Original Research Article

Endotyping Cellular and Humoral Immunoreactivity against Hen's Egg White. A Retrospective Study in Allergic Patients.

:

ABSTRACT

Background: Hypersensitivity to chicken eggs is the second most common food allergy, presenting IgE-mediated and non-IgE-mediated hypersensitivity mechanisms. While IgE-mediated hypersensitivity is easily detected, non-IgE-mediated endotypes present challenging obstacles to diagnosis.
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 hen's egg white (albumen).
Study Design: We retrospectively examined the medical charts of two cohorts of patients clinically diagnosed with diverse allergic phenotypes with clinical suspicion of non–IgE-mediated hypersensitivity against hen's egg white, who were investigated with the help of TTP or LAIT.
Methodology: The registered results of the semi-quantitative serum TTP against 1 mg/mL albumen extract (TTP cohort) as well as the registered results of the Leukocyte Adherence Inhibition (LAI) percentage promoted by the *ex vivo* challenges against 1 mg/mL albumen extract (LAIT cohort) were distributed through a cascade distribution chart to outline the variability of the results. The statistical characteristics inside each cohort were calculated.
Results: The cascade distribution for TTP with hen's egg extract showed a relatively uniform distribution. There were eight negative results. The mean was estimated at 1:156; the median was 1:64; the standard deviation was estimated at 1:185; the mode was 1:512 (appeared eighteen times). The cascade distribution for LAIT with hen's egg extract showed a relatively uniform distribution. There were twenty and one negative results. The LAI ranged from 0% to 98%. The mean was 44.2%; the median was 48.5%; the standard deviation was 32.2%; the mode was 0% (appeared 21 times).
Conclusion: Our preliminary results support that the TTP and LAIT performed with hen's albumen extract may discriminate diverse humoral and cellular immunoreactivity degrees in patients suffering from diversified allergic phenotypes.

Keywords: Albumen; Endotype; Hen's Egg White; Hypersensitivity; Leukocyte Adherence Inhibition Test; Non–IgE-mediated Immunoreactivity; Phenotype; Precipitins.

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

1. INTRODUCTION

The domestic chicken *Gallus domesticus* resulted from hybridizing and domesticating several Asiatic wild *Gallus* species that started around eight thousand years ago [1]. The extensive creation of chicken for broilers or the production of eggs destined for human nutrition is nowadays one of the many contributors to the reconfiguration of the biosphere [2]. Hens are female adult chickens that lay eggs even if not fertilized. Eggs are organic vessels oviparous

animals produce to incubate an embryo outside the animal's body [3]. After being laid, eggs may be a target of predators in nature for their high nutritional content [4]. Chicken eggs are constituted by a protective shell, a yolk (or vitellus), an egg white (or albumen), and an embryo (when fertilized) [5]. Egg white mainly consists of water (88%), protein (10.5%), carbohydrate (0.5%), ash (0.8%), and lipids (0.2%) [6]. The most abundant protein in albumen is ovalbumin (54%), a phosphoglycoprotein that converts to s-ovalbumin (a less reactive form of albumin) during storage [7]. Ovotransferrin (also known as conalbumin, an iron-binding glycoprotein) is the second most abundant protein (12%), followed by ovomucoid (11%), a heat-resistant major allergen which functions as a trypsin inhibitor [8, 9]. Other proteins were also quantified: ovomucin (3.5%), lysozyme (3.5%), ovoinhibitor (1.5%), ovoglycoprotein (1.0%), ovoflavoprotein (0.8%), ovomacroglobulin (0.5%), avidin (0.5%) and about more thirty proteins found in decrescent proportions [10, 11].

In addition to their nutritional values, albumen's proteins present several functional properties explored by the artisanal culinary and food industry [12]. Usually referred to in cosmetic ingredients as albumen, egg white proteins are used to enhance skin and hair properties, enhance skin tone, and reduce wrinkles. It is incorporated into face masks, cleansers, and skincare formulations [13].

Hypersensitivity to egg proteins is the second most common food allergy, with a self-report prevalence of up to 7% of the inquired population [14]. Allergy to egg white proteins was one of the first thoroughly investigated reported cases of food allergy, successfully treated with desensitization in 1912 [15]. Patients may present cutaneous manifestations (urticaria, angioedema, atopic and contact dermatitis), gastrointestinal symptoms (allergic proctocolitis, eosinophilic esophagitis, food-protein induced enterocolitis syndrome), respiratory inflammations (rhinitis, bronchitis) and anaphylaxis [16, 17].

The Allergen Nomenclature Sub-Committee of the World Health Organization and International Union of Immunological Societies (WHO/IUIS) has recognized ten allergens related to *Gallus domesticus*, from which four are abundant in albumen according to their official nomenclature: Gal d 1 (ovomucoid), Gal d 2 (ovalbumin), Gal d 3 (ovotransferrin) and Gal d 4 (lysozyme C) [18].

Bird-egg syndromes are symptomatic allergic cross-reactivities among egg proteins, poultry meat, and aeroallergens from feathers [19]. Egg white proteins may also function as aeroallergens, producing respiratory allergic phenotypes [20].

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 other food allergens with the help of Tube Titration of Precipitins (TTP) [21-23]. These tools are evaluated as laboratory tools to endotype immunoreactivity conditions as a Personalized Medicine strategy [24].

We routinely employ the LAIT and the TTP in our facilities as a triage to evaluate non–IgE-mediated immunoreactivity against suspected allergens before performing more exhaustive *in vivo* provocation tests [25-31]. To evaluate the potential of the LAIT and TTP to endotyping non–IgE-mediated cellular and humoral immunoreactivity against hen's eggs albumen extracts, we retrospectively compiled the electronic medical charts of patients investigated by one of these assays.

The present study is a proof-of-concept that hypothesizes that LAIT and the TTP may differentiate diverse degrees of cellular and humoral immunoreactivity against hen's egg white proteins in allergic patients.

2. MATERIALS AND METHODS

2.1 Subjects

After receiving Institutional Review Board approval from the Instituto Alergoimuno de Americana (Brazil; 9/2024), we reviewed the electronic chart of 9,600 outpatients who attended our facility from January 2018 to November 2024.

A cohort of 100 consecutive outside patients (TTP cohort) had been simultaneously submitted to TTP with hen's egg white for presenting in patients with non–IgE-mediated allergic phenotypes (urticaria, angioedema, atopic and contact dermatitis, allergic proctocolitis, eosinophilic esophagitis, food-protein induced enterocolitis, rhinitis, and bronchitis). This cohort counted 23 males; mean age 42.7 years; SD 20.3 years; range 4 to 92 years; median 42.5 years; modes = 35 and 38 years (each appeared five times).

A cohort of 100 consecutive outside patients (LAIT cohort) had been simultaneously submitted to TIAL with hen's egg white for presenting in patients with non–IgE-mediated allergic phenotypes (urticaria, angioedema, atopic and contact dermatitis, allergic proctocolitis, eosinophilic esophagitis, food-protein induced enterocolitis, rhinitis, and bronchitis). This cohort counted 27 males; mean age 39.9 years; SD 23.4 years; range 0 to 85 years; median 41.5 years; mode = 1 (appeared five times).

This study did not include patients under biological and/or systemic anti-inflammatory therapy. These procedures were offered to patients with clinical suspicion of hen's egg white hypersensitivity who demonstrated a non-reactive or inconclusive skin test against hen's egg white [32].

2.2 Hen's egg white extract

Hen's egg white was 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 [33]. The protein quantification of the allergen extract was done according to Bradford's protein-dye binding methodology [34]. The solution was diluted in 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 hen's egg white extract solution was used for allergic skin tests, TTP, and LAIT. All relevant and mandatory laboratory health and safety measures have been complied with during the experiments.

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

2.3.1 LAIT: Procedure for allergen *ex vivo* challenging

We performed LAIT as previously described [35-41]. Shortly, each donor's fresh plasma was divided into two parts and used in parallel *ex vivo* challenging tests with the hen's egg white extract 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 [42-44]. 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 hen's egg extract 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 titers (the highest dilution factor that yields a positive reading) were recorded [45].

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 cascade distribution for the TTP cohort with hen's egg extract showed a relatively uniform distribution (Fig 1). There were eight negative results. The mean was estimated at 1:156; the median was 1:64; the standard deviation was estimated at 1:185; the mode was 1:512 (appeared eighteen times). Some patients showed low or moderate concentrations of precipitins against hen's egg white. In contrast, others showed high concentrations of precipitins against the hen's egg white, which could reflect the participation of hen's egg white allergens in these patients' non–IgE-mediated hypersensitivity conditions.

The cascade distribution for the LAIT cohort with hen's egg extract showed a relatively uniform distribution (Fig 2). There were twenty and one negative results. The LAI ranged from 0% to 98%. The mean was 44.2%; the median was 48.5%; the standard deviation was 32.2%; the mode was 0% (appeared 21 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 hen's egg white allergens in these patients' non–IgE-mediated hypersensitivity conditions.

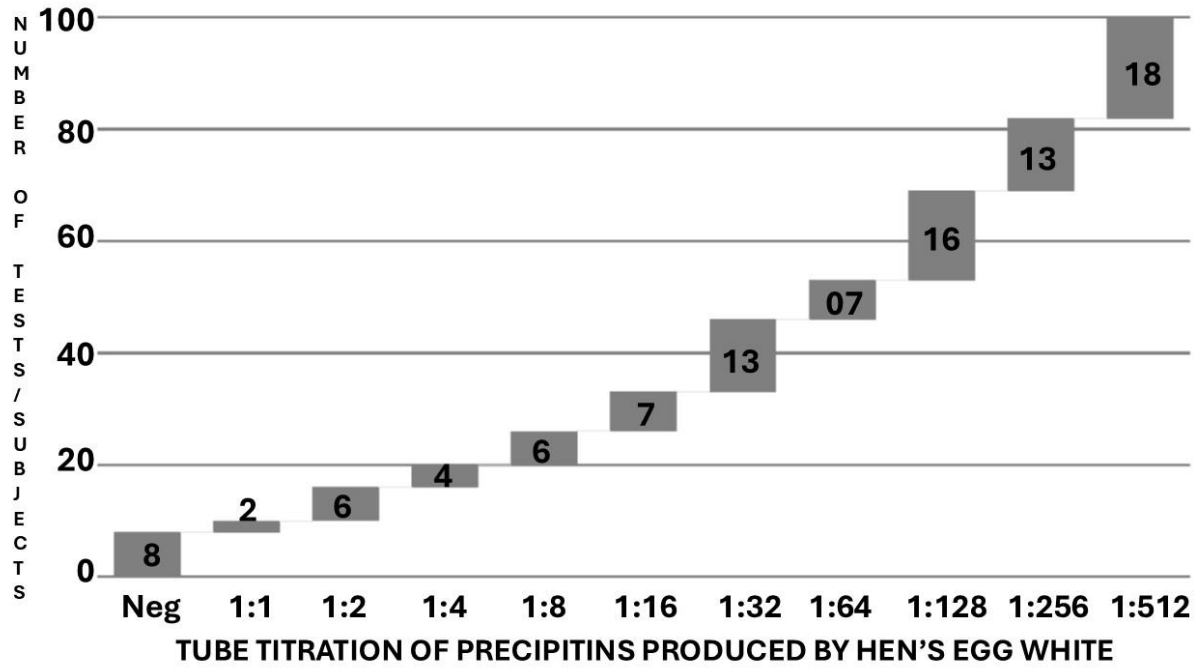

Fig. 1. Cascade distribution chart of the tube titration of precipitins (x-axis %) resulting from the hen's egg white extract against the serum of the TTP cohort of 100 tests/subjects (y-axis).

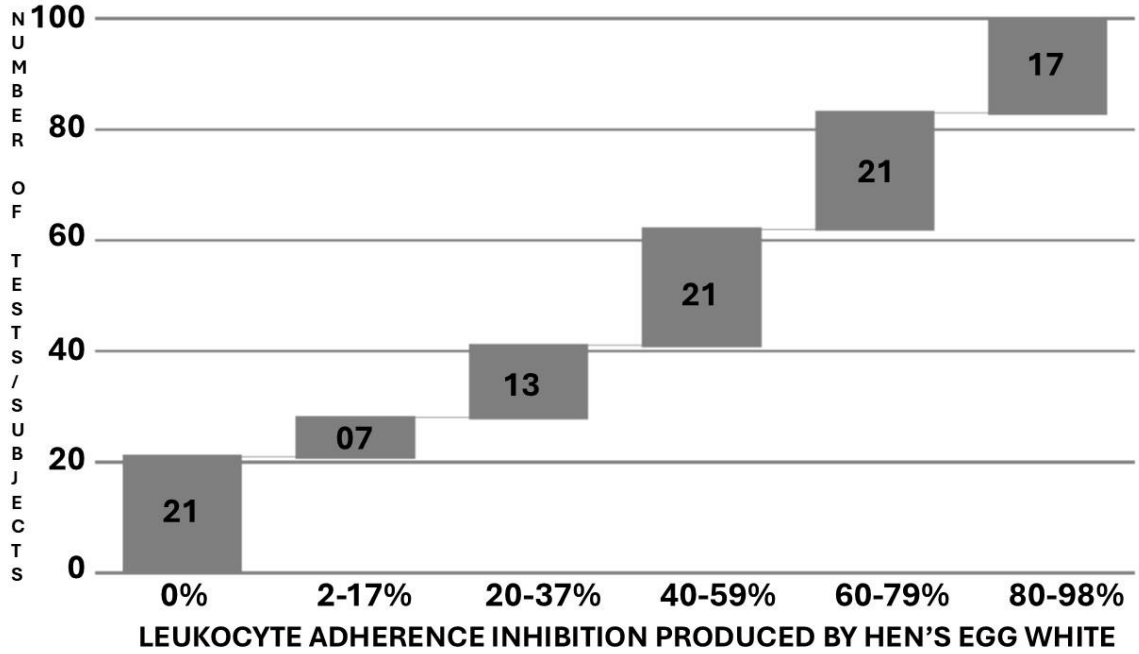

Fig. 2. Cascade distribution chart of the range groups of Leukocyte Adherence Inhibition (LAI) results (x-axis %) of the *ex vivo* challenge test against hen's egg white extract monitored by the Leukocyte Adherence Inhibition Test (LAIT), according to the respective number of outcomes over the LAIT cohort with 100 tests/subjects (y-axis).

4. DISCUSSION

When scientists need an animal model of chronic allergy to egg proteins, they administer mice subcutaneous injections of 10 µg of ovalbumin with an adjuvant such as aluminum hydroxide [46].

In 1931, Woodruff and Goodpasture reported the first technique employing chicken eggs to propagate viruses [47]. Nowadays, several immunization vaccines (such as for measles, mumps, rubella, influenza, yellow fever, Q fever, and rabies) are prepared from viruses propagated in eggs and present residual or significative contents of egg proteins, originating controversy about vaccination in patients with egg allergy [48-50]. Most concerns and safety evaluations about human parenteral egg components are always taken looking for immediate anaphylactic responses. However, no effective surveillance has ever been done about the possibility of worsening of the immunoreactivity after further immunogenic stimulus, or ever the risk presented by the parenteral administration of a food allergen protein to a non-yet-sensitized individual and the risk of developing further hypersensitization.

Laboratory allergy diagnosis to hen's egg white proteins is yet far from ideal, and continuous research is being done to improve the laboratory tools by developing new techniques [51].

LAIT and TTP performed with egg proteins seem to be promising tools for evaluating cellular and humoral responses after immunization with egg-propagated virus vaccines to evaluate the risk of development of eventual hypersensitivity reactions.

The primary strategy of personalized medicine is to use endotype biomarkers for cellular and humoral immunoreactivity to personalize treatments for allergic patients [52]. The semi-quantitative titration of precipitins is a pioneering laboratory exam upon which the fundamental bases of Immunology were constructed [53]. Precipitating antibodies suggest the presence of a humoral immune response against the tested antigens [54]. Before the discovery of IgE, researching precipitins against food allergens was the primary way to realize *in vitro* diagnosis of humoral immunoreactivity [55, 56].

The LAIT is an *ex vivo* challenge test performed with a viable leukocyte buffy coat that can theoretically explore most known immune pathways as it allows the interaction of all immune-circulating participants with the allergens [57]. Several immune pathways can produce the final leukocyte adherence inhibition [58-61].

The present study is a proof-of-concept that hypothesizes that LAIT and the TTP may differentiate diverse degrees of cellular and humoral immunoreactivity against hen's egg albumen among patients suffering from several non–IgE-mediated allergic phenotypes (urticaria, angioedema, atopic and contact dermatitis, allergic proctocolitis, eosinophilic esophagitis, food-protein induced enterocolitis, rhinitis, and bronchitis. 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 the albumen extract. These immunoassays provide evidence about cellular and humoral immunoreactivity distributed into an extensive spectral range that may suggest immune tolerance or hypersensitivity.

This preliminary retrospective survey demonstrated extensive results from the TTP and the *ex vivo* challenge test monitored by LAIT against hen's eggs albumen in two cohorts of patients with diverse non–IgE-mediated allergic phenotypes. 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 evaluated for immunoreactivity against several chemical and biological allergens, demonstrating positive results for some of them. Our results suggest that some of our patients may impair their allergic symptoms by additional immunoreactivity against hen's egg albumen allergens.

5. LIMITATIONS

This study is a retrospective analysis of data collected over six years and ten 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 a 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 by anamnesis, physical examination, undetectable specific IgE results, and non-reactive or inconclusive skin tests. The study lost the follow-up of many of these patients, making it impossible to establish a relationship between the immunoassays' results and the patient's clinical outcome. Unfortunately, it was not possible 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 hen's egg albumen in patients clinically diagnosed with diverse non–IgE-mediated allergic phenotypes (rhinoconjunctivitis, rhinosinusitis, bronchitis, atopic dermatitis, and urticaria). This methodology may be easily incorporated into specialized centers since the technologies to perform TIAL and TTP are relatively inexpensive and can be performed with minimum laboratory equipment. However, the technique depends on trained biomedical personnel performing artisanal and time-consuming laboratory procedures. As preliminary results, the propaedeutic meaning of these results and the possibility of interferents must be yet established [62]. 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 hen's egg white allergens [63].

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 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 etiologic diagnosis from their patients, as well as determine the endotypes (mechanisms) of hypersensitivity, in order to choose more convenient and personalized therapies for them.

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 [64].

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.

REFERENCES

1. Lawal RA, Martin SH, Vanmechelen K, et al. The wild species genome ancestry of domestic chickens. BMC Biology. 2020;18(1):13.
2. Bennett CE, Thomas R, Williams M, et al. The broiler chicken as a signal of a human reconfigured biosphere. 2018;5(12):180325.
3. Blackbum DG. Viviparity and oviparity: evolution and reproductive strategies. Encyclopedia of reproduction. 1999;4:994-1003.
4. Riber AB. Gregarious nesting—An anti-predator response in laying hens. Applied Animal Behaviour Science. 2012;138(1):70-78.
5. Sugino H, Nitoda T, Juneja L. General chemical composition of hen eggs. In: Hen eggs. CRC Press; 2018:13-24.
6. Campbell L, Raikos V, Euston SR. Modification of functional properties of egg-white proteins. Nahrung. 2003;47(6):369-376.
7. Smith M, F Back J. Studies on Ovalbumin II. The Formation and Properties of S-Ovalbumin, a More Stable Form of Ovalbumin %J Australian Journal of Biological Sciences. 1965;18(2):365-377.
8. Plancken IV, Van Remoortere M, Van-Loey A, Hendrickx ME. Trypsin Inhibition Activity of Heat-Denatured Ovomucoid: A Kinetic Study. Biotechnology Progress. 2004;20(1):82-86.
9. Chen X, Zhang X, Wu Y, Tong P, Gao J, Chen H. Effects of Iron Saturations on the Physicochemical Properties and Potential Physiological Functions of Ovotransferrin: Based on Structure-Activity Relationship. J Agric Food Chem. 2024;72(46):25648-25660.
10. Gossett PW, Rizvi S, Baker R. Quantitative analysis of gelation in egg protein systems. J Food Technology. 1984;38(5):67-96.
11. Johnson TM, Zabik ME. Gelation Properties of Albumen Proteins, Singly and in Combination. Poultry Science. 1981;60(9):2071-2083.
12. Razi SM, Fahim H, Amirabadi S, Rashidinejad A. An overview of the functional properties of egg white proteins and their application in the food industry. Food Hydrocolloids. 2023;135:108183.
13. Cristiano L, Guagni M. Zooceuticals and Cosmetic Ingredients Derived from Animals. 2022;9(1):13.
14. Rona RJ, Keil T, Summers C, et al. The prevalence of food allergy: A meta-analysis. Journal of Allergy and Clinical Immunology. 2007;120(3):638-646.

15. Schloss OM. A Case of Allergy to Common Foods. Am J Dis Child. 1912;3(6):341-362.
16. Hasan SA, Wells RD, Davis CM. Egg hypersensitivity in review. Paper presented at: Allergy Asthma Proc2013.
17. Tey D, Heine RG. Egg allergy in childhood: an update. Curr Opin Allergy Clin Immunol. 2009;9(3):244-250.
18. Allergen-Nomenclature-Sub-Committee. The official list of allergens from Gallus Domesticus. https://www.allergen.org/search.php?allergensource=Gallus&searchsource=Search. Accessed November 23, 2024.
19. Popescu FD. Cross-reactivity between aeroallergens and food allergens. World Journal of Methodology. 2015;5(2):31-50.
20. Quirce S, Díez-Gómez ML, Eiras P, Cuevas M, Baz G, Losada E. Inhalant allergy to egg yolk and egg white proteins. Clin Exp Allergy. 1998;28(4):478-485.
21. Olivier CE, Pinto DG, Teixeira APM, Santana JLS, Santos RAPG, Lima RPS. Self-imposed food restriction and oral food challenges are correlated with precipitin's accuracy in the diagnosis of non-IgE-mediated food-related adulthood acute episodes of urticaria. J Allergy Ther. 2021;12(8):1-8.
22. Olivier CE, Pinto DG, Teixeira APM, Santana JLS, Santos RAPGS, Lima RPS. Contribution of the Leukocyte Adherence Inhibition Test for the evaluation of immunoreactivity against gluten extracts in non—IgE-mediated / non-autoimmune Gluten-Related Disorders. European Journal of Clinical Medicine. 2022;3(2):1-7.
23. Olivier CE, Pinto DG, Teixeira APM, Santana JLS, Santos RAPGS, Lima RPS. Leukocyte Adherence Inhibition Test to the Assessment of Immunoreactivity Against Cow's Milk Proteins in Non—IgE-Mediated Gastrointestinal Food Allergy. Eur J Clin Med. 2022;3(2):38-43.
24. Olivier CE, Pinto DG, Lima RPS, et al. Assessment of Immunoreactivity against Therapeutic Options Employing the Leukocyte Adherence Inhibition Test as a Tool for Precision Medicine. European Journal of Clinical Medicine. 2021;2(3):40-45.
25. Kuratsuji T. Studies on leukocyte adherence inhibition test. Part II. Clinical applications of LAI test to detect delayed type hypersensitivity in infants and children. Keio J Med. 1981;30(2):65-69.
26. Olivier CE, Pinto DG, Teixeira APM, et al. Evaluating Non-IgE-mediated Allergens' Immunoreactivity in Patients with "Intrinsic" Persistent Rhinitis with Help of the Leukocyte Adherence Inhibition Test. Eur J Med Health Sci. 2023;5(1):17-22.
27. Olivier CE, Pinto DG, Teixeira APM, et al. Evaluating Non-IgE-Mediated Allergens' Immunoreactivity in Patients Formerly Classified as "Intrinsic" Asthmatics with Help of the Leukocyte Adherence Inhibition Test. Eur J Clin Med. 2023;4(2):1-7.
28. Olivier CE, Pinto DG, Teixeira APM, et al. Contribution of the Leukocyte Adherence Inhibition Test to the Diagnosis of Innate Non–IgE-mediated Immunoreactivity against *Alternaria alternata*. Asian J Immunol. 2023;6(1):243-251.
29. Olivier CE, Pinto DG, Teixeira APM, Santana JLS, Santos RAPG, Lima RPS. Contribution of the Leukocyte Adherence Inhibition Test to the Diagnosis of Innate Non–IgE-mediated Immunoreactivity against *Saccharomyces cerevisiae*. Asian J Immunol. 2023;6(1):234-241.
30. Olivier CE, Pinto DG, Teixeira APM, et al. Contribution of the Leukocyte Adherence Inhibition Test to the Diagnosis of Non–IgE-mediated Immunoreactivity against *Candida albicans* in Patients with Atopic Dermatitis. Asian J Immunol. 2023;6(1):268-276.
31. Olivier CE, Pinto DG, Teixeira APM, et al. Contribution of the Leukocyte Adherence Inhibition Test in Diagnosing Non–IgE-Mediated Immunoreactivity against *Aspergillus fumigatus* in Patients with Allergic Rhinitis and Asthma. Asian J Immunol. 2024;7(1):12-20.

32. Olivier CE, Argentão DGP, Santos RAPG, Silva MD, Lima RPS, Zollner RL. Skin scrape test: an inexpensive and painless skin test for recognition of immediate hypersensitivity in children and adults. Open Allergy J. 2013;6:9-17.
33. Coca AF. Studies in Specific Hypersensitiveness V. The Preparation of Fluid Extracts and Solutions for Use in the Diagnosis and Treatment of the Allergies with Notes on the Collection of Pollens. J Immunol. 1922;7(2):163-178.
34. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976;72:248-254.
35. Olivier CE, Lima RPS, Pinto DG, et al. In search of a tolerance-induction strategy for cow's milk allergies: significant reduction of beta-lactoglobulin allergenicity via transglutaminase/cysteine polymerization. Clinics. 2012;67(10):1171-1179.
36. Olivier CE, Santos RAPG, Lima RPS, Argentão DGP, Silva GKM, Silva MD. A Novel Utility for an Old Method: The Leukocyte Adherence Inhibition Test Is an Easy Way to Detect the Immunoreactive Interference of the Collection Tube Anticoagulant on Cellular Immunoassays. Journal of Cell Adhesion. 2014;Article ID 860427(http://dx.doi.org/10.1155/2014/860427):1-6.
37. Olivier CE, Pinto DG, Santos RAPG, Lima RPS. Dextran's interference over the Leukocyte Adherence Inhibition Test. Academia Letter. 2021; Article (number):3792.
38. Olivier CE, Pinto DG, Teixeira APM, Santana JLS, Santos RAPGS, Lima RPS. Immunoreactivity against Dermatophagoides pteronyssinus Assessed by the Leukocyte Adherence Inhibition Test in Patients with Intrinsic Atopic Dermatitis and Correlated "Intrinsic" Non–IgE-mediated Allergic Conditions. Eur J Clin Med. 2021;2(6):45-50.
39. Olivier CE, Pinto DG, Teixeira APM, Santana JLS, Santos RAPGS, Lima RPS. Contribution of the Leukocyte Adherence Inhibition Test to the Evaluation of Cellular Immunoreactivity against Latex Extracts for Non—IgE-Mediated Latex-Fruit-Pollen Syndrome in Allergic Candidates to Exclusion Diets and Allergic Desensitization. Eur J Clin Med. 2022;3(1):11-17.
40. C.E. Olivier, D.G. Pinto, A.P.M. Teixeira, C.S. Miguel, R.A.P.G. Santos, J.L.S. Santana, R.P.S. Lima, Endotyping Cellular and Humoral Immunoreactivity Against Tartrazine in Allergic Patients: A Retrospective Study, Asian J Immunol, 7 (2024) 217-227.
41. CE O, DG P, APM T, JLS S, RAPG S, 63-70. LRAJoI. Exploring the Role of Leukocyte Adherence Inhibition Test in Assessing Non-IgE Mediated Immunoreactivity to Benzoic Acid in Allergic Patients. Asian J Immunol. 2024;7(1):63-70.
42. Olivier CE, Pinto DG, Teixeira APM, Santana JLS, Santos RAPGS, Lima RPS. Intrinsic Atopic Dermatitis: Titration of Precipitins in the Screening of Food Allergens for Prescription of Elimination Diets and Desensitization Strategies. Eur J Clin Med. 2021;2(6):1-9.
43. Olivier CE, Pinto DG, Teixeira APM, et al. Endotyping Non-IgE-Mediated Immunoreactivity to *Dermatophagoides farinae*: Implications for Allergic Patients. Asian Journal of Immunology. 2024;7(1):90-99.
44. Olivier CE, Pinto DG, Teixeira APM, Santana JLS, Santos RAPG, Lima RPS. Endotyping Cellular and Humoral Immunoreactivity against Aluminum in Allergic Patients: A Retrospective Study. Asian J Immunol. 2024;7(1):149-158.
45. Williams CA, Chase MW. CHAPTER 13 - Precipitation Reactions. In: Reactions of Antibodies with Soluble Antigens. Vol 3. Academic Press. ; 1971:1-102.
46. Saldanha JCS, Gargiulo DL, Silva SS, et al. A model of chronic IgE-mediated food allergy in ovalbumin-sensitized mice. Brazilian Journal of Medical and Biological Research. 2004;37.
47. Woodruff AM, Goodpasture EW. The Susceptibility of the Chorio-Allantoic Membrane of Chick Embryos to Infection with the Fowl-Pox Virus. Am J Pathol. 1931;7(3):209-222.205.
48. Kelso JM, Yunginger JW. Immunization of egg-allergic individuals with egg- or chicken-derived vaccines. Immunol Allergy Clin. 2003;23(4):635-648.

49. Li JT, Rank MA, Squillace DL, Kita H. Ovalbumin content of influenza vaccines. J Allergy Clin Immunol. 2010;125(6):1412-1413; author reply 1413-1414.
50. Zeiger RS. Current issues with influenza vaccination in egg allergy. J Allergy Clin Immunol. 2002;110(6):834-840.
51. Zhang RW, Yuan DD, Yang X, et al. Detection of egg white allergy in children by specific IgE microarray chemiluminescence immunoassay. Clin Chim Acta. 2025;565:119966.
52. Agache I, Akdis CA. Endotypes of allergic diseases and asthma: An important step in building blocks for the future of precision medicine. Allergology International. 2020;65(3):243-252.
53. Wells HG. Studies on the chemistry of anaphylaxis (III). Experiments with isolated proteins, especially those of the hen's egg. Journal of Infectious Diseases. 1911;9:147-171.
54. Gell PGH, Harington CR, Rivers RP. The antigenic function of simple chemical compounds; production of precipitins in rabbits. Brit J Exp Pathol. 1946;27(5):267-286.
55. Cunningham-Rundles C, Brandeis WE, Good RA, Day NK. Milk precipitins, circulating immune complexes, and IgA deficiency. Proc Nat Acad Sci USA. 1978;75(7):3387-3389.
56. Ferguson A, Carswell F. Precipitins to dietary proteins in serum and upper intestinal secretions of coeliac children. Br Med J. 1972;1(5792):75-77.
57. Olivier CE, Lima RPdS, Pinto DG, Santos RAPGd. The Plasma Preincubation with Papain Before the Assay Suggests that a Gell and Coombs Type II Reaction is Been Demonstrated by the Leukocyte Adherence Inhibition Test. Biom J Sci Tech Res. 2021;36(3):28647 - 28655.
58. Thomson DMP. Assessment of immune status by the leukocyte adherence inhibition test. New York: Academic Press; 1982.
59. Tong AW, Burger DR, Finke P, Barney C, Vandenbark AA, Vetto RM. Assessment of the mechanism of the leukocyte adherence inhibition test. Cancer Res. 1979;39(2 Pt 2):597-603.
60. Fink A, Heller L, Eliraz A, et al. Allergen-specific leukocyte adherence inhibition (LAI) assay: sensitivity, specificity and mechanism. Immunol Lett. 1987;16(1):65-70.
61. Halliday WJ, Maluish A, Miller S. Blocking and unblocking of cell-mediated anti-tumor immunity in mice, as detected by the leucocyte adherence inhibition test. Cell Immunol. 1974;10(3):467-475.
62. Anouar S, Hazim R, Brahim A. Interferences in Immunological Assays: Causes, Detection, and Prevention. Asian J Immunol. 2024;7(1):71-78.
63. Chiarentin L, Gonçalves C, Augusto C, Miranda M, Cardoso C, Vitorino C. Drilling into "Quality by Design" Approach for Analytical Methods. Crit Rev Anal Chem. 2023:1-42.
64. WMA. World Medical Association Declaration of Helsinki: ethical principles for medical research involving human subjects. JAMA. 2013;310(20):2191-2194.