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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

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ABSTRACT

Background: **Due to the lack of standardized laboratory procedures able to demonstrate specific immune responses against the culprit allergens, the non—IgE-mediated allergy syndromes are a group of conditions diagnosed mostly by clinical examination and exclusion criteria.**

Objective: **To evaluate the opportunity of the Leukocyte Adherence Inhibition Test (LAIT) to discriminate specific immunoreactivity against *Dermatophagoides pteronyssinus* (*Dp*) in a group of patients with non–IgE-mediated chronic allergic conditions.**

Methods: ***Ex vivo*** **challenge tests performed with *Dp* were monitored by LAIT in patients presenting diverse non–IgE-mediated allergic conditions: intrinsic Atopic Dermatitis (iAD), intrinsic Allergic Rhinitis (iAR), intrinsic Ocular Allergy (iOA), intrinsic Chronic Pharyngitis (iCP), and intrinsic Asthma (iAS).**

Results: **The mean LAI of the control group was 7%; the mean LAI of the iAR group was 34%; the mean LAI of the iCP group was 44%; the mean LAI of the iAS group was 45%; the mean LAI of the iOA group was 47%; the mean LAI of the iAD group was 55%. The non-parametric Wilcoxon-Mann-Whitney U test comparing the control group with each other group showed significance with p-value < $\alpha = 0.05$ for all groups.**

Conclusion: **The Leukocyte Adherence Inhibition Test is an easy, quick, and inexpensive *ex vivo* immunoassay with the potential to predict individual immunoreactivity against HDM allergens in real-world patients with non–IgE-mediated allergies.**

Keywords: Allergens, allergic conjunctivitis, allergic rhinitis, antigen, asthma, atopic dermatitis, *Dermatophagoides pteronyssinus,* leukocyte adherence inhibition test, immunoassay, hypersensitivity, pharyngitis.

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I. Introduction

There are several ways to classify allergies [1]. In real-world clinical practice, most allergic diseases are initially classified as IgE-mediated or non–IgE-mediated [2]. Some conditions are understood as having a mixed-physiopathology, with concomitant IgE-mediated and non–IgE-mediated mechanisms [3]. This pragmatic classification is done just by the fact that the research of specific IgE in serum is a broadly disseminated technology, easily accessible to most clinical laboratories. However, most IgE-mediated diseases present an equivalent clinical condition in which there is no detectable serum IgE [4]. One of the most remarkable conditions is Atopic Dermatitis (AD), which is classified as "extrinsic" (when there is evidence of serum IgE), and "intrinsic" when there is no evidence of serum IgE. Remarkably, on the clinical examination, there is no physical distinction between intrinsic Atopic Dermatitis (iAD) and extrinsic Atopic Dermatitis (eAD). Most remarkable is the fact that the serum circulating IgE, as detectable by the routine immunoassays, are not the real responsible for the allergic disease, since the Gell and Coombs' type I allergic reaction is produced by the IgE linked to the high-affinity IgE-receptor FcεRI on the surface of the effector cells, such as mast cells, basophils, neutrophils, eosinophils, monocytes, platelets, and dendritic cells [5]-[8]. Alongside the high-affinity IgE-receptor, there is the low-affinity IgE-receptor, (FcεRII or CD23) that is found in lymphocytes, macrophages, eosinophils, dendritic cells, and epithelial cells [9]. It is for this reason that Chinese researchers have proposed to detect

IgE, not in the serum, but in the plasma, collected with EDTA, describing a simple method to dissociate cell-bound IgE with lactic acid [10]. Through this technology, some patients initially classified as "intrinsic" could be re-allocated as "extrinsic". While the high-affinity IgE-receptor binds preferentially to free IgE, the low-affinity IgE-receptor binds preferentially to IgE immunocomplexes [11], [12]. This knowledge also suggests that the IgE may also participate in Gell and Coombs' type II and type III hypersensitivity reactions. Equivalent to IgE-mediated Allergic Rhinitis (AR), the non-systemic IgE-mediated condition, the Local Allergic Rhinitis (LAR) is also associated with House Dust Mite (HDM) hypersensitivity, as proven by *in vivo* challenge tests performed with *Dermatophagoides pteronyssinus* (*Dp*) extracts (nasal provocation tests) and the research of *Dp*-specific IgE on nasal secretions collected after that [13]. The association of HDM systemic IgE-mediated hypersensitivities manifested as eAD, AR, Ocular allergy (OA), and/or Asthma, are well-known medical conditions [14]-[17] Similarly, it has been found that *Dp,* as well other aeroallergens, might be relevant pathogens for non–IgE-mediated conditions, as suggested by the atopy patch tests, immunoblot, and epidermal dendritic cell phenotyping [18]. In fact, the immunoreactivity against *Dermatophagoides* extracts, demonstrated by atopy patch tests, is greater in iAD (66.6%) than in eAD (47.4%), and both significantly greater than in healthy subjects (12.2%) [19]. Another way employed to study the cellular immunoreactivity of HDM extracts is the Leukocyte Adherence Inhibition Test (LAIT), as designed by Halliday [20]. Preliminary studies demonstrated that the results of the LAIT were specific for the HDM antigens responsible for delayed-type reactions verified 48 hours-reading after intradermic injections [21]. However, the study of non–IgE-mediated hypersensitivity reactions against HDM allergens is a very complex subject, since the HDM extracts possess a myriad of biologically active proteins which interfere in the host immune physiology. One of the HDM's main allergens is *Der p1*, a cysteine protease responsible for epithelial cell desquamation [22]. This and other proteases produced by the HDM are responsible for immune-independent pathogenic mechanisms that facilitate the transport of allergens across the host's epithelial barriers [23]. Besides the rupture of tight junctions, these proteases also interfere in the leukocytes mechanisms of defense, such as the polymorphonuclear migration, by degrading the epithelial-derived neutrophil attractant (ENA)-78/CXCL5 [24]. With these facts in mind, one must consider that the cellular responses provoked by *ex vivo* challenges with HDM extracts are just the final result of the biologic interaction of these active enzymes with specific and unspecific host's immune mechanisms. We must also consider that these immune mechanisms are produced, exactly to neutralize the effect of these HDM enzymes. Specific *ex vivo* challenges tests, such as the LAIT, besides the cellular involvement, also allow the participation of antibodies and immune complexes, mimetizing a type II and/or a type III Gell and Coombs' hypersensitivity immune response [25]-[28]. These complexes interactions make it mandatory that the interpretation of the *ex vivo* tests, done with HDM extracts, be always done in accordance with the clinical evaluation of the patient diagnosed with the studied conditions, since the isolated demonstration of immunoreactivity against a given antigen, does not mean, necessarily, the presence of an allergic disease. Motivated by these questions, we invited a heterogeneous group of allergic outpatients, that possessed in common the inexistence of detectable serum specific IgE against Dp (and other allergens), to donate blood to evaluate the performance of the *ex vivo* challenge test with *Dp* monitored by the LAIT. These patients were grouped according to their clinical diagnosis and theirs tests compared with the results of an asymptomatic control group.

II. Methods

A. Subjects

After receiving Institutional Review Board approval, from the Instituto Alergoimuno de Americana (Brazil), 467 outpatients (120 male; 18-91 years old; mean age = 49.7 years, SD = 17.2 years) were invited, with informed consent formularies, to voluntarily provide blood samples to perform *ex vivo* challenge tests, according to the principles of Helsinki and the International Committee of Medical Journals Editors requirements of privacy [29]. All patients presented clinical signals and symptoms of allergic diseases temporally related with exposition to house dust and clinically compatible with HDM hypersensitivity. The control group (n = 21, male = 8; mean age = 47.7 years; range = 19-77 years; SD = 16.4 years) did not present any allergic symptoms. Patients and control-group individuals had non-detectable serum-specific IgE and non-reactive skin tests against Dp and at least 20 other diverse respiratory and food allergens [30]. 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 Atopic Dermatitis, 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 = 161; male = 48; mean age = 50.6 years; range:18-86 years; SD = 17.8 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 = 102; male = 25; mean age = 47.4 years; range 18-87, SD = 17.7 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=19; male = 5; mean age = 48.6 years; range 19-91 years; SD = 19.3 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 = 50; male = 12; mean age = 50.7 years; range 18-80 years; SD = 17.2 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 = 47; male = 8; mean age = 50.3 years; range = 18-88 years; SD = 17.6 years).

6) Intrinsic Chronic Pharyngitis (iCP)

Chronic Pharyngitis is a poorly studied condition that affects a group of patients that attends the otolaryngologists [31], [32]. Most of them are empirically diagnosed as carriers of gastroesophageal reflux, according to the aspect of the pharynges, and despite the lifestyle changes and antacids, they persist to present itchy sore throat and dry cough. Some of these patients also have Allergic Rhinitis and improve their itchy throat symptoms with the treatment of their respiratory allergies. We separated a group of patients presenting purely with an itchy sore throat and dry cough. They presented without respiratory, dermatologic, ocular, gastrointestinal signs and/or symptoms; and without evidence of blood serum IgE-mediated hypersensitivity. They were classified in the intrinsic Chronic Pharyngitis (iCP) 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. The clinical sign defining this condition was the presence of hyperemic elevated plaques of reactive lymphoid tissue in the oropharynx (n = 67; male = 14; mean age = 50.5 years; range 50-82 years; SD = 13.7 years) [33].

C. Leukocyte Adherence Inhibition Test

Plasma samples were collected in heparinized collection tubes. The *ex vivo* challenge tests were performed as described previously [34]. Shortly, each donor's fresh plasma was divided into two parts and used in a paralleled *ex vivo* challenging tests with *Dp* depigmented 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 (10 µL of a solution with 1 mg/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 (%).

D. 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 number and frequency (%) distribution of the tests among the range of results of each group (figures 2 to 8). The data of the independent groups were compared group by group by the non-parametric Wilcoxon-Mann-Whitney U test [35], [36].

III. Results

TABLE I: Non-Parametric Wilcoxon-Mann-Whitney U Test (WMWUT) Comparing the Control Group with Each Other Patient's Group and Comparing the Patients' Groups between Each Other Patient's Group

Group	Group	U	*z*-score	*p*-value
Control	iAR	546.6	3.5221	0.00044
Control	iCP	283	4.11148	< 0.00001
Control	iAS	182,5	4.30879	< 0.00001
Control	iOA	38	-4.36052	< 0.00001
Control	iAD/iAR	119.5	4.9579	< 0.00001
Control	iAD	340	5.94533	< 0.00001
iAR	iAD	5,286.5	4.86467	< 0.00001
iAR	iAD/iAR	1,793	-2.46532	0.01352
iAR	iCP	2,844	-1.83994	0.06576 (n. s.)
iAR	iAS	2,064.5	-1.90196	0.05744 (n. s.)
iAR	iOA	732.5	-1.6813	0.09296 (n. s.)

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

The mean LAI of the control group was 7%; the mean LAI of the iAR group was 34%; the mean LAI of the iCP group was 44%; the mean LAI of the iAS group was 45%; the mean LAI of the iOA group was 47%; the mean LAI of the iAD group was 55%. The non-parametric Wilcoxon-Mann-Whitney U test (WMWUt) comparing the control group with each other patient's group showed significance with *p*-value < α = 0.05 for all comparisons (Table I).

The non-parametric Wilcoxon-Mann-Whitney U test comparing the patients' groups between each other patient's group showed significance with *p*-value < α = 0.05 for only

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two comparisons: between iAR versus iAD and between iAR versus iAD/iAR. The comparisons between the other groups were not statistically significant (Table I).

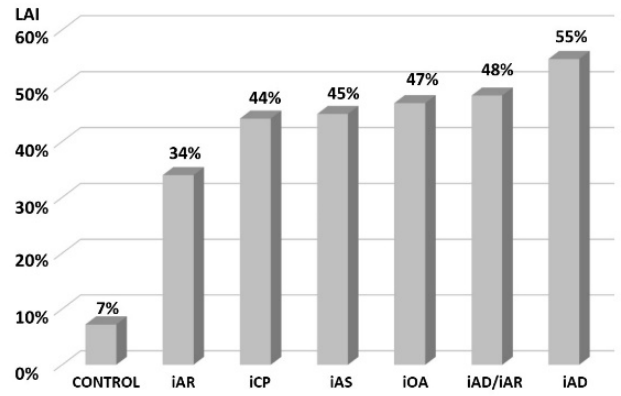

Fig. 1. Column comparison chart with the control group of the average Leukocyte Inhibition (%) of the ex vivo challenge tests performed with D. pteronyssinus extract, monitored by Leukocyte Adherence Inhibition Tests, grouped according to the clinical symptoms.

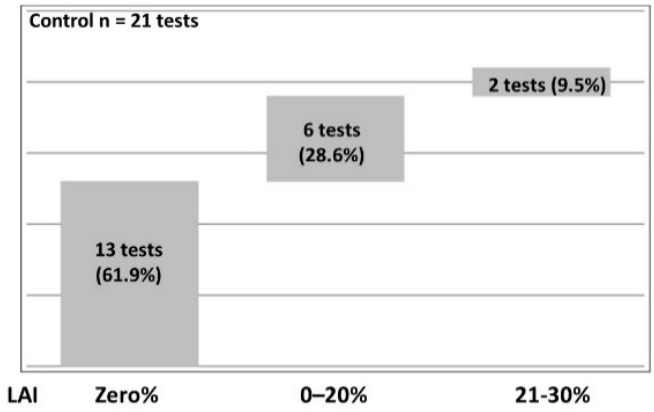

Fig. 2. Cascade distribution chart of the number (and percentage) of *ex vivo* challenge tests performed with *D. pteronyssinus* extract, monitored by Leukocyte Adherence Inhibition Tests, according to the range of results (%) of Leukocyte Adherence Inhibition (LAI) in 21 control individuals, presenting no allergic-related symptoms.

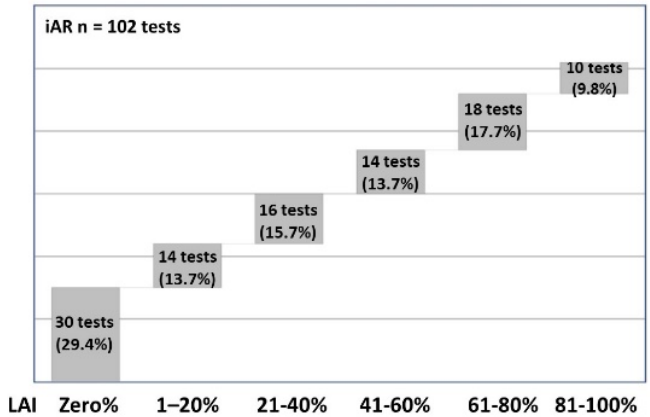

Fig. 3. Cascade distribution chart of the number (and percentage) of *ex vivo* challenge tests performed with *D. pteronyssinus* extract, monitored by Leukocyte Adherence Inhibition Tests, according to the range of results (%) of Leukocyte Adherence Inhibition (LAI) in 102 individuals with non–IgE-mediated intrinsic Allergic Rhinitis (iAR).

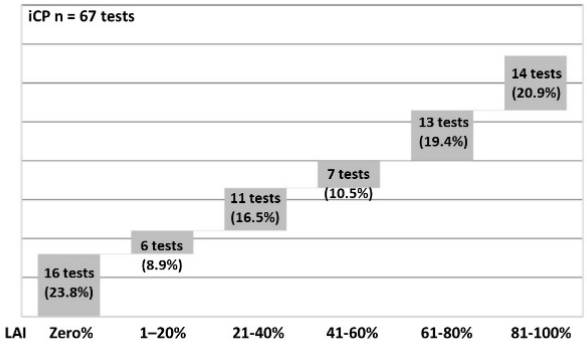

Fig. 4. Cascade distribution chart of the number (and percentage) of *ex vivo* challenge tests performed with *D. pteronyssinus* extract, monitored by Leukocyte Adherence Inhibition Tests, according to the range of results (%) of Leukocyte Adherence Inhibition (LAI) in 67 individuals with non–IgE-mediated intrinsic Chronic Pharyngitis (iCP).

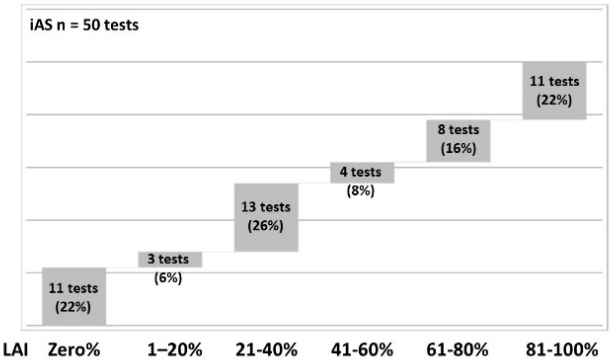

Fig. 5. Cascade distribution chart of the number (and percentage) of *ex vivo* challenge tests performed with *D. pteronyssinus* extract, monitored by Leukocyte Adherence Inhibition Tests, according to the range of results (%) of Leukocyte Adherence Inhibition (LAI) in 50 individuals with non–IgE-mediated intrinsic Asthma (iAS).

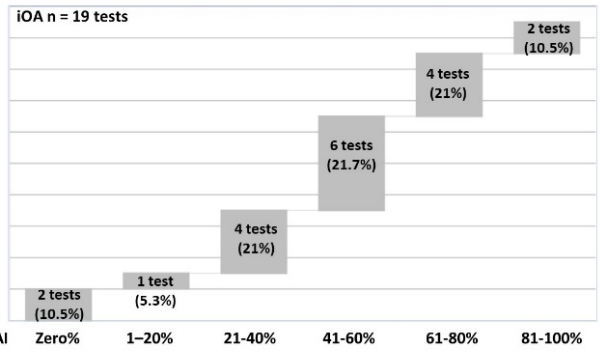

Fig. 6. Cascade distribution chart of the number (and percentage) of *ex vivo* challenge tests performed with *D. pteronyssinus* extract, monitored by Leukocyte Adherence Inhibition Tests, according to the range of results (%) of Leukocyte Adherence Inhibition (LAI) in 19 individuals with non–IgE-mediated intrinsic Ocular Allergy (iOA).

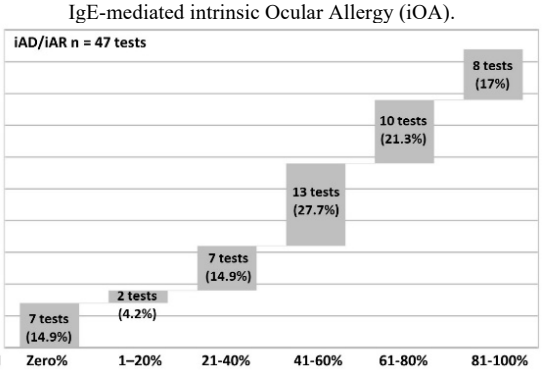

Fig. 7. Cascade distribution chart of the number (and percentage) of *ex vivo* challenge tests performed with *D. pteronyssinus* extract, monitored by Leukocyte Adherence Inhibition Tests, according to the range of results (%) of Leukocyte Adherence Inhibition (LAI) in 47 individuals with non–IgE-mediated combined intrinsic Atopic Dermatitis and intrinsic Allergic Rhinitis (iAD/iAR).

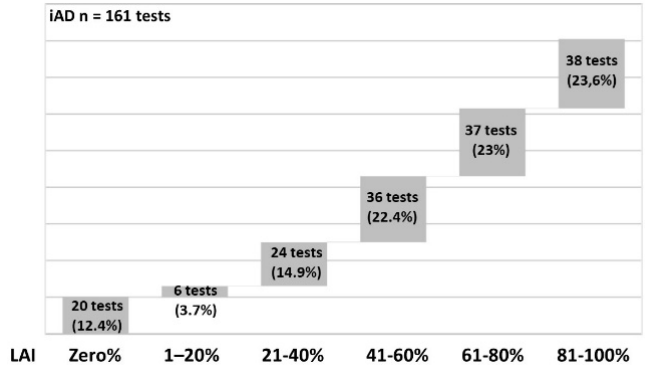

Fig. 8. Cascade distribution chart of the number (and percentage) of *ex vivo* challenge tests performed with *D. pteronyssinus* extract, monitored by Leukocyte Adherence Inhibition Tests, according to the range of results (%) of Leukocyte Adherence Inhibition (LAI) in 161 individuals with non—IgE-mediated intrinsic Atopic Dermatitis (iAD).

IV. Discussion

The significant difference between the mean LAI of the control group and the patient groups demonstrated that the *ex vivo* challenge test performed with *Dp*, monitored by the LAIT, can differentiate the specific immunoreactivity between the groups. Considering only these data, if we elect a LAI = 20,0% as the reference value to the laboratory evidence of immunoreactivity, then the false-positive rate of the test is 10%. However, if we elect a LAI > 30% as the cut-off reference value to the laboratory evidence of immunoreactivity, then the false-positive rate becomes 0,0%. Once the clinical symptoms may be produced by diverse hypersensitivities besides HDM allergy, the false-negative rate of the test cannot be determined by these data.

The diagnosis of the culprits responsible for the allergic reaction syndromes produced by non—IgE-mediated hypersensitivity is yet a great challenge to clinicians. The suspicion of a hypersensitivity reaction is usually based on the patients' perception of a relationship between the allergen and the symptoms and confirmed, when feasible, with the *in vivo* challenge tests, that do not demonstrate, necessarily, the immune mechanisms responsible for the symptoms. The inhibition of the leukocytes' glass adherence just indicates the release of cytokines after the immune recognition of the challenged antigen [37]. The LAIT may be considered a rudimentary or a triage test, when compared with other modern *ex vivo* challenge tests monitored by the quantification of the cytokines, such as the interferons and interleukins [38]. However, the LAIT is a more feasible, inexpensive, and faster test, easily adaptable to the routine of a medical facility dedicated to the diagnosis and/or the treatment of allergic patients [39], [40]. Our results demonstrated that the LAIT is potentially a useful tool to be employed clinically to predict *ex vivo* immunoreactivity against HDM in allergic patients with undetectable serum specific IgE.

Abbreviations

Dermatophagoides pteronyssinus: *Dp*
eAD: extrinsic Atopic Dermatitis
iAD: intrinsic Atopic Dermatitis
iAR: intrinsic Allergic Rhinitis
iAS: intrinsic Asthma
iCP: intrinsic Chronic Pharyngitis
iOA: intrinsic Ocular Allergy
LA: Leukocyte Adherence
LAR: Leukocyte Adherence Ratio
LAI: Leukocyte Adherence Inhibition
LAIT: Leukocyte Adherence Inhibition Test
Wilcoxon-Mann-Whitney U test: WMWUt

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