cited 2020 Sep 22];68(3):235–7. Available from: <https://pubmed.ncbi.nlm.nih.gov/7264104/>
3. Bublin M, Pfister M, Radauer C, Oberhuber C, Bulley S, DeWitt ÅM, et al. Component-resolved diagnosis of kiwifruit allergy with purified natural and recombinant kiwifruit allergens. *J Allergy Clin Immunol*. 2010;125(3):687–95.
4. Brehler R, Theissen U, Mohr C, Luger T. “Latex-fruit syndrome”: Frequency of cross-reacting IgE antibodies. *Allergy Eur J Allergy Clin Immunol* [Internet]. 1997 [cited 2020 Sep 6];52(4):404–10. Available from: <https://pubmed.ncbi.nlm.nih.gov/9188921/>
5. Eriksson NE, Werner S, Foucard T, Möller C, Berg T, Kiviloog J, et al. Self-reported hypersensitivity to exotic fruit in birch pollen-allergic patients. *Allergol Int*. 2003;52(4):199–206.
6. Pastorello EA, Pravettoni V, Ispano M, Farioli L, Ansaloni R, Rotondo F, et al. Identification of the allergenic components of kiwi fruit and evaluation of their crossreactivity with timothy and birch pollens. *J Allergy Clin Immunol* [Internet]. 1996 [cited 2020 Sep 6];98(3):601–10. Available from: <https://pubmed.ncbi.nlm.nih.gov/8828538/>
7. Bublin M, Dennstedt S, Buchegger M, Antonietta Ciardiello M, Bernardi ML, Tuppo L, et al. The performance of a component-based allergen microarray for the diagnosis of kiwifruit allergy. *Clin Exp Allergy*. 2011;41(1):129–36.

8. Allergome [Internet]. [cited 2020 Sep 6]. Available from:
http://www.allergome.org/script/search_step2.php
9. Asero R. Allergy to kiwi: Is component-resolved diagnosis in routine clinical practice really impossible? *Eur Ann Allergy Clin Immunol.* 2012;44(2):42–7.
10. Sirvent S, Cantó B, Cuesta-Herranz J, Gómez F, Blanca N, Canto G, et al. Act d 12 and Act d 13: Two novel, masked, relevant allergens in kiwifruit seeds. *J Allergy Clin Immunol.* 2014;133(6).
11. Sirvent S, Cantó B, Gómez F, Blanca N, Cuesta-Herranz J, Canto G, et al. Detailed characterization of Act d 12 and Act d 13 from kiwi seeds: Implication in IgE cross-reactivity with peanut and tree nuts. *Allergy Eur J Allergy Clin Immunol.* 2014;69(11):1481–8.
12. Muraro A, Roberts G, Worm M, Bilò MB, Brockow K, Fernández Rivas M, et al. Anaphylaxis: Guidelines from the European Academy of Allergy and Clinical Immunology. *Allergy Eur J Allergy Clin Immunol.* 2014;69(8):1026–45.
13. Dreborg S, Frew A. Position Paper: Allergen standardization and skin tests. *Allergy* [Internet]. 1993 [cited 2020 Sep 6];48:49–54. Available from:
<https://onlinelibrary.wiley.com/doi/full/10.1111/j.1398-9995.1993.tb04756.x>
14. Palacin A, Rodriguez J, Blanco C, Lopez-Torrejon G, Sánchez-Monge R, Varela J, et al. Immunoglobulin e recognition patterns to purified kiwifruit (*Actinidia deliciosa*) allergens in patients sensitized to kiwi with different clinical symptoms. *Clin Exp Allergy.* 2008;38(7):1220–8.
15. Bublin M. Kiwifruit Allergies [Internet]. 1st ed. Vol. 68, *Advances in Food and Nutrition Research.* Elsevier Inc.; 2013. 321–340 p. Available from:
<http://dx.doi.org/10.1016/B978-0-12-394294-4.00018-3>
16. Lucas JSA, Grimshaw KEC, Collins K, Warner JO, Hourihane JOB. Kiwi fruit is a

significant allergen and is associated with differing patterns of reactivity in children and adults. *Clin Exp Allergy*. 2004;34(7):1115–21.

17. Ciardiello MA, Giangrieco I, Tuppo L, Tamburrini M, Buccheri M, Palazzo P, et al. Influence of the natural ripening stage, cold storage, and ethylene treatment on the protein and ige-binding profiles of green and gold Kiwi fruit extracts. *J Agric Food Chem*. 2009;57(4):1565–71.

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Table 1. Clinical and demographical data of the patients and controls. ND: not done. LTP, lipid transfer protein; OAS, oral allergy syndrome; SPT, skin prick test.

		Patients (n=36)	Controls (n=31)
Gender (n, % male)		12/36 (33.3)	6 (19.4)
Age, mean (min-max)		27 (6-62)	33 (18-59)
Clinical symptoms, n (%)	Contact urticaria	1/36 (2.8)	-
	OAS	7/36 (19.4)	-
	Systemic symptoms	11/36 (30.6)	-
	Anaphylaxis	17/36 (47.2)	-
SPT, n (%)	Peach LTP (Bial)	7/36 (19.4)	3/31 (9.7)
	<i>Betula verrucosa</i> (ALK-Abelló)	15/36 (41.7)	4/31 (12.9%)
	Profilin (ALK-Abelló)	9/36 (25)	3/31 (9.7)
sIgE, n (%)	ISAC Pru p 3 (LTP)	7/36 (19.4)	ND
	ISAC Bet v 1 (PR-10)	6/36 (16.7)	ND
	ISAC Phl p 12 (Profilin)	8/36 (22.2)	ND

Table 2. Patients challenged with whole kiwifruit seeds. IB, immunoblotting; ND, not done; OAS, oral allergy symptoms; SBOFC, single blind oral food challenge; SPT, skin prick test.

	Allergy symptoms with kiwifruit before SBOFC	Bands at 51 kDa and/or 12 kDa in IB (20 µg/strip) of kiwifruit extract	SPT kiwifruit seed extract	Result of SBOFC	Allergy symptoms with three-nuts and seeds	Storage proteins sensitization in ISAC and FABER
Patient 17	OAS	(-)	(+)	(-)	Systemic symptoms with almond, walnut, hazelnut, cashew, chestnut, peanut	ISAC and FABER: (-)
Patient 26	Anaphylaxis	(-)	(+)	(-)	(-)	ISAC: (-) FABER: ND
Patient 23	OAS	Band at 51 kDa	(-)	(-)	(-)	ISAC and FABER (-)
Patient 25	Systemic symptoms	(-)	(+)	(-)	(-)	ISAC: (-) FABER: Ara h 3 (20.67 FU/ml)
Patient 24	Systemic symptoms	Band at 12 kDa	(+)	(-)	Systemic symptoms with peanut, almond, walnut, chestnut; OAS with pistachio and cashew.	ISAC: rJug r 1 (1.8 ISU) FABER: ND
Patient 4	Anaphylaxis	(-)	(+)	(-)	(-)	ISAC and FABER: (-)
Patient 10	Systemic symptoms	(-)	(+)	(-)	(-)	ISAC and FABER: (-)
Patient 15	OAS	(-)	(+)	(-)	(-)	ISAC and FABER: (-)

Table 3. Results of skin prick test with kiwifruit extracts. IQR, interquartile range. SPT, skin prick test.

		Positive sensitization, n (%)	Median (mm), (IQR)	Specificity, n (%)
SPT, n (%)	Kiwifruit pulp extract	22/36 (61.1)	4.25 (1-8)	24/25 (96%)
	Kiwifruit seed extract	17/36 (47.2)	2.25 (0-4.25)	25/25 (100%)
	Commercial extract kiwifruit Diater	24/36 (66.7)	4.75 (1-7)	28/31 (90.32%)
	Commercial extract kiwifruit Bial	23/36 (63.9)	4.75 (0-7.5)	30/31 (96.8%)
	Commercial extract kiwifruit Leti	22/36 (61.1)	5 (0-7.75)	30/31 (96.8%)
	Commercial extract kiwifruit ALK-Abelló	19/36 (52.8)	3 (0-6)	30/31 (96.8%)
	Prick-prick kiwifruit	18/22 (81.8)	7.5 (3.5-9.5)	16/17 (94.1%)

Table 4. Clinical characteristics of sensitized and not-sensitized patients to kiwifruit seeds.

GI, gastrointestinal; IQR, interquartile rank; OAS, oral allergy syndrome; R, respiratory.

		Patients with negative SPT to kiwifruit seed extract (n=19)	Patients with positive SPT to kiwifruit seed extract (n=17)	<i>P</i> value
Age, mean (max-min)		30.6 (6-62)	22.2 (4-46)	0.095
Gender (n, % male)		4/18 (22.2)	7/17 (41.2)	0.345
Clinical symptom severity ranking	Contact urticaria	1/19 (5.3)	0/17	0.019
	OAS	6/19 (31.6)	1/17 (5.9)	
	Systemic symptoms (urticaria, GI, R)	6/19 (31.6)	5/17 (29.4)	
	Anaphylaxis	6/19 (31.6)	11/17 (64.7)	
Debut age, median (IQR)		26 (12-39)	14 (6-23)	0.068
Eliciting dose (n=17), n (%)	1/32	4/11 (36.4)	4/6 (66.7)	0.302
	1/16	5/11 (45.5)	1/6 (16.7)	
	1/8	0	1/6 (16.7)	
	1/4	1/11 (9.1)	0	
	1/2	1/11 (9.1)	0	
sIgE Kiwifruit extract CAP, n (%)		8/19 (42.1)	15/17 (88.2)	0.006
sIgE kiwifruit extract CAP (kUA/L), median (IQR)		0.28 (0.12-2.53)	2.25 (1.05-3.96)	0.029
sIgE Act d 1 ISAC, n (%)		7/19 (36.8)	14/17 (82.3)	0.008
sIgE Act d 1 ISAC (ISU), median (IQR)		0.12 (0-0.69)	1.36 (0.59-4.25)	0.004

Figure 1. Immunoblotting of kiwifruit seed extract (20 microgr/strip), in sera of patients (n=36).

