Original Research Article

Endotyping Cellular and Humoral Immunoreactivity against Pollen and Citrus Fruits in patients with Non–IgE-mediated Rhinoconjunctivitis.

ABSTRACT

Background: Allergies to citrus fruits are often associated with pollinosis due to cross-reactivity among pollen and food allergens (fruit-pollen syndrome) displayed in patients with several conditions, such as allergic rhinoconjunctivitis.
Aim: To evaluate the potential of the Tube Titration of Precipitins (TTP) and the Leukocyte Adherence Inhibition Test (LAIT) to discriminate cellular and humoral immunoreactivity against two *Citrus* spices (orange and lemon) and a pollen extract in patients with non–IgE-mediated allergic rhinoconjunctivitis.
Study Design: We retrospectively examined the medical charts of two cohorts of patients clinically diagnosed with non–IgE-mediated rhinoconjunctivitis with clinical suspicion of hypersensitivity against Citrus spices and pollen, who were investigated with the help of TTP or LAIT, simultaneously tested against individual extracts of orange, lemon and pollen.
Methodology: The registered results of TTP and LAIT were distributed in ranges through a cascade distribution chart to outline the variability of the results. Dispersion graphs plotting the results of LAIT between the results of each pair of allergens were presented. The statistical significances were calculated.
Results: The TTP for the pollen, orange, and lemon extracts showed a distribution concentrated on the higher dilutions, precluding an adequate differentiation among patients' immunoreactivities. On the contrary, the LAIT results showed a wide distribution of results, demonstrating a better potential to differentiate patients and predict hypersensitivity. While the TTP results showed a slight correlation between the paired tests (Pearson's correlation coefficient between r = 0.007 to 0.11), the LAIT results demonstrated a significant moderate correlation between the paired assays, projecting a better potential to predict cross-reactivity among the allergens (Pearson's correlation coefficient between r = 0.43 to 0.56).
Conclusion: Our preliminary results support that the TTP and LAIT performed with orange, lemon, and pollen extracts can potentially endotype (or discriminate) diverse degrees of humoral and cellular immunoreactivity in non–IgE-mediated allergic rhinoconjunctivitis patients.

Keywords: Conjunctivitis; Hypersensitivity; Lemon; Leukocyte Adherence Inhibition Test; Orange; Pollen; Precipitins; Rhinitis.

Abbreviations:
LAI: Leukocyte Adherence Inhibition
LAIT: Leukocyte Adherence Inhibition Test
TTP: Tube Titration of Precipitins

1. INTRODUCTION

Citrus is a genus of wild and domesticated fruit plants in the *Rutaceae* family in which several cultivars are classified, such as oranges (e.g., *Citrus sinensis*), mandarins (e.g., *Citrus reticulata*),

grapefruits (e.g., *Citrus paradisi*), pomelos (e.g., *Citrus maxima*), limes (e.g., *Citrus latifolia*), and lemons (e.g., *Citrus limon*) [1].

A survey performed by the Good Housekeeping Institute pointed out citrus fruits among the top ten foods perceived by food-allergic people as responsible for their symptoms [2].

Allergies to citrus fruits are often associated with pollinosis due to a phenomenon of cross-reactivity among pollen and food allergens (fruit-pollen syndrome), displayed in patients with combined conditions such as allergic rhinitis, allergic conjunctivitis, allergic bronchitis, oral allergy syndrome, urticaria, angioedema, digestive symptoms and anaphylaxis [3-8]. Nasal provocation with pollen extracts is an uncomplicated way to diagnose pollen hypersensitivity [9]. Fruit-pollen syndrome is also frequently associated with latex allergies, sometimes called latex-pollen-fruit syndrome or latex-fruit-pollen syndrome [10, 11]. Pollen-derived products such as honey and royal jelly were described as triggers for anaphylaxis and urticaria in patients with pollinosis [12, 13].

The Allergen Nomenclature Sub-Committee of the World Health Organization and International Union of Immunological Societies (WHO/IUIS) has recognized so far four allergens weighting from 8 to 23 kDa, identified from the sweet orange (*Citrus sinensis),* according to their official nomenclature: Cit s 1 (Germin-like protein), Cit s 2 (Profilin), Cit s 3 (Non-specific lipid-transfer protein type 1) and Cit s 7 (Gibberellin regulated protein) [14]. The same Sub-Committee listed one allergen from lemon (*Citrus limon*): Cit l 3, a 9.6 KDa non-specific lipid-transfer protein type 1 [15], as well as one allergen from mandarin (*Citrus reticulata*): Cit r 3, a 9 KDa non-specific lipid-transfer protein type 1 [16].

The orange profilin, Cit s 2, has an amino acid sequence similar to pollen profilins, such as the birch Bet v 2 (73% identity) [17]. Profilins are plant pan-allergens responsible for cross-sensitization between pollen and plant-derived foods [18]. Pollen and plant food profilin allergens show equivalent IgE and IgG reactivity, are quickly inactivated by gastric digestion, and are commonly involved in polysensitization of allergic patients [19]. Profilin hypersensitivity is common in patients with cross-reactivity to pollen and fruits such as oranges, pineapples, melons, watermelons, tomatoes, and bananas [20, 21].

Gibberellin-regulated proteins (GRP) are a group of emergent allergens described in orange, Japanese apricots, sweet cherries, pomegranates, bell peppers, strawberries, and also in pollen from the *Cupressaceae* tree (cypress) family [22]. GRP hypersensitivities are clinically associated with severe adverse reactions, such as a case of orange-induced anaphylaxis [23, 24]. Cystatin-like proteins found in freshly squeezed orange juice also produced angioedema, dysphonia, and dyspnea [25]. Cystatins are enzymes playing several roles in microorganisms, plants, pollens, animals, and humans already considered autoallergens since they were implicated in autoallergies through cross-reaction IgE-mediated and T-cell mediated hypersensitivities [26, 27].

Citrus seeds also contain reaginic proteins that may produce allergic reactions when mixed with whole-fruit-crushed juices or when accidentally ingested, producing anaphylaxis, urticaria, and respiratory and digestive symptoms in patients who otherwise tolerate squeezed juices [28-30]. Reactions to citrus seeds are related to hypersensitivity to citrin, an 11S globulin belonging to the cupin superfamily, which cross-react with cashew and pistachio allergens [31]. Citrus-induced phytophotodermatitis is associated with the presence of coumarins and furocoumarins (psoralens, xanthotoxins, and bergaptens) in the peel or the juice of the fruit, involving cutaneous photosensitivity, phototoxicity, and/or photoallergy [32, 33]. Sensory hypersensitivity (photophobia and osmophobia) in patients with migraine are also related to citrus fruits [34].

Non–IgE-mediated cellular immunoreactivity against food allergens had already been reported by our group with the help of the Leukocyte Adherence Inhibition Test (LAIT), as well as humoral immunoreactivity against food allergens with the help of Tube Titration of Precipitins (TTP) [35-40]. We routinely employ the LAIT and the TTP in our facilities as triage to evaluate non–IgE-mediated immunoreactivity against suspected allergens before performing more exhaustive *in vivo* provocation tests [41-47]. To evaluate the potential of the LAIT and TTP to endotyping non–IgE-mediated cellular and humoral immunoreactivity against orange, lemon, and pollen extracts, we retrospectively compiled the electronic medical charts of patients diagnosed with non–IgE-mediated rhinoconjunctivitis who were investigated simultaneously for immunoreactivity against these three allergens by one of these assays.

The present study is a proof-of-concept that hypothesizes that LAIT and the TTP may endotype (or differentiate) diverse degrees of cellular and humoral immunoreactivity against orange, lemon, and pollen allergens among patients suffering from non–IgE-mediated rhinoconjunctivitis. As the tests were performed simultaneously with the same venous sample with the three allergens, it is possible to calculate two-sample paired t-tests between each pair of LAIT results (since they refer to the same

quantitative variable), as well to calculate correlation scores and present dispersion graphs between them to distinguish some order of cross-reactivity [48].

2. MATERIALS AND METHODS

2.1 Subjects

After receiving Institutional Review Board approval from the Instituto Alergoimuno de Americana (Brazil; 01/2025), we reviewed the electronic chart of 9,900 outpatients who attended our facility from January 2018 to January 2025.

A cohort of 100 consecutive outside patients (TTP cohort) had been simultaneously submitted to TTP with orange extract, lemon extract, and pollen extract for presenting non–IgE-mediated allergic rhinoconjunctivitis. This cohort counted 28 males; mean age 37.8 years; SD 20.4 years; range 4 to 88 years; median 32.5 years; modes = 29 (appeared seven times); geometric mean = 29.4 years.

A cohort of 100 consecutive outside patients (LAIT cohort) had been simultaneously submitted to TIAL with orange extract, lemon extract, and pollen extract for presenting Non–IgE-mediated allergic rhinoconjunctivitis. This cohort counted 35 males; mean age 42.9 years; SD 18.7 years; range 8 to 76 years; median 38 years; modes = 9 and 38 years (each appeared five times); geometric mean = 32.2 years.

This study did not include patients under biological and/or systemic anti-inflammatory therapy. These procedures were offered to patients with clinical suspicion of Citrus spices hypersensitivity who demonstrated a non-reactive or inconclusive skin test against sodium bisulfite, orange, and lemon extracts [49].

2.2 Extracts

2.2.1 Orange extract

The whole orange (pulp, peel, and seeds) was crushed, homogenized, and then left for 48 hours in a Coca-based extractor solution (propylparaben 0.5g, methylparaben 1g, sorbitol 30g, NaCl 5g, $NaHCO_3$ 2.5g, 1,000mL H_2O) at 4 °C for protein extraction before centrifugation and separation of the water-soluble fraction from solid particles and oily fraction [50]. The protein quantification of the allergen extracts was done according to Bradford's protein-dye binding methodology [51]. The solution was diluted in an antigen dilution solution (NaCl 10g; KH_2PO_4 0.72g; Na_3PO_4 2.86g; methylparaben 1g; propylparaben 0.5g; glycerin 400mL; H_2O 600mL) to an estimated protein concentration of 1 mg/mL and stored at 4 °C into amber opaque glass vials. The orange extract solution was used to perform allergic skin tests, TTP, and LAIT. All relevant and mandatory laboratory health and safety measures have been complied with during the experiments.

2.2.2 Lemon extract

The lemon extract solution was prepared using a similar technique employed for the orange extract.

2.2.3 Pollen extract

The pollen's protein extraction was performed as follows: in a beaker, 5g of dehydrated beekeeping pollen, acquired from a local provider, was added to the Coca-based extractor solution to cover the amount of pollen. The sample was crushed and then left for 48 hours at 4 °C. The sample was centrifuged (4,500 rpm for 10 min) and filtered. The protein concentration was estimated spectrophotometrically and diluted to 1 mg/mL in antigen dilution solution (NaCl 10g, KH2PO4 0.72g, Na3PO4 2.86g, methylparaben 1g, propylparaben 0.5g, glycerin 400 mL, H2O 600mL) and used to perform the LAIT and allergic skin tests.

2.3. LAIT: *Ex vivo* Investigation: Leukocyte Adherence Inhibition Test

2.3.1 LAIT: Procedure for allergen *ex vivo* challenging

We performed the LAIT as previously described [52-58]. Shortly, each donor's fresh plasma was divided into two parts and used in parallel *ex vivo* challenging tests with the orange or lemon extracts and the unchallenged plasma (added with antigen dilution solution as a control). We collected plasma with high leukocyte content (buffy coat) from the heparinized tube after one hour of sedimentation at 37 °C. Then, we distributed aliquots of 100 µL into Eppendorf tubes with (or without) the challenging extract and kept them under agitation for 30 minutes (200 rpm at 37 °C).

2.3.2 LAIT: Procedure for adherence assay

After incubation, the plasma was allocated into a standard Neubauer hemocytometer counting chamber with a plain, non-metallic glass surface and left to stand for 2 hours at 37 °C in the humidified atmosphere of the covered water bath to allow leukocytes to adhere to the glass. Next, we counted the leukocytes, removed the coverslip, and washed the chamber by immersion in a beaker with phosphate buffer saline (PBS) at 37 °C. Then, we added a drop of PBS to the hemocytometer's chamber and allocated a clean coverslip over it. The remaining cells were counted in the same squares as previously examined.

2.3.3 LAIT: Procedure for calculation

The percentage of Leukocyte Adherence (LA) of each assay was estimated as: (the number of leukocytes observed on the hemocytometry chamber after washing divided by the number of leukocytes observed on the hemocytometry chamber before washing) and multiplied by 100 (%). The Leukocyte Adherence Ratio (LAR) was estimated based on the ratio between the LA from the antigen-specific challenged plasma and the LA from the unchallenged control plasma: LAR = LA of the challenged sample divided by LA of unchallenged control plasma multiplied by 100 (%). To further calculate the Leukocyte Adherence Inhibition (LAI), we subtracted the LAR from 100 (%). We employed the LAI results for the cascade distribution chart and the statistics calculations, both performed with the help of the Microsoft Excel® statistical package.

2.4 TTP: *In vitro* Investigation: Tube Titration of Precipitins

As previously reported, the semi-quantitative TTP against the aluminum solution was performed in a transparent vitreous tube array [59-61]. Shortly, the patient's blood was collected in a clot-activator collecting tube. After separation, the serum was centrifugated at 2,000 rpm for 10 minutes. Each allergen extract was allocated in sets of eleven glass tubes at progressive duplicated serum dilutions. The progressive dilutions were combined with the 15 µL of the antigen (1 mg/mL) with 250 µL of the patient's serum, progressively diluted into physiological saline solution (NaCl 0,9%) in the dilution ratios of 1:1; 1:2; 1:4; 1:8; 1:16; 1:32; 1:64; 1:128; 1:256; and 1:512. One tube was a blank control done with the water and serum to observe occasional spontaneous precipitation (Sia Test). After 24 hours, the tubes were examined, and the titters (the highest dilution factor that yields a positive reading) were recorded [62].

3. RESULTS

As a retrospective survey, there was no research protocol; therefore, we report the incidental immune investigation as registered in the digital medical charts.

The TTP for the orange extract showed a distribution concentrated on the higher dilutions (Fig 1). There were no negative results. There were no positive results in the range from 1:1 to 1:32 dilutions. The mean was estimated at 1:347; the median was 1:256; the standard deviation was estimated at 1:169; the mode was 1:512 (appeared 49 times).

The TTP for the lemon extract showed a distribution concentrated on the higher dilutions (Fig 2). There were no negative results. There were no positive results in the range from 1:1 to 1:32 dilutions. The mean was estimated at 1:358; the median was 1:256; the standard deviation was estimated at 1:157; the mode was 1:512 (appeared 49 times).

The TTP for the pollen extract showed a distribution concentrated on the higher dilutions (Fig 3). There were no negative results. There were no positive results in the range from 1:1 to 1:32 dilutions. The mean was estimated at 1:387; the median was 1:512; the standard deviation was estimated at 1:155; the mode was 1:512 (appeared 59 times).

The LAIT for the orange extract showed a wide distribution range of results (Fig. 4). Most results were concentrated in the more immunoreactive groups. There were five negative results. The LAI ranged from 0% to 98%. The mean was 62.6%; the median was 68.5%; the standard deviation was 26.9%; the mode was 0% (appeared five times). Some patients showed low or moderate immunoreactivity during the *ex vivo* challenge test. In contrast, others displayed strong immunoreactivity, which could reflect the participation of orange allergens in a Non–IgE-mediated hypersensitivity condition in these patients.

The LAIT for lemon extract showed a wide distribution range of results (Fig. 5). Most were concentrated in the more immunoreactive groups. There were six negative results. The LAI ranged from 0% to 99%. The mean was 54%; the median was 56.5%; the standard deviation was 27.7%; the mode was 0% (appeared six times). Some patients showed low or moderate immunoreactivity during the ex vivo challenge test. In contrast, others displayed strong immunoreactivity, which could reflect the participation of lemon allergens in a Non–IgE-mediated hypersensitivity condition.

The LAIT for pollen extract showed a wide distribution range of LAI results (Fig. 6). There were three negative results. The LAI ranged from 0% to 98%. The mean was 55.9%; the median was 60.5%; the standard deviation was 27.5%; and the mode was 79% (appeared four times). Some patients showed low or moderate immunoreactivity in response to the *ex vivo* challenge test. Most displayed strong immunoreactivity, which could reflect the participation of pollens in the non–IgE-mediated hypersensitivity of these patients.

The paired-t test indicated a non-significant slight difference between the results of lemon TTP and orange TTP ($p = .640$). Pearson's correlation indicated a non-significant small positive relationship between lemon TTP and orange TTP results: $r(98) = .00798$, p-value = .937.

The paired-t test indicated a non-significant slight difference between pollen TTP and orange TTP results (p-value = .077). Pearson's correlation indicated a non-significant small positive relationship between pollen TTP and orange TTP results: $r(98) = .0761$, p-value = .452.

The paired-t test indicated a non-significant slight difference between pollen TTP and lemon TTP results (p-value = .172). Pearson's correlation indicated a non-significant small positive relationship between pollen and lemon TTP results: $r(98) = .114$, p-value = .259.

The paired t-test indicated a significant difference between orange and lemon LAIT results (p-value = 0.02741). However, Pearson's correlation indicated a significantly moderate positive relationship between the orange and lemon LAIT results: $r(98) = .45$, p-value < .001.

The paired t-test indicated a significant difference between pollen and orange LAIT results (p-value = 0.08241). However, Pearson's correlation indicated a significant positive relationship between pollen and orange LAIT results: $r(98) = .56$, p-value < .001.

The paired t-test indicated a significant difference between pollen and lemon LAIT results (p-value = 0.6322). However, Pearson's correlation indicated a significant positive relationship between pollen and lemon LAIT results: $r(98) = .43$, p-value < .001.

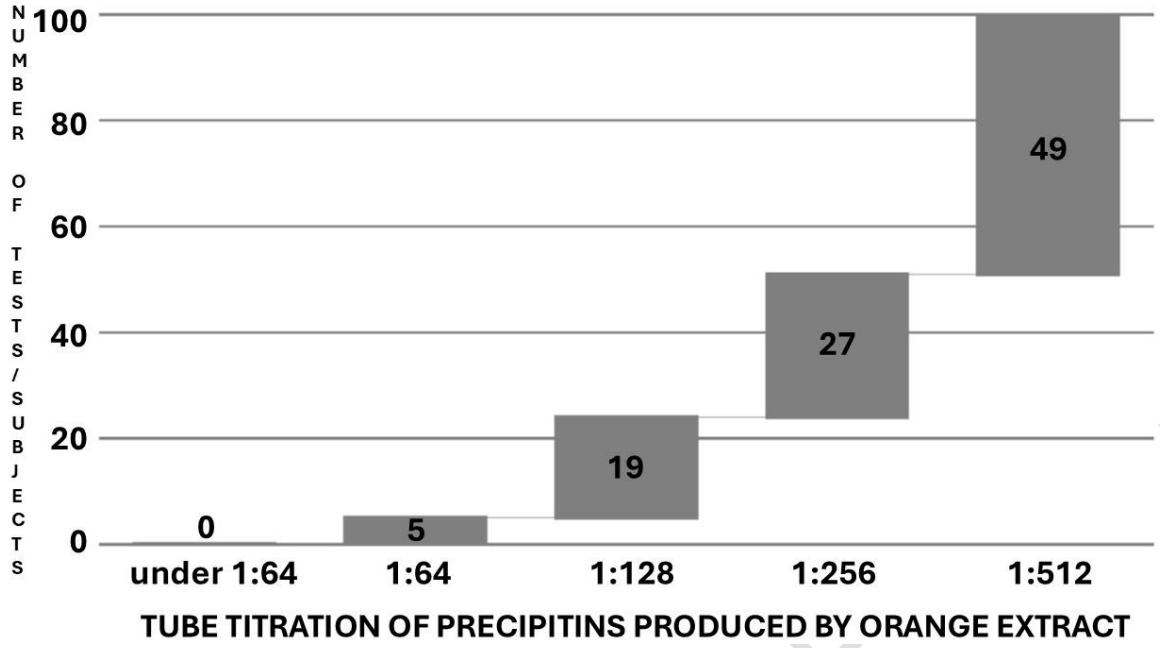

Fig. 1. Cascade distribution chart of the tube titration of precipitins (x-axis %) resulting from the orange extract against the serum of the TTP cohort of 100 tests/subjects (y-axis).

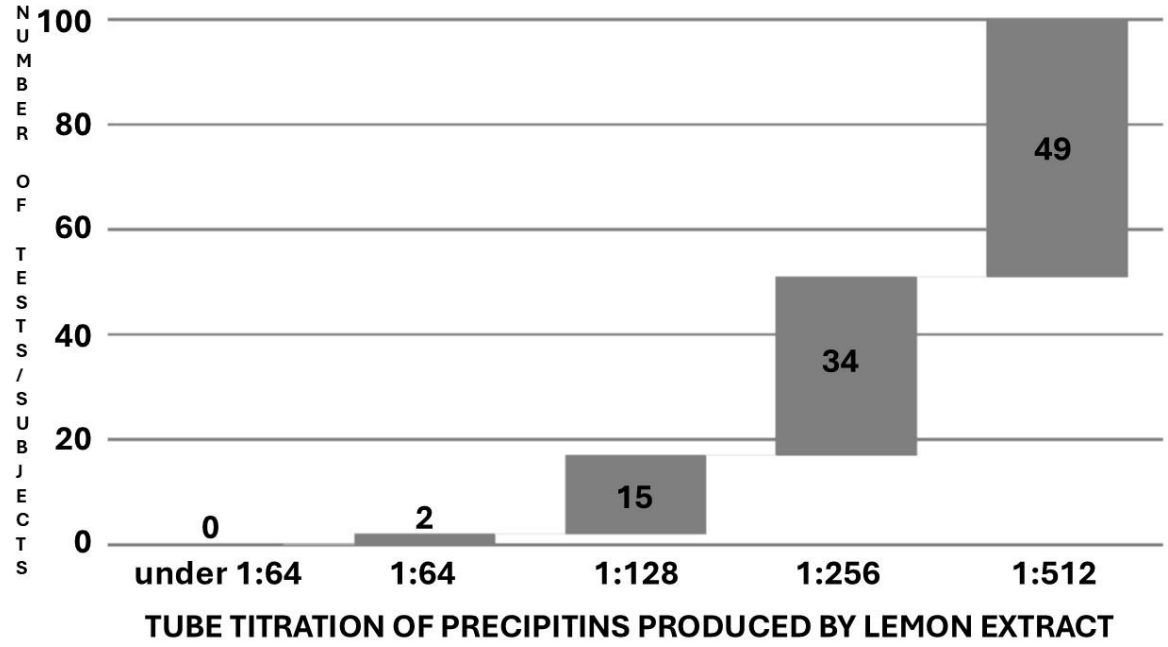

Fig. 2. Cascade distribution chart of the tube titration of precipitins (x-axis %) resulting from the lemon extract against the serum of the TTP cohort of 100 tests/subjects (y-axis).

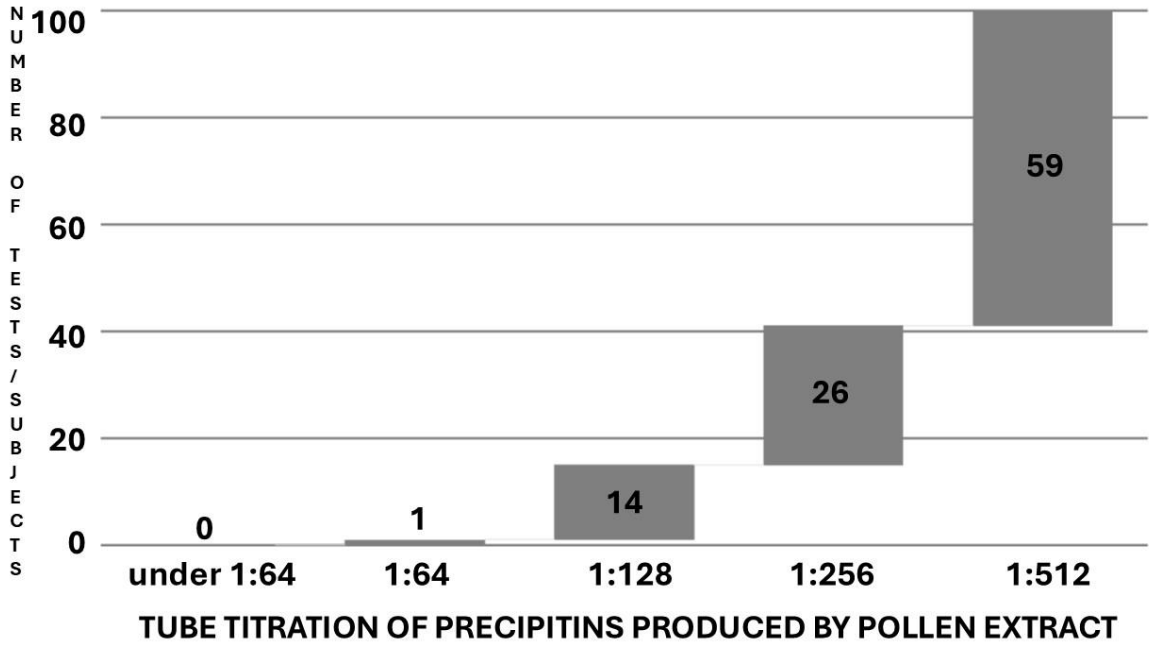

Fig. 3. Cascade distribution chart of the tube titration of precipitins (x-axis %) resulting from the pollen extract against the serum of the TTP cohort of 100 tests/subjects (y-axis).

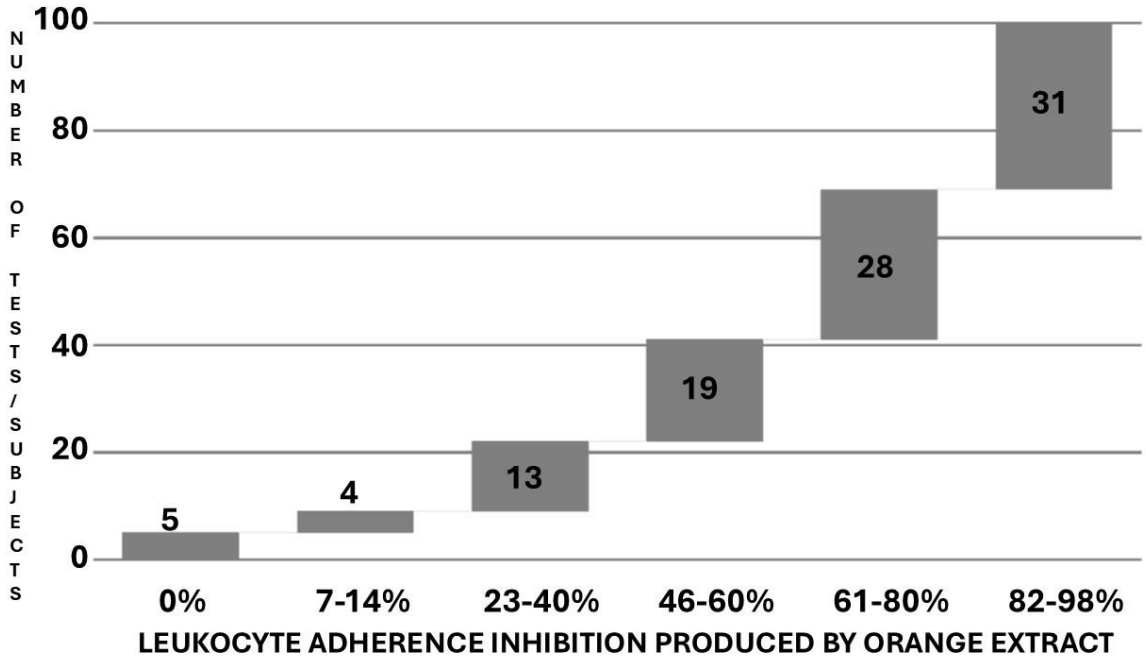

Fig. 4. Cascade distribution chart of the range groups of Leukocyte Adherence Inhibition (LAI) results (x-axis %) of the *ex vivo* challenge test against orange extract monitored by the Leukocyte Adherence Inhibition Test (LAIT), according to the respective number of outcomes over the LAIT cohort with 100 tests/subjects (y-axis).

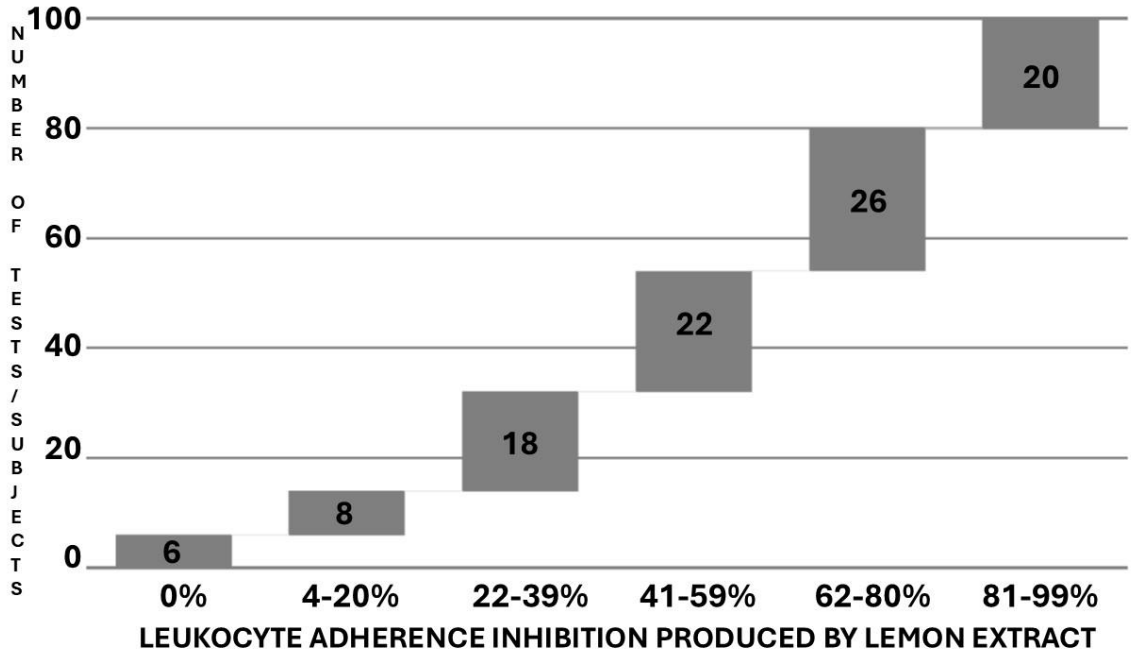

Fig. 5. Cascade distribution chart of the range groups of Leukocyte Adherence Inhibition (LAI) results (x-axis %) of *ex vivo ex vivo* challenge test against lemon extract monitored by the Leukocyte Adherence Inhibition Test (LAIT), according to the respective number of outcomes over the LAIT cohort with 100 tests/subjects (y-axis).

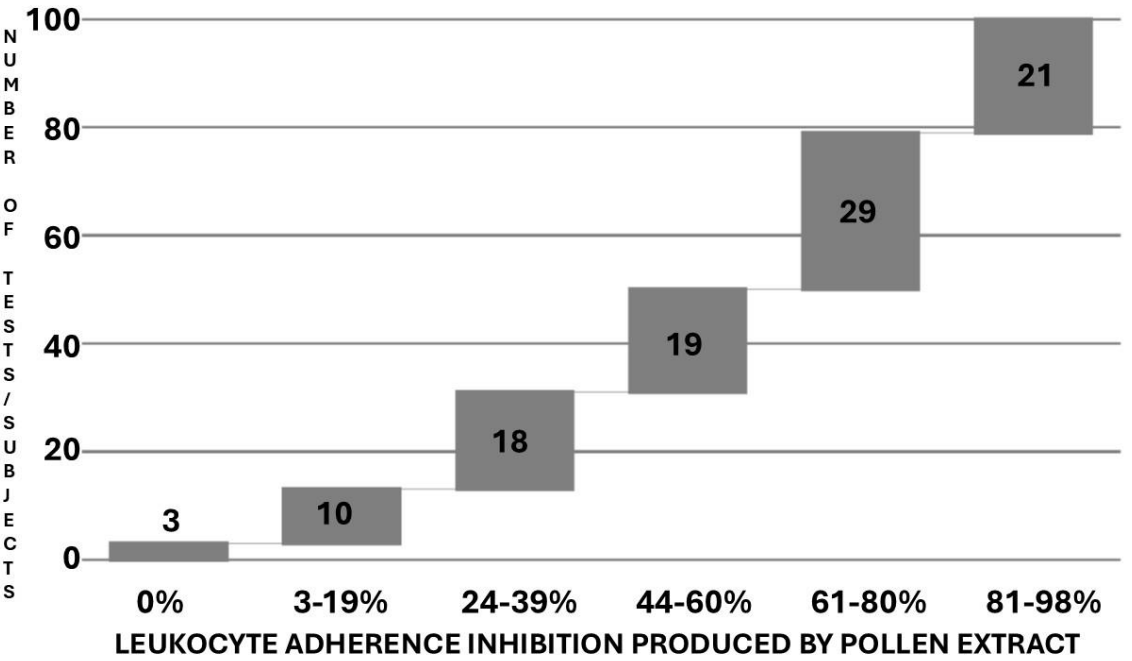

Fig. 6. Cascade distribution chart of the range groups of Leukocyte Adherence Inhibition (LAI) results (x-axis %) of *ex vivo* challenge test against pollen extract monitored by the Leukocyte Adherence Inhibition Test (LAIT), according to the respective number of outcomes over the LAIT cohort with 100 tests/subjects (y-axis).

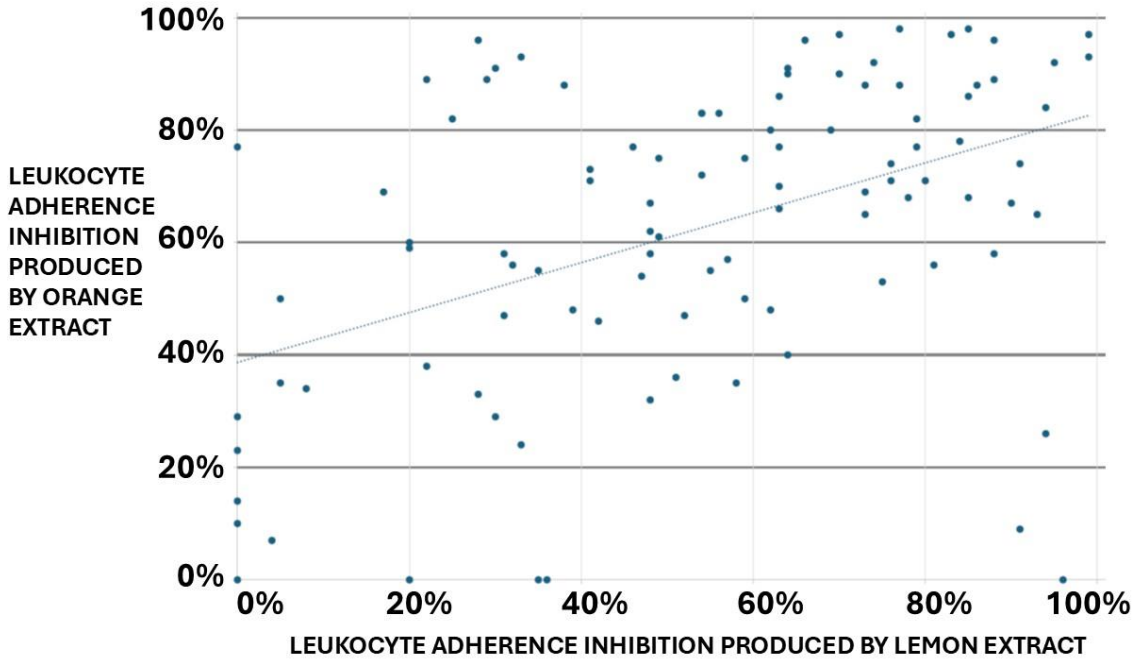

Fig. 7. Dispersion chart of the Leukocyte Adherence Inhibition (LAI) results of the *ex vivo* challenge test against lemon extract (x-axis %), plotted against the paired LAI results of the *ex vivo* challenge test against orange extract (y-axis %).

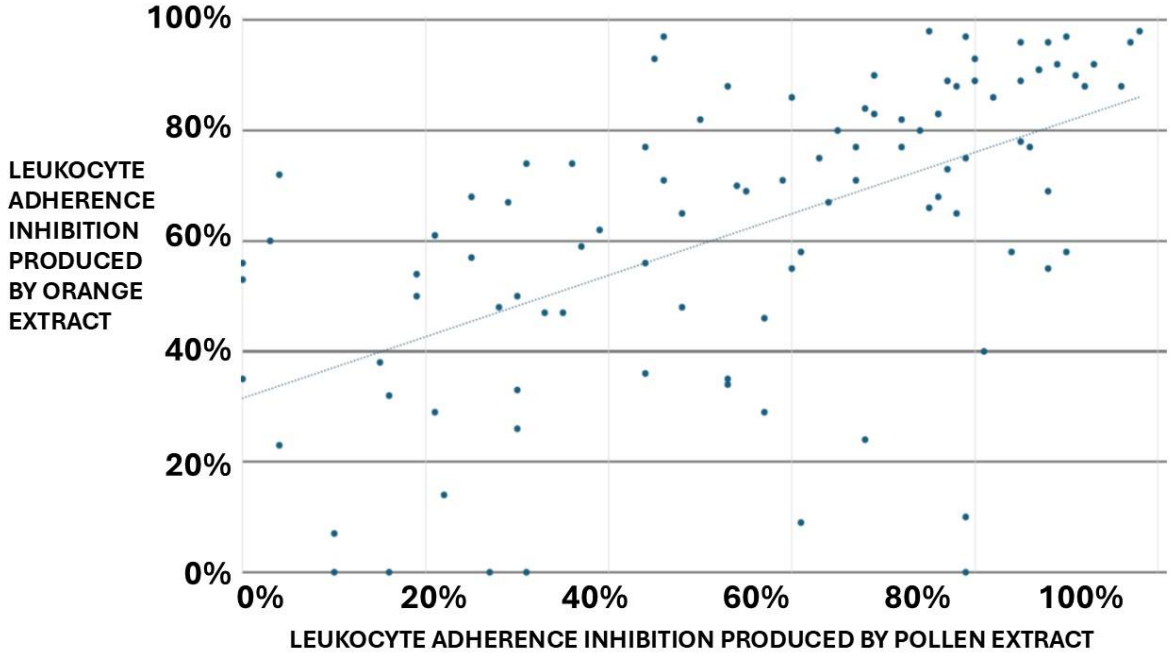

Fig. 8. Dispersion chart of the Leukocyte Adherence Inhibition (LAI) results of the *ex vivo* challenge test against pollen extract (x-axis %), plotted against the paired LAI results of the *ex vivo* challenge test against orange extract (y-axis %).

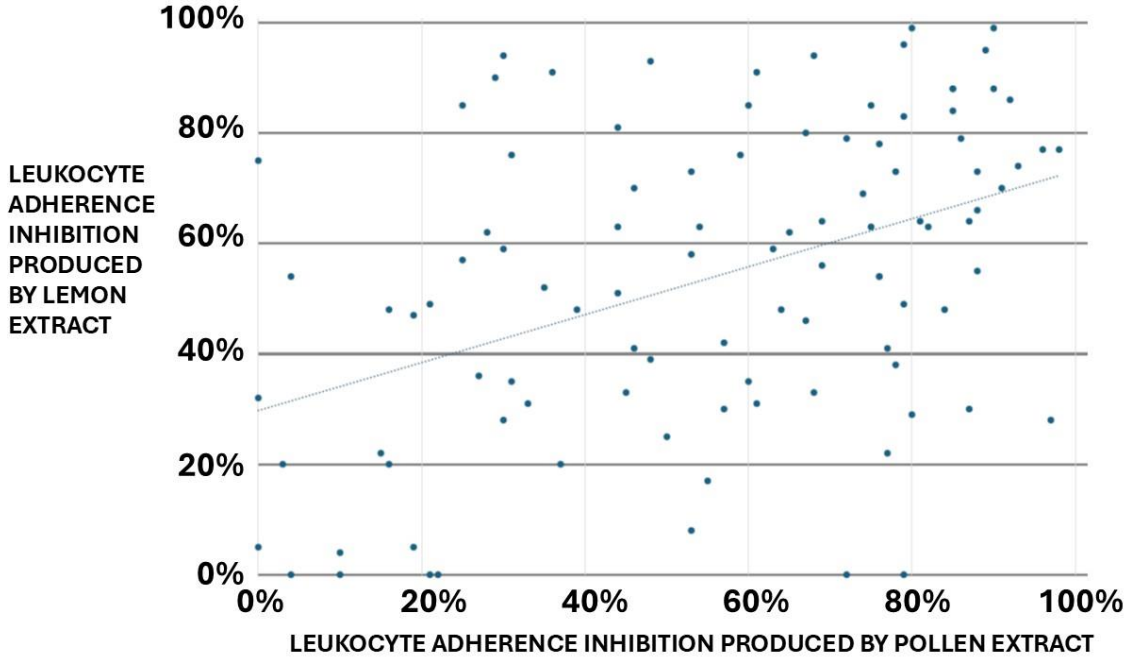

Fig. 9. Dispersion chart of the Leukocyte Adherence Inhibition (LAI) results against pollen extract (x-axis %), plotted against the paired LAI results of the *ex vivo* challenge test against lemon extract (y-axis %).

4. DISCUSSION

Charles Blackley reported the first diagnosis of pollinosis in 1873 when conceiving the skin provocation test to associate the respiratory symptoms of his patients with pollen inhalation [63].

Pollinosis is preferably referred to as pollen allergy or, by extension, allergic rhinoconjunctivitis due to the frequent association of nasal and conjunctival symptoms [64]. Except for desertic or icy regions, pollinosis is a disease that occurs worldwide [65-72]. Pollinosis in Brazil has regional characteristics dependent on local flora and regional crops [73-76]. Our facility borders a city called "Limeira" due to its excellent production of lime oranges, from where several of our patients come.

The two major phenotypes related to allergic rhinoconjunctivitis are "seasonal" and "perennial." Perennial allergic rhinoconjunctivitis is usually related to house dust mites, and seasonal allergic rhinoconjunctivitis is usually related to pollinosis (at least in regions with well-defined climatic stations) [77]. Atmospheric pollen concentration is a standard parameter regularly measured by air controller agencies and is strongly related to pollinosis symptoms [78]. Allergic rhinoconjunctivitis is a prototype model of disease that may be classically produced by IgE-mediated hypersensitivity and non–IgE-mediated hypersensitivity [79, 80]. Longitudinal Clustering Analysis has recently characterized novel rhinoconjunctivitis phenotypes; however, several questions have not yet been answered [81].

A well-studied non–IgE-mediated hypersensitivity endotype responsible for pollen-related allergic conjunctivitis and allergic blepharitis is the Macrophage Migration Inhibitory Factor, a cytokine responsible for eosinophil accumulation in the conjunctivas and eyelid dermis exposed to pollen [82]. Migration Inhibition Factors (MIFs) were the first lymphokines related to delayed hypersensitivity [83]. MIFs are pluripotent cytokines essential in non–IgE-mediated allergic inflammation, recruiting reaginic cells, such as macrophages and eosinophils, to the inflammatory site [84]. MIFs are essential cytokines for T cell activation and sustainment of innate proinflammatory responses [85]. LAIT is an easy and affordable way to put in evidence the possible participation of leukocyte (or macrophage) inhibition cytokines in *ex vivo* challenges tests with allergens [86, 87].

Despite being considered a respiratory condition, proteins bearing correlated pollen epitopes are usually eaten through fruits and vegetables, producing cross-reacting allergic reactions [88, 89].

Endotyping biomarkers of cellular and humoral immunoreactivity and cross-reactivity are essential to build better strategies to impersonate treatments for allergic patients [90]. At the clinical set, diagnosis of IgE-mediated hypersensitivity is an easy task, accomplished by anamnesis, skin tests, and the laboratory research of specific IgE; however, to diagnose non–IgE-mediated hypersensitivity, it is necessary to employ a multi-omics approach to differentiate the particularities of the variety of clinical phenotypes and immune endotypes responsible for allergic diseases [91-93]. The concept of immune dysregulation is evolving, and besides the major primary immunodeficiencies, there are secondary immunodeficiencies following inflammatory conditions raised by immune hypersensitivities, clinically known as allergies [94].

The semi-quantitative research and titration of precipitins is a pioneering laboratory exam upon which the fundamental bases of Immunology were constructed [95]. Precipitating antibodies suggest the presence of a humoral immune response against the tested antigens [96]. Before the discovery of IgE, the research of precipitins against pollen and mold allergens was the leading way to realize *in vitro* diagnostic of immunoreactivity against these agents [97, 98].

Precipitins to pollen allergens are obtained after sensitization of guinea pigs with *Phleum pratense* and *Dactylis glomerata* pollens, producing antiserum reactive against these pollens and cross-reactive against *Festuca pratensis* and *Cynodon dactylon* [99].

The LAIT is an *ex vivo* challenge test performed with a viable leukocyte buffy coat that can theoretically explore the most well-known immune pathways as it allows the interaction of all immune-circulating participants with the allergens [100]. Several immune pathways can produce the final leukocyte adherence inhibition [101-104].

The present study is a proof-of-concept that hypothesizes that LAIT and the TTP may endotype (or differentiate) diverse degrees of cellular and humoral immunoreactivity against orange, lemon, and pollen allergens among patients suffering from non–IgE-mediated rhinoconjunctivitis. As the tests were performed simultaneously with the same venous sample with the three allergens, it was possible to calculate a paired t-test to distinguish some order of cross-reactivity between them.

The retrospective compilation of our data showed a large distribution of results when we ascertained the results of TTP and TIAL to explore humoral and cellular immunoreactivity against two *Citrus* allergens and a pollen extract. These immunoassays did not precisely identify the mechanisms responsible for clinical conditions. Instead, they provide evidence about cellular and humoral immunoreactivity distributed into an extensive spectral range that may suggest immune tolerance or hypersensitivity.

The TTP for the pollen, orange, and lemon extracts showed a distribution concentrated on the higher dilutions, precluding an adequate differentiation among patients' immunoreactivities. Further studies performed with assays extended to higher dilutions are needed to achieve more reliable conclusions. On the contrary, the LAIT results showed a wide distribution of results, demonstrating a better potential to endotype (or differentiate) patients with hypersensitivity. While the TTP results showed a slight correlation between the paired tests, the LAIT results demonstrated a moderate correlation between the paired assays, projecting a better potential to predict cross-reactivity among the allergens.

On the contrary, the LAIT results showed a wide distribution of results, demonstrating a better potential to endotype (or differentiate) patients and predict hypersensitivity. While the TTP results showed a slight correlation between the paired tests (Pearson's correlation coefficient between $r = 0.007$ to 0.11), the LAIT results demonstrated a significant moderate correlation between the paired assays, projecting a better potential to predict cross-reactivity among the allergens (Pearson's correlation coefficient between $r = 0.43$ to 0.56).

This preliminary retrospective survey demonstrated extensive results from the TTP and the *ex vivo* challenge test monitored by LAIT against pollen and two Citrus species in two cohorts of non–IgE-mediated rhinoconjunctivitis patients. TTP and LAIT are complementary triage tests used at our facilities to select worthwhile antigens to proceed with more laborious *in vivo* provocation tests when the specific IgE is undetectable. None of our patients presented an exclusive reaction to these allergens. Every patient was simultaneously tested for several chemical and biological allergens, demonstrating positive results for some of them. Our results suggest that pollen-allergic patients may impair their symptoms by additional cross-immunoreactivity against *Citrus* allergens.

5. LIMITATIONS

This study is a retrospective analysis of data collected over six years and nine months. There was no protocol research, and the subject's data was limited to the essentials available on our electronic sheets. Therefore, we could not establish a cross-comparison between positive and negative controls to validate the results. The number of subjects is appropriate for preliminary study; however, future studies must be more comprehensive. The lack of a research protocol implies the possibility of a bias produced by the point of view of the physician who indicated the exam (CEO) based on a clinical suspicion led purely by the anamnesis and physical examination. The study lost many of these patients to follow-up, so assuring the relationship between the immunoassay results and the patient's clinical outcome is not possible yet. Unfortunately, it was impossible to compare the two procedures with paired tests because they were taken from distinct groups of patients.

6. CONCLUSION

Our preliminary results show that the LAIT and TTP may differentiate diverse degrees of immunoreactivity against pollen, orange, and lemon extracts in patients clinically diagnosed with non–IgE-mediated allergic rhinoconjunctivitis. TIAL and TTP are inexpensive, can be performed with minimum laboratory equipment, and can be incorporated into strategies to address health disparities in respiratory and food allergy [105]. As a preliminary report, the propaedeutic meaning of the presented results and the possibility of interferents must be yet established [106]. More studies focused on the quality-by-design approach with prospective larger double-blind cohorts need to evaluate the potential contribution of LAIT and TTP for endotyping cellular and humoral immunoreactivity in patients suspected of hypersensitivity against citrus fruits and pollen allergens [107].

7. FUTURE DIRECTIONS AND RECOMMENDATIONS FOR CLINICAL PRACTICE

The primary intended use of *in vitro* or *ex vivo* allergen challenge tests is to spare the patients from being submitted to unnecessary, exhaustive, and dangerous *in vivo* challenge tests. Exploring the humoral and the cellular arms of immune systems, the TTP and TIAL alone or combined may represent, in the near future, a tool for allergists to construct an etiological diagnosis from their patients, as well as determine the endotypes (mechanisms) of hypersensitivity, in order to choose more convenient and personalized therapies for them. Adding data provided by TTP and TIAL may also contribute to streamlining biomedical research and improving tools such as Large Language Models, usually used by clinicians as a decision support system to enhance diagnostic accuracy [108].

CONSENT

As a retrospective survey of results recorded *in cognito*, consent was given collectively by the institution's ethics committee following the principles of the Declaration of Helsinki [109].

ETHICAL APPROVALS

The authors have collected and preserved written ethical approval *per* international standards.

Disclaimer (artificial intelligence)

Authors hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

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