

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

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ABSTRACT

Background: Due to the lack of standardized laboratory procedures able to demonstrate specific non—IgE-mediated immune responses against latex allergens, these conditions are diagnosed mostly by clinical criteria based on empiric exclusion prescriptions monitored by *in vivo* challenge tests.

Objective: To evaluate the opportunity of an *ex vivo* challenge immunoassay, the Leukocyte Adherence Inhibition (LAI) Test (LAIT), to discriminate non—IgE-mediated latex-specific immunoreactivity.

Methods: *Ex vivo* challenge tests performed with *Hevea Brasiliense*'s latex extract, monitored by LAIT, were assayed in an asymptomatic control group and a group of patients with diverse respiratory and cutaneous non—IgE-mediated allergic conditions, clinically diagnosed by a certified allergologist.

Results: The mean LAI of the control group was 8.3%. The mean LAI of the complete patients' group was 41.1%. The non-parametric Wilcoxon-Mann-Whitney U (WMWU) test comparing the control group with the whole patient's group showed significance with a *p*-value < 0.00001. The WMWU test comparing the control group with each other patient's group showed significance with a *p*-value < α = 0.05 for all comparisons. The WMWU test comparing the patients' groups between each other did not show any significant *p*-value.

Conclusion: Several patients from the diverse non—IgE-mediated allergic phenotypes presented variable immunoreactivity against the latex extract, as demonstrated by the LAIT, which proved to be an easy, quick, and inexpensive *ex vivo* immunoassay with the potential to predict individual immunoreactivity against *Hevea brasiliensis* latex allergens in real-world patients with non—IgE-mediated allergies.

Keywords: Allergic conjunctivitis, allergic rhinitis, asthma, atopic dermatitis, *Hevea brasiliensis*, hypersensitivity, latex, leukocyte adherence inhibition test, pharyngitis, urticaria.

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I. INTRODUCTION

The *Hevea brasiliensis* (*Hb*), also known as rubber tree, is a member of the Euphorbiaceae family that possesses a great latex regenerative capacity allowing its renewable exploitation at an industrial scale [1]. Latex gloves have begun to be used at the beginning of the 1900s by medical surgeons, and until now, they are recognized by their nickname: "surgical gloves" [2]. Until the eighties, the use of latex gloves was not a strong habit among health professionals, being restricted to the operatory field. Little procedures were usually performed with bare hands. Until

then, there were sparse reports of allergic reactions to latex [3]. Initially, the individuals that developed latex allergy usually were medical surgeons or patients submitted to multiple surgical procedures [4]. After the appearance of the HIV epidemic, there was an overspread use of disposable protective devices, turning the natural rubber allergy more common among other health workers, as well the appearance of latex allergy among condom users [5], [6]. After the improvement of the industrial techniques, and the implementation of controlled rubber tree cultivation areas around the world, the disposable latex gloves became more accessible, and their use become more common among non-

health professionals, such as the hairdressers, the food handlers, and the children's caretakers, changing the profile of the latex-allergic individuals [7], [8]. The appearance of the COVID-19 pandemic has intensified the use of protective gloves, however, nowadays, there are several options to replace latex in the production of disposable gloves, such as vinyl or nitrile.

The *Hb* latex is the cytoplasm of the laticifers, specialized cells that produce a diversity of biopolymers of $(C_5H_8)_n$ isoprene units (natural rubber) to heal traumatic injuries against the tree [9]. Secreted with the natural rubber, there is a high variety of allergenic proteins and enzymes, that share common epitopes with other vegetal compounds [10]. The Allergen Nomenclature Sub-Committee of the World Health Association and the International Union of Immunological Societies cataloged, until now, 15 major groups of allergens from the *Hb* latex (Hev b 1 to 15) [11]. Several of these are pan-allergens, such as the Heveins (Hev b 6), the Patatin homolog (Hev b 7), the Profilins (Hev b 8), the Chitinases (Hev b 11), and the Lipid Transport Protein (LTP-Hev b 12), that share homologous epitopes with proteins of several fruits, tubers, and pollens [12], [13]. The cross-reactivity among latex allergens and proteins of pollens and several edible fruits and tubers began to be described by the nineties, originating the designation "latex-fruit-pollen syndrome" [14], [15]. Latex allergens may be responsible for IgE-mediated, non-IgE-mediated, and mixed hypersensitivity reactions [13]. The great variety of allergens of the *Hb* latex and the diversity of hypersensitivity reactions and cross-reactions turn the allergy to natural latex proteins into a complex immune condition requiring a multi-parametric approach [10]. The main strategy to treat these latex-fruit-pollen syndromes is avoidance and sublingual desensitization, a practice first described in 1901 with natural pollens and nowadays with their allergoids [16]-[24]. The diagnosis of the Gell and Coombs' type I IgE-mediated hypersensitivity is a relatively easy task done with the help of allergic skin tests or immunoassays designed to detect latex-specific IgE. The type IV Gell and Coombs' cellular hypersensitivity reactions usually are diagnosed by *in vivo* challenge tests such as the contact tests [25]. To better comprehend the type II Gell and Coombs' non-IgE-mediated hypersensitivity immune mechanisms around the latex hypersensitivity, we perform the Leukocyte Adherence Inhibition Test (LAIT) in outpatients with diverse non-IgE-mediated allergic conditions. The main objective was to evaluate the possibility of unsuspected participation of latex hypersensitivity in the patients' symptoms that could suggest a further clinical investigation inside a management strategy, considering a diagnostic/therapeutic exclusion diet and/or the indication of allergen desensitization.

The Leukocyte Adherence Inhibition Test (LAIT) is an *ex vivo* challenge test designed by Halliday, in 1972, to evaluate the inhibitory effect of specific antigens on the glass adherence of leukocytes [26]-[31]. When not activated, leukocytes kept in live conditions possess the natural capacity to adhere to glass. When challenged by specific antigens to which they are sensitized, the leukocytes release paracrine soluble factors that interfere with glass adherence of nearby leukocytes, a nonspecific phenomenon, that can be quantified with a concomitant assay done with unchallenged plasma

[32]-[36]. Besides the leukocyte participation, the specific inhibition of the glass adherence also requires the engagement of specific antibodies, suggesting a type II Gell and Coombs antibody-dependent cellular-mediated immune response [37]-[40].

II. METHODS

A. Subjects

After receiving Institutional Review Board approval, from the Instituto Alergoimuno de Americana (Brazil), a group of 456 patients (112 male; 18-90 years old; mean age = 49.1 years, SD = 16,7 years) and a control group of 16 non-allergic subjects (4 male; 25-70 years old; mean age = 47.8 years, SD = 13,4 years) were invited, with informed consent formularies, to voluntarily be submitted to allergy skin tests and provide blood samples to research specific IgE antibodies and to perform *ex vivo* challenge tests, according to the principles of Helsinki and the International Committee of Medical Journals Editors requirements of privacy [41]. The control did not present any allergic symptoms. All patients presented clinical signals and symptoms of allergic diseases, classified in groups, as described below. Patients and control-group individuals had non-detectable serum-specific IgE and non-reactive skin tests against latex extracts and at least 20 other diverse respiratory and food allergens [42]. The study was descriptive, retrospective, and did not interfere with the patient's treatment or the assistant physician's diagnosis. All relevant and mandatory laboratory health and safety measures have been complied with, within the complete course of the experiments.

B. Clinical Groups

1) Intrinsic Atopic Dermatitis Group (iAD)

Patients presenting exclusively signals and symptoms of intrinsic Atopic Dermatitis (iAD), without blood serum evidence of IgE-mediated hypersensitivity, and not showing other signs and/or respiratory or ocular symptoms were classified in the iAD group (n = 134; male = 34; mean age = 51.5 years; range: 18-87 years; SD = 18.1 years).

2) Intrinsic Allergic Rhinitis Group (iAR)

Patients presenting purely with signals and symptoms of Allergic Rhinitis, without blood serum evidence of IgE-mediated hypersensitivity, and not showing other signs and/or cutaneous or respiratory symptoms were classified in the intrinsic Allergic Rhinitis (iAR) group. The nickname "intrinsic" was just added to emphasize the fact that there was no evidence of systemic IgE mediation, similarly to what is already established concerning the iAD (n = 75; male = 19; mean age = 47 years; range 18-85 years, SD = 17 years).

3) Intrinsic Ocular Allergy group (iOA)

Patients referred by their ophthalmologists, with a clinical diagnosis of Ocular Allergy, without blood serum evidence of serum IgE-mediated hypersensitivity, and not showing other signs and/or cutaneous or respiratory symptoms were classified in the intrinsic Ocular Allergy (iOA) group. The nickname "intrinsic" was just added to emphasize the fact that there was no evidence of systemic IgE mediation, similarly to what is already established concerning the iAD (n = 16; male

= 5; mean age = 46.1 years; range = 19-71 years; SD = 16.7 years).

4) Intrinsic Asthma group (iAS)

Patients presenting signals and symptoms of Asthma, without evidence of blood serum IgE-mediated hypersensitivity, with or without symptoms of allergic rhinitis, and not showing other signs and/or cutaneous or ocular symptoms were classified in the intrinsic Asthma (iAS) group. The nickname “intrinsic” was just added to emphasize the fact that there was no evidence of systemic IgE mediation, similarly to what is already established concerning the iAD (n = 39; male = 10; mean age = 50.2 years; range = 18-80 years; SD = 15.6 years).

5) Intrinsic Atopic Dermatitis / Intrinsic Allergic Rhinitis group (iAD/iAR)

Patients presenting conjoint signals and symptoms of Allergic Rhinitis and Atopic Dermatitis, without evidence of blood serum IgE-mediated hypersensitivity, and not showing other respiratory signs and/or ocular symptoms were classified in the iAD/iAR group (n = 36; male = 5; mean age = 46.1 years; range = 18-82 years; SD = 16.2 years).

6) Intrinsic Chronic Urticaria Group (iCU)

Patients with persistent or recurrent urticaria for more than 2 months, with no evidence of IgE-mediated sensitization, were classified in the intrinsic Chronic Urticaria (iCU) group. The nickname “intrinsic” was just added to emphasize the fact that there was no evidence of systemic IgE mediation, similarly to what is already established concerning the iAD (n = 103; male = 25; mean age = 47 years; range = 18-99 years; SD = 16 years).

7) Intrinsic Chronic Pharyngitis (iCP)

Most patients with Chronic Pharyngitis search the allergists due to concomitant symptoms of Allergic Rhinitis. The clinical sign defining this condition is the presence of hyperemic elevated plaques of reactive lymphoid tissue in the oropharynx [43]-[45]. They were classified in the intrinsic Chronic Pharyngitis (iCP) group with the nickname “intrinsic” just to emphasize the fact that there was no evidence of systemic IgE mediation, similarly to what is already established concerning the iAD. (n = 53; male = 14; mean age = 52.3 years; range 18-83 years; SD = 13 years).

C. Latex extraction

The *Hevea brasiliensis* centrifugated latex was bought from a local supplier (COLITEX – Poloni – São Paulo - Brazil) in liquid form (60% latex: 1% ammonia). The latex was extracted in Coca's solution at 4 °C for 48 hours, before five steps of centrifugation and filtration for separation of the water-soluble fraction from solid particles [46]. The protein quantification of the allergen extracts was done according to Bradford's protein-dye binding methodology [47]. The *Hb* latex extract was diluted to a protein concentration of 1 mg/mL and stored at 4 °C. All relevant and mandatory laboratory health and safety measures have been complied with in the complete course of the experiments.

D. Leukocyte Adherence Inhibition Test

Plasma samples were collected in heparinized collection tubes. The *ex vivo* challenge tests were performed as described previously [48]. Shortly, each donor's fresh plasma

was divided into two parts and used in paralleled *ex vivo* challenging tests with *Hb* latex extract and the unchallenged plasma assay. The plasma with high leukocyte content (buffy coat) was collected from the heparinized tube after one hour of sedimentation at 37 °C and aliquots of 100 µL were distributed into Eppendorf tubes kept under agitation for 30 minutes (200 rpm at 37 °C) with (or without, as used as control) antigen extract (10µL of a solution with 1mg/mL and pH 7.5). 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, leukocytes were counted, the coverslip was removed, and the chamber was washed by immersion in a beaker with PBS at 37 °C. A drop of PBS was added to the hemocytometer chamber and a clean coverslip was placed over it. The remaining cells were counted in the same squares as previously examined. 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 groups and the LA from the unchallenged control group: LAR = LA of the challenged sample divided by LA of unchallenged control sample; multiplied by 100 (%). To further calculate the Leukocyte Adherence Inhibition (LAI) the LAR was subtracted from 100 (%).

E. Graphic Presentation of Data and Statistics

A column graph was plotted with the mean LAIT results of each group (Fig. 1). Cascade graphs were assembled according to the distribution of the tests among the range of results of each group (Fig. 2 to 9). The data of the patients' groups were compared with the control group by the non-parametric Wilcoxon-Mann-Whitney U test (Table I) [49], [50].

III. RESULTS

TABLE I: NON-PARAMETRIC WILCOXON-MANN-WHITNEY U TEST (WMWUT) COMPARING THE CONTROL GROUP WITH EACH OTHER PATIENT'S GROUP

Group	Group	U	z-score	p-value
Control	Patients	1,421.5	415.09	< 0.00001
Control	iAR	286.5	3.30	0.00104
Control	iAS	133	330.78	0.00094
Control	iCP	192.5	3.30	0.00104
Control	iCU	261	4.38	< 0.00001
Control	iAD/iAR	109	3.53	0.0004
Control	iAD	400	4.08	< 0.00001
Control	iOA	39.5	(-) 3.31	0.0009

The level of significance adopted was the p-value < α = 0.05.

The mean LAI of the control group was 8.3% (range = 0 – 53%; SD = 14.7%). The mean LAI of the complete patients' group was 41.1% (range = 0–100%; SD = 31.3%). The mean LAI of the iAR group was 35.9% (range = 0–100%; SD = 31.2%). The mean LAI of the iAS group was 38.1% (range = 0–94%; SD = 30.8%). The mean LAI of the iCP group was

38.2% (range = 0–99%; SD = 31.6%). The mean LAI of the iCU group was 42.3% (range = 0–100%; SD = 28.9%). The mean LAI of the iAD/iAR group was 42.9% (range = 0–100%; SD = 32%). The mean LAI of the iAD group was 44% (range = 0–100%; SD = 32.6%). The mean LAI of the iOA group was 46.9% (range = 0–88%; SD = 29.3). The non-parametric Wilcoxon-Mann-Whitney U (WMWU) test comparing the control group with the whole patient's group showed significance with a p -value < 0.00001 . The WMWU test comparing the control group with each patient's group showed significance with a p -value $< \alpha = 0.05$ for all comparisons. The non-parametric Wilcoxon-Mann-Whitney U test comparing the patients' groups between each other did not show any significant p -value $< \alpha = 0.05$. The cascade graphs visually showed that most subjects of the control group did not present significant immunoreactivity against the latex extract, while there was a heterogeneous distribution of results inside each patients group, demonstrating that several patients from the diverse allergic phenotypes groups presented a significant immunoreactivity against the latex extract, as demonstrated by the TIAL.

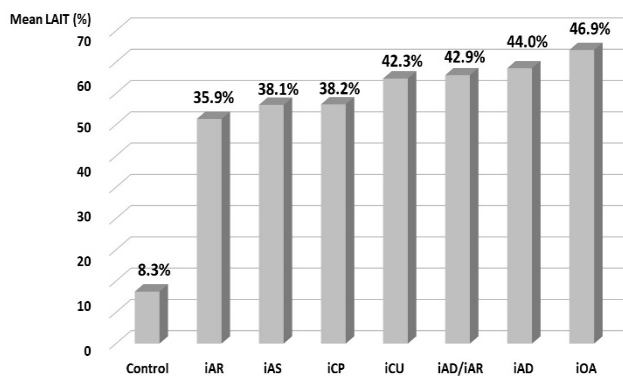


Fig. 1. Column comparison chart with the average Leukocyte Inhibition (%) of the *ex vivo* challenge tests performed with *Hevea brasiliensis* latex extract, monitored by Leukocyte Adherence Inhibition Tests, grouped according to the control group and clinical symptoms of patients' groups. iAR: intrinsic Allergic Rhinitis; iAS: intrinsic Asthma; iCP: intrinsic Chronic Pharyngitis; iCU: intrinsic Chronic Urticaria; iAD/iAR: combined intrinsic Atopic Dermatitis and intrinsic Allergic Rhinitis; iAD: intrinsic Atopic Dermatitis; iOA: intrinsic Ocular Allergy.

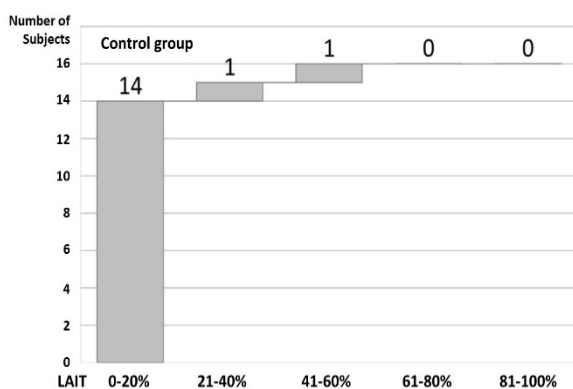


Fig. 2. Cascade distribution chart of the number of *ex vivo* challenge tests performed with *Hevea brasiliensis* latex extract monitored by Leukocyte Adherence Inhibition Tests, according to the range of results (%) of Leukocyte Adherence Inhibition (LAI) of 16 control subjects, presenting no allergic-related symptoms.

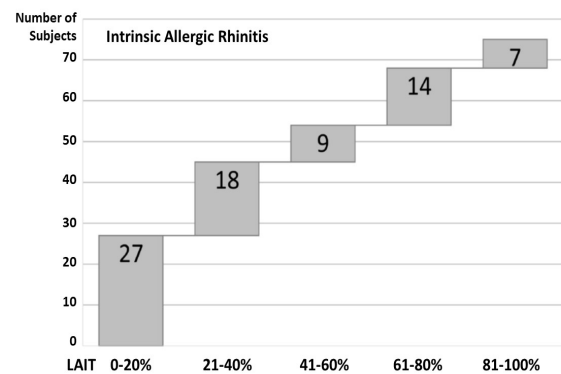


Fig. 3. Cascade distribution chart of the number of *ex vivo* challenge tests performed with *Hevea brasiliensis* latex extract monitored by Leukocyte Adherence Inhibition Tests, according to the range of results (%) of Leukocyte Adherence Inhibition (LAI) of 75 patients with non-IgE-mediated intrinsic Allergic Rhinitis (iAR).

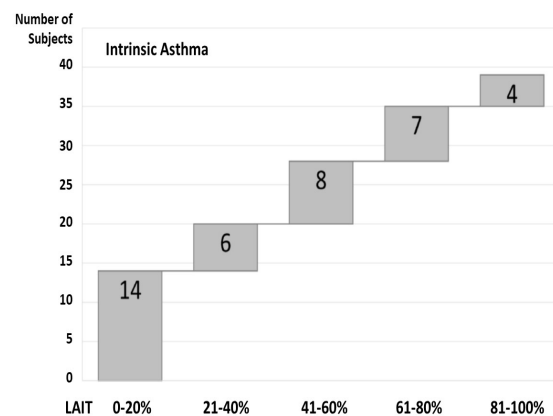


Fig. 4. Cascade distribution chart of the number of *ex vivo* challenge tests performed with *Hevea brasiliensis* latex extract monitored by Leukocyte Adherence Inhibition Tests, according to the range of results (%) of Leukocyte Adherence Inhibition (LAI) of 39 patients with non-IgE-mediated intrinsic Asthma (iAS).

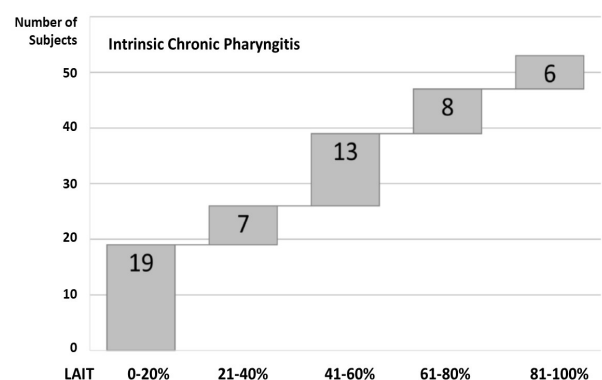


Fig. 5. Cascade distribution chart of the number of *ex vivo* challenge tests performed with *Hevea brasiliensis* latex extract monitored by Leukocyte Adherence Inhibition Tests, according to the range of results (%) of Leukocyte Adherence Inhibition (LAI) of 53 patients with non-IgE-mediated intrinsic Chronic Pharyngitis (iCP).

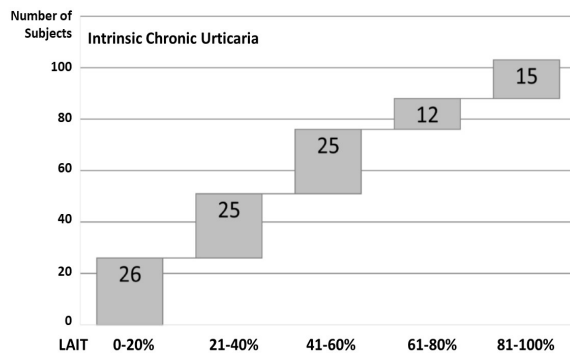


Fig. 6. Cascade distribution chart of the number of *ex vivo* challenge tests performed with *Hevea brasiliensis* latex extract monitored by Leukocyte Adherence Inhibition Tests, according to the range of results (%) of Leukocyte Adherence Inhibition (LAI) of 103 patients with non—IgE-mediated intrinsic Chronic Urticaria (iCU).

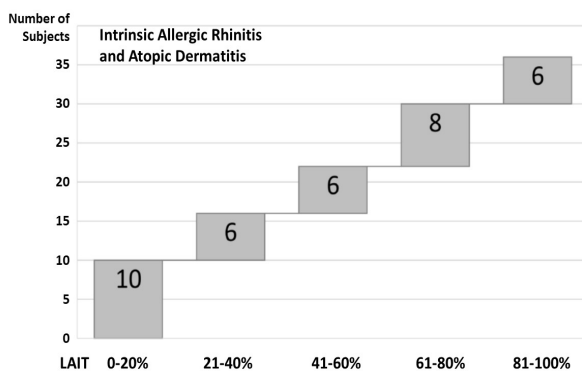


Fig. 7. Cascade distribution chart of the number of *ex vivo* challenge tests performed with *Hevea brasiliensis* latex extract monitored by Leukocyte Adherence Inhibition Tests, according to the range of results (%) of Leukocyte Adherence Inhibition (LAI) of 36 patients with non—IgE-mediated combined intrinsic Atopic Dermatitis and intrinsic Allergic Rhinitis (iAD/iAR).

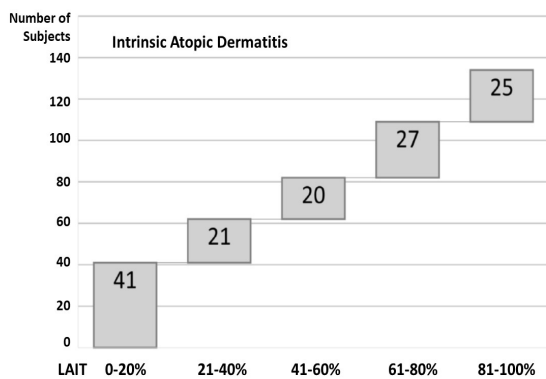


Fig. 8. Cascade distribution chart of the number of *ex vivo* challenge tests performed with *Hevea brasiliensis* latex extract monitored by Leukocyte Adherence Inhibition Tests, according to the range of results (%) of Leukocyte Adherence Inhibition (LAI) of 134 patients with non—IgE-mediated intrinsic Atopic Dermatitis (iAD).

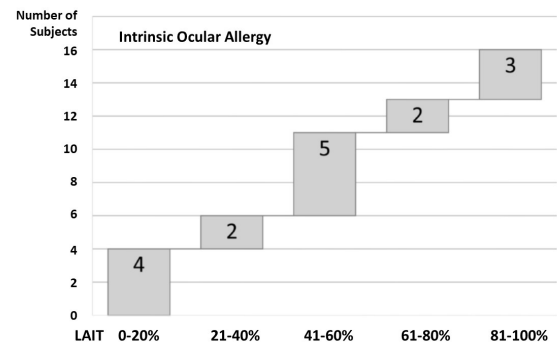


Fig. 9. Cascade distribution chart of the number of *ex vivo* challenge tests performed with *Hevea brasiliensis* latex extract monitored by Leukocyte Adherence Inhibition Tests, according to the range of results (%) of Leukocyte Adherence Inhibition (LAI) of 16 patients with non—IgE-mediated intrinsic Ocular Allergy (iOA).

IV. DISCUSSION

When classifying the diverse kinds of hypersensitivity reactions, Gell and Coombs described three distinct non—IgE-mediated groups of immune mechanisms that could produce clinically significant syndromes. The Gell and Coombs classification is rather a broader vision of four groups of immune interactions, according to the main participants of the sequential chain of events producing the disease. However, the immune system is not so simplistically compartmentalized, and the existence of one mechanism of hypersensitivity does not exclude the others. The complex immune interactions, as we know nowadays, speak more in favor that rather than a single mechanism producing allergic disease, there are instead “mixed” mechanisms participating in the physiopathology. When searching for a culprit for an allergic symptom, the physicians usually stop the search when it is found an IgE-mediated hypersensitivity, however, the patient may also be under the influence of uncovered non—IgE-mediated hypersensitivities. The discrepancy between two groups of patients with absolutely the same clinical presentation and different IgE profiles, gives rise to the concept of “extrinsic” and “intrinsic” allergy, as a reference to two phenotypes of patients according to the evidence (or not) of IgE-mediated hypersensitivity [51]-[54]. The significant difference between the mean LAI of the control group and the patients’ groups demonstrated that the *ex vivo* challenge test performed with the *Hb* latex extract, monitored by the LAIT, can differentiate the specific immunoreactivity between the groups. The largest LAI found in the control group was 53%, which is possibly due to an asymptomatic sensitization since most *ex vivo* challenge tests from the control group resulted in the LAI = 0%. This fact also states that the finding of immunoreactivity against an allergen as demonstrated by the LAIT does not mean necessarily the existence of an allergic disease. Anyway, the link between the immunoreactivity demonstrated by the LAIT and the effective participation of the allergen in the pathophysiology of each allergic patient may only be determined by a careful *in vivo* challenge test initiated with an exclusion diet and a controlled re-introduction of the allergen through an Oral Challenge Test. The lack of clinically available immunoassays to predict the non—IgE-mediated hypersensitivities turns, literally, the diagnosis of these conditions, a challenge to most physicians. The *in vivo*

challenge tests are laborious, costly, time-wasting, and depend on a previous successful exclusion diet to allow the resurgence of the allergic symptoms. The feasibility of an *ex vivo* challenge test able to select a group of antigens to proceed with the exclusion diet and the further *in vivo* oral challenges tests is a highly desirable tool. The inhibition of the leukocytes' glass adherence is an indicator of the existence of a specific immunoreactivity against a given antigen. It does not diagnosis a clinical disease but may point out some suspects to be appreciated by the judgmental clinical eye. The LAIT may be used as a triage test that just indicates the release of cytokines after the encounter with a specific antigen.[55] The LAIT is a feasible test, easily adaptable to the routine of a medical facility dedicated to the diagnosis and/or the treatment of allergic patients. In our series, several patients from the diverse non—IgE-mediated allergic phenotype presented variable immunoreactivity against the *Hb* latex extract, as demonstrated by the LAIT, which proved to be an easy, quick, and inexpensive *ex vivo* immunoassay with the potential to predict individual immunoreactivity against latex allergens in real-world patients with non—IgE-mediated allergies.

ABBREVIATIONS

Hb: *Hevea brasiliensis*
iAR: intrinsic Allergic Rhinitis
iAS: intrinsic Asthma
iAD: intrinsic Atopic Dermatitis
iCP: intrinsic Chronic Pharyngitis
iCU: intrinsic Chronic Urticaria
iOA: intrinsic Ocular Allergy
LA: Leukocyte Adherence
LAR: Leukocyte Adherence Ratio
LAI: Leukocyte Adherence Inhibition
LAIT: Leukocyte Adherence Inhibition Test
WMWU test: Wilcoxon-Mann-Whitney U test

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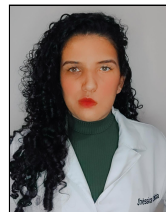
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