

Evaluating Non-IgE-Mediated Allergens' Immunoreactivity in Patients Formerly Classified as "Intrinsic" Asthmatics with Help of the Leukocyte Adherence Inhibition Test

Celso Eduardo Olivier, Daiana G. Pinto, Ana P. M. Teixeira, Jh ssica L. S. Santana, Raquel A. P. G. Santos, Regiane P. S. Lima, and Everton S. Monteiro

ABSTRACT

Background: The ancient concept of "intrinsic" and "extrinsic" asthma has evolved in line with growing scientific knowledge and the use of new clinical diagnostic tools.

Objective: The present study aims to evaluate the non-IgE-mediated immunoreactivity against common allergens, in asthmatic patients without evidence of IgE-mediated hypersensitivity against these allergens.

Methods: A group of 127 "intrinsic" asthmatic outpatients, were submitted to *ex vivo* Leukocyte Adherence Inhibition Tests (LAIT) with extracts of *Dermatophagoides pteronyssinus*, *Hevea brasiliensis* latex, dog dander, cat dander, cow's milk proteins, beekeeping pollen and a mixture of fungal extracts.

Results: Cascade graphs were assembled according to the distribution of the LAIT results among the range of results of Leukocyte Adherence Inhibition (LAI) inside each group. A column graph was plotted with the mean LAI results for each antigen. The non-parametric Wilcoxon-Mann-Whitney U-test performed between the results of each test was significant only between the results of the LAIT performed with the dog extract and the mixed fungal extract ($U = 1,179.5$; the z -score was -2.64109 ; the p -value was 0.0083). All other Wilcoxon-Mann-Whitney U-test calculations between LAIT performed with the other allergens were not significant at $p < 0.01$.

Conclusion: The evidence of non-IgE-mediated immunoreactivity in allergic patients has been reported in the medical literature since the beginning of the 19th century and has been increasing since then. This descriptive article presents the contribution of the LAIT as a tool to quantify the overall non-IgE-mediated cellular/humoral immunoreactivity against common allergens in patients formerly classified inside the "intrinsic" asthma endotype.

Keywords: Allergy, asthma, bronchitis, cats, dogs, *Dermatophagoides pteronyssinus*, *Hevea brasiliensis*, hypersensitivity, non-IgE-mediated hypersensitivity, leukocyte adherence inhibition test.

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C. E. Olivier*

Instituto Alergoimuno de Americana, Brazil.

(e-mail: celso@alergoimuno.med.br)

D. G. Pinto

Instituto Alergoimuno de Americana, Brazil.

(e-mail: daianaguedes85@gmail.com)

A. P. M. Teixeira

Instituto Alergoimuno de Americana, Brazil.

(e-mail: monezzi88@gmail.com)

J. L. S. Santana

Instituto Alergoimuno de Americana, Brazil.

(e-mail: ljhessica31@gmail.com)

R. A. P. G. Santos

Instituto Alergoimuno de Americana, Brazil.

(e-mail: raquel.cuidados@gmail.com)

R. P. S. Lima

Instituto Alergoimuno de Americana, Brazil.

(e-mail: regianepatussi@gmail.com)

E. S. Monteiro

Laboratory of Immunology, Heart Institute, School of Medicine, University of S o Paulo (InCor, FMUSP), Brazil.

(e-mail: everton.monteiro@hc.fm.usp.br)

***Corresponding Author**

I. INTRODUCTION

The association of respiratory symptoms, such as cough, wheezing, and expiratory dyspnea is a condition already described under several names, such as "asthmatic bronchitis", "bronchial asthma", "asthmatic syndrome", or shortly, "asthma" [1]. The main physiopathological determinant of this condition is the bronchial spasm, also known as "bronchospasm". Asthmatic syndromes are not a disease per se but may be produced by a variety of conditions, such as allergies, viral infections, parasitic infestations, or bronchial aspiration [2], [3]. "Asthma" is not an etiologic diagnosis or even an individualized disease, but a resultant condition that deserves proper investigation to discover its causes. Sometimes, saying "somebody has asthma" is something like saying "somebody has a fever"

[4]. Once there was an ancient medical aphorism that said: "all is not allergy that wheezes", which was said at a time when most cases of asthma were attributed to hypersensitivity to aeroallergens and/or to foods [5], [6]. The beginning of the twentieth century saw the publication of several scientific articles associating the onset of asthma attacks with the inhalation of horse dandruff, plant pollens, and dust, as well as with the ingestion of eggs, berries, fish, and cereals [7], [8]. Before the "IgE-era", the diagnosis of sensitization was aided by allergic skin tests, Complement fixation assays, and the research of precipitins [9], [10]. Almost three decades before the association of the reaginic activity of IgE with allergic symptoms, medical authors had already classified bronchial asthma in two overlapping categories: A) "extrinsic", when there was evidence of hypersensitivity against at least one environmental or food

trigger, and B) “intrinsic”, when there was no clear association with any perceived trigger [11]-[14]. After the establishment of the IgE as the Gell & Coombs type I hypersensitivity reaction, the “extrinsic” bronchial asthma was associated with IgE-mediated hypersensitivity and the “intrinsic” bronchial asthma was linked with the non-IgE-mediated hypersensitivities, while an “intermediate” phenotype was defined for the patients with an increase in total IgE levels, but no known association with any allergen [15], [16]. A century after the first elaboration of the concept of what would be the “intrinsic” phenotype of asthma, there is still no universal consensus on its definition, with a lot of questions waiting to be answered, mainly when both IgE-mediated and non-IgE-mediated mechanisms seem to be present in the same patient [17], [18]. One of the many mechanisms proposed for the installation of “intrinsic” asthma is the presence of superantigens. Microorganisms, such as *Staphylococcus aureus* can invade the bronchial mucosa and release exotoxins, which act as superantigens with the capacity to directly stimulate T and B cell proliferation [19]. Despite the absence of detectable local or systemic IgE, there is increasing evidence that patients with the “intrinsic” phenotype present an intense innate and adaptive immune activity nearby the bronchial mucosa, with the detection of eosinophils, neutrophils, lymphocytes, adaptive immune cytokines, chemokines, and increase production of mRNA for their receptors [20]. These pieces of evidence suggest that the IgE-mediated hypersensitivity reaction is not the only one responsible for allergic diseases [21]-[24].

The Leukocyte Adherence Inhibition Test (LAIT) is an immunoassay designed by Halliday to detect cellular immunoreactivity against diverse antigens associated with allergic and non-allergic conditions [25]-[29]. When put in contact with an immunoreactive antigen, leukocytes lost their ability to adhere to glass, showing a non-specific immunoreactivity against this specific substance [30]. Several mediators were already described as involved in this immunoassay, suggesting that a Gell & Coombs type II reaction is at play [31]-[33]. In this descriptive work, we compile the data supplied by the LAIT performed with fresh blood of “intrinsic” asthmatic outpatients to provide an overview of their relative non-IgE-mediated immunoreactivity against some common allergens.

II. METHODS

A. Subjects

After receiving Institutional Review Board approval, from the Instituto Alergoimuno de Americana (Brazil), a group of 127 asthmatic outpatients, or their responsible (34 male; 0 - 82 years old; mean age = 38.7 years, SD = 25.8 years), were informed and invited to voluntarily provide blood samples to perform *ex vivo* Leukocyte Adherence Inhibition Tests, according to the principles of the World Medical Association Declaration of Helsinki and the International Committee of Medical Journals Editors requirements of privacy [34]. The clinical diagnosis of the “intrinsic” asthmatic condition was done according to the criteria discussed previously [35], [36]. All individuals had

normal-range total IgE, non-detectable specific IgE, and non-reactive skin tests against the studied allergens. None of the patients had a history or evidence of any concomitant rheumatologic auto-immune condition. The study was descriptive, and 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 were complied with within the complete course of the experiments.

B. Antigens Extraction

The pollen’s proteins extraction was performed as follows: in a beaker, 5 g of dehydrated beekeeping pollen, acquired from a local provider, were added to the Coca-based extractor solution (propylparaben 0.5 g, methylparaben 1 g, sorbitol 30 g, NaCl 5 g, NaHCO₃ 2.5 g, 1,000 mL H₂O) added to cover the amount of pollen [37]. 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 0.72 g, Na₃PO₄ 2.86 g, methylparaben 1 g, propylparaben 0.5 g, glycerin 400 mL, H₂O 600 mL) and used to perform the LAIT and allergic skin tests [38], [39].

The dog’s and cat’s hair were provided by a local pet service center, which collected the material directly from the animal with a proper shaving machine. The hair was treated with acetone to remove the fat. After this, the acetone was removed from the sample using the autoclave. The sample was grounded for 48 hours at 4 °C with a Coca-based extractor solution added to cover the amount of antigen. 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 to perform the LAIT and allergic skin tests.

The *Dermatophagoides pteronyssinus* extracts were obtained from frozen cultures. The contents of the bottles containing mites (adults, nymphs, larvae, feces, and eggs) and culture medium were weighed and left at a rate of 10 ml of PBS buffer per gram of material (10%) by gentle magnetic stirring (1,000 rpm) for four hours at 4 °C. The material was centrifuged at 5,000 rpm for 30 minutes. The supernatant was kept apart. The sediment was resuspended in the same conditions as the previous step and kept stirring at 4 °C for 2 hours. The centrifugation was repeated, and the supernatants were mixed into an Erlenmeyer flask. The solution was filtered through a double paper filter and later through a 0.2 µm pore-size filter with the help of a vacuum flask and a vacuum pump. The extract was dialyzed with aid of the TermoFisher SnakeSkin™ 88244 Dialysis Tubing in distilled water (1:50 ratio) with 3 water changes for 24 hours, to eliminate molecules of low molecular mass (< 5,000 Da). After dialysis, the extract was centrifuged at 10,000 rpm for 30 minutes at 4 °C and frozen at -40 °C. The protein concentration was estimated spectrophotometrically and diluted to 500 µg/mL in antigen dilution solution to perform the LAIT and allergic skin tests.

Cultures of *Alternaria alternata*, *Aspergillus fumigatus*, *Trichophyton rubrum*, and *Candida albicans* were weighted and mixed in equal parts and treated by the same technique

described previously for *D. pteronyssinus*. The protein concentration was estimated spectrophotometrically and diluted to 500 µg/mL in antigen dilution solution to perform the LAIT and allergic skin tests.

The *Hevea brasiliensis* latex proteins extraction was performed accordingly as described elsewhere [40]. The protein concentration was estimated spectrophotometrically and diluted to 1 mg/mL in antigen dilution solution to perform the LAIT and allergic skin tests.

Cow's milk protein extraction was performed accordingly described elsewhere [41]. The protein concentration was estimated spectrophotometrically and diluted to 1 mg/mL in antigen dilution solution to perform the LAIT and allergic skin tests.

All relevant and mandatory laboratory health and safety measures have been complied with in the complete course of the experiments.

C. Leukocyte Adherence Inhibition Test

Leukocyte Adherence Inhibition Tests were performed as described previously [42].

D. Graphic Presentation of Data and Statistics

Cascade graphs were assembled according to the distribution of the tests among the range of results of each group (Fig. 1 to 7).

The x-axis was divided into six groups of results. The first grouped the tests with no Leukocyte Adherence Inhibition (LAI) after the *ex vivo* challenge (0%). The second was the group of tests with 1 to 20% of LAI. The third was the group of tests with 21 to 40% of LAI. The fourth was the group of tests with 41 to 60% of LAI. The fifth was the group of tests with 61 to 80% of LAI. The sixth was the group of tests with 81 to 100% of LAI. The y-axis presents the percentage of each group inside the total of tests for each antigen.

A column graph was plotted with the mean LAIT results of each antigen (Fig. 8). The data of the LAIT allergen groups were compared with each other by the non-parametric Wilcoxon-Mann-Whitney U test [43], [44].

III. RESULTS

The mean LAI of the 113 tests performed with *D. pteronyssinus* extract was 47.4% (range = 0-100%; Median = 48%; Mode = 0%, which appeared 21 times; SD = 32.9%). See Fig. 1.

The mean LAI of the 59 tests performed with mixed fungal extract was 36.9% (range = 0-100%; Median = 35%; Mode = 0% which appeared 17 times; SD = 32.1%). See Fig. 2.

The mean LAI of the 56 tests performed with the pollens extract was 42.5% (range = 0-97%; Median = 48.5%; Mode = 0%, which appeared 11 times; SD = 31.1%). See Fig. 3.

The mean LAI of the 84 tests performed with the *Hevea brasiliensis* latex extract was 45.1% (range = 0-97%; Median = 44%; Mode = 0%, which appeared 14 times; SD = 29.7%). See Fig. 4.

The mean LAI of the 55 tests performed with the dog dander extract was 53.7% (range = 0-100%; Median = 57%;

Mode = 0%, and 83% which appeared 3 times; SD = 27.4%). See Fig. 5.

The mean LAI of the 42 tests performed with the cat dander extract was 42.7% (range = 0-100%; Median = 47.5%; Mode = 0%, which appeared 8 times; SD = 28.4%). See Fig. 6.

The mean LAI of the 40 tests performed with the cow's milk extract was 42.5% (range = 0-94%; Median = 46%; Mode = 0%, which appeared 9 times; SD = 33.1%). See Fig. 7.

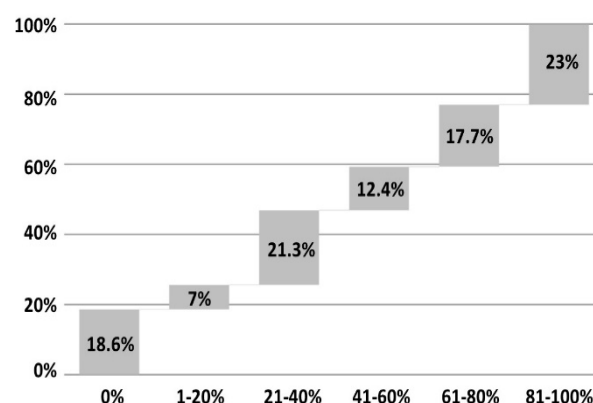


Fig. 1. Cascade distribution chart of the range groups of Leukocyte Adherence Inhibition (x-axis) of *ex vivo* *Dermatophagoides pteronyssinus* extract challenges monitored by Leukocyte Adherence Inhibition Tests, according to the percentage calculated over 113 patients (y-axis) with non-IgE-mediated "intrinsic" Asthma.

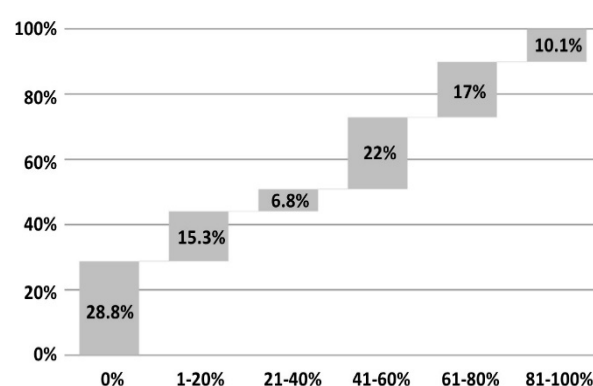


Fig. 2. Cascade distribution chart of the range groups of Leukocyte Adherence Inhibition (x-axis) of *ex vivo* mixed fungal extract challenges monitored by Leukocyte Adherence Inhibition Tests, according to the percentage calculated over 59 patients (y-axis) with non-IgE-mediated "intrinsic" Asthma.

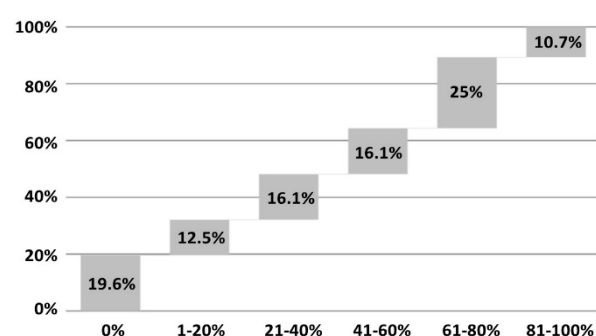


Fig. 3. Cascade distribution chart of the range groups of Leukocyte Adherence Inhibition (x-axis) of *ex vivo* beekeeping pollen extract challenges monitored by Leukocyte Adherence Inhibition Tests, according to the percentage calculated over 56 patients (y-axis) with non-IgE-mediated "intrinsic" Asthma.

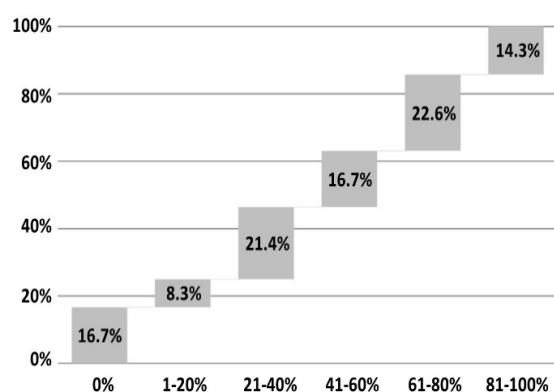


Fig. 4. Cascade distribution chart of the range groups of Leukocyte Adherence Inhibition (x-axis) of *ex vivo* *Hevea brasiliensis* latex extract challenges monitored by Leukocyte Adherence Inhibition Tests, according to the percentage calculated over 84 patients (y-axis) non-IgE-mediated "intrinsic" Asthma.

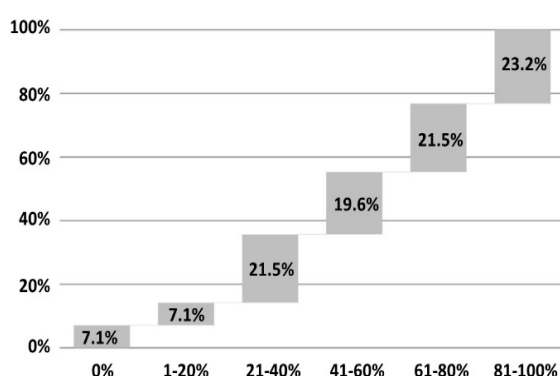


Fig. 5. Cascade distribution chart of the range groups of Leukocyte Adherence Inhibition (x-axis) of *ex vivo* dog dander extract challenges monitored by Leukocyte Adherence Inhibition Tests, according to the percentage calculated over 55 patients (y-axis) with non-IgE-mediated "intrinsic" Asthma.

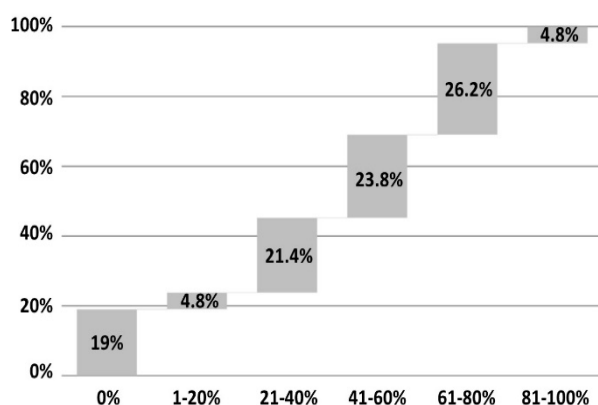


Fig. 6. Cascade distribution chart of the range groups of Leukocyte Adherence Inhibition (x-axis) of *ex vivo* cat dander extract challenges monitored by Leukocyte Adherence Inhibition Tests, according to the percentage calculated over 42 patients (y-axis) with non-IgE-mediated "intrinsic" Asthma.

The Wilcoxon-Mann-Whitney test performed between the results of each test was significantly different only between the results of the LAIT performed with the dog extract and the mixed fungal extract ($U = 1,179.5$; z -score = -2.64109 ; p -value = 0.0083). This difference was mainly due to the low quantity of tests with no Leukocyte Inhibition with the dog dander extracts (7.1% of the tests) and the high quantity of tests with no Leukocyte Inhibition with the mixed fungal

extract (28.8% of the tests). All other Wilcoxon-Mann-Whitney tests calculations between the others LAIT allergens groups were not significant (p -value < 0.01). The mean LAI of each tested allergen was plotted in Fig. 8.

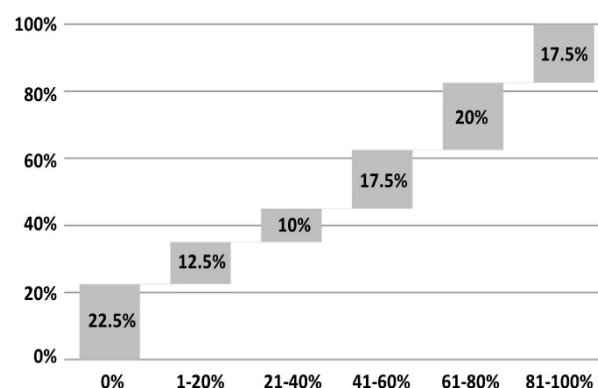


Fig. 7. Cascade distribution chart of the range groups of Leukocyte Adherence Inhibition (x-axis) of *ex vivo* cow's milk extract challenges monitored by Leukocyte Adherence Inhibition Tests, according to the percentage calculated over 40 patients (y-axis) with non-IgE-mediated "intrinsic" Asthma.

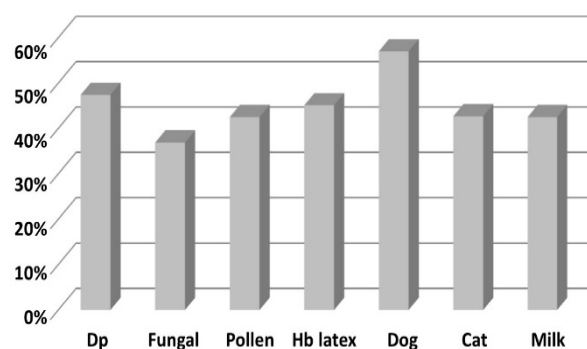


Fig. 8. Mean Leukocyte Adherence Inhibition (%) performed with *Dermatophagoides pteronyssinus* extract (Dp); mixed fungal extracts, beekeeping pollen extracts, *Hevea brasiliensis* (Hb) latex extracts, dog dander extracts, cat dander extracts, and cow's milk proteins extracts.

IV. DISCUSSION

The ancient concept of "intrinsic" and "extrinsic" asthma is evolving according to the increasing scientific knowledge and the use of new clinical diagnostic tools. The concept of an "intrinsic" endotype for asthmatic patients, in opposition to an "extrinsic" endotype, was already considered, at least, controversial [45]. The term "intrinsic", in the past, was already described as "misleading" because it could produce the assumption that the "absence of evidence" of an external agent could somehow be equivalent to the "existence of evidence" that no external agent was triggering the hypersensitivity reaction. When a physician labeled the patient with the diagnosis of "intrinsic asthma", it was somehow assumed that there was no need for further investigation. Now a day, it seems that an increasing number of medical doctors automatically label all their asthmatic patients as "intrinsic", since few physicians care to extensively investigate the hypersensitivity triggers, once provided that the bronchospasm stays well-controlled with the inhaled steroids and bronchodilators. This attitude is somehow stimulated by the pharmaceutical emphasis of the

clinical algorithms recommended by authoritative medical societies [46]. In our opinion, the more reasonable would be to classify the already investigated asthmatic patients as follow: A) patients with asthmatic syndromes due to Known non-allergic related causes, such for instance Loeffler's syndrome, bronchial aspiration, and viral/bacterial infections; B) asthmatic patients with evidence of IgE-mediated immune hypersensitivity; C) asthmatic patients with evidence (or strong suspicion) of non-IgE-mediated immune hypersensitivity against specific allergens; and D) asthmatic patients, that after an extensive investigation did not demonstrate any evidence of hypersensitivity against any allergen (the true "intrinsic" asthmatic patient). The investigation of an asthmatic patient, in our opinion, must initially be done by a careful anamnesis and physical exam, considering initially the less invasive and most accessible complementary assays. A thorough allergic skin test battery can provide a good triage in skin-reactive patients and be followed by the serum quantification of specific IgE against the most common allergens. The investigation of the non-IgE-mediated hypersensitivities; however, is not an easy task for pragmatic physicians used to reasoning with the easy-to-request automatized lab exams. Pediatricians are more used to diagnose non-IgE-mediated hypersensitivities since it is relatively easy to substitute the cow's milk formula used by weaned babies for standard amino acid formulations and infer the diagnosis of a non-IgE-mediated cow's milk allergy as responsible for the asthmatic symptoms of their patients. For older people, with a diversified diet, there is a need for an additional effort to perform an exclusion diet followed by an Oral Food Challenge, to provide evidence that a non-IgE-mediated food allergy is producing the wheezing symptoms. However, this discovery is the first step to dramatically improving the patient's quality of life, which can also be followed by a further step such as the planning of a desensitization strategy. Among the universe of possibilities, the existence of clinical tools to select the more probable allergens to begin the investigation is highly desirable. The LAIT is studied here no to perform a definitive diagnosis of the etiologic cause of the disease but to raise clues above the more probable agents triggering the asthmatic condition. In our experience, a negative TIAL is a strong piece of evidence that the patient's immune system, simply "ignores" the presence of the allergen, and with exception of the immune exhaustion states (when there is a very low or no adherence in the control assay), it scarcely produces any clinical hypersensitivity reaction at all. When positive, the LAIT raises a reasonable suspicion to pursue a more throughout investigation strategy such as the provocation tests, saving time and effort before the prescription of live-changing habits and, perhaps, a desensitization strategy.

ABBREVIATIONS

LAI: Leukocyte Adherence Inhibition
LAIT: Leukocyte Adherence Inhibition Test

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CONFLICT OF INTEREST

The authors declare that they do not have any conflict of interest.

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C. E. Olivier. Born in Brazil in 1963. Physician graduated from the Faculty of Medicine of the State University of Campinas (1986). Medical residency at the Faculty of Medicine of the State University of Campinas (1988). Specialist in Allergy and Immunology from the Brazilian Association of Allergy and Immunology (2011). Certified in Pediatric Allergy and Immunology by the Brazilian Society of Pediatrics (2015). Specialist in Clinical Analysis by the Faculty of Health Sciences of the Methodist University of Piracicaba (2012). Doctorate (Ph.D.) in Clinical Medicine (under the guidance of Prof. Dr. Ricardo de Lima Zollner) concluded at the Department of Allergy and Immunology of the Faculty of Medicine of the State University of Campinas (2012).

He is the Senior Researcher of the Americana's Alergoimuno Institute (Instituto Alergoimuno de Americana). He has experience in the field of Medicine, with an emphasis on Allergy and Immunology and Clinical Analysis.

Dr. Olivier is a member of the Brazilian Association of Allergy and Immunopathology. His *curriculum vitae* can be accessed at <http://lattes.cnpq.br/7035870789320492>. ORCID: 0000-0001-7541-3182



D. G. Pinto. Born in Brazil in 1985. Biomedic graduated from the Faculty of Americana (2010). She is a researcher at the Americana's Alergoimuno Institute (Instituto Alergoimuno de Americana).

She has experience in the field of laboratory Medicine, acting mainly on the following themes: preparation of allergens and allergoids for in vivo, ex vivo, and in vitro diagnosis of allergy, leukocyte adherence inhibition tests, precipitins, and immunoblot.

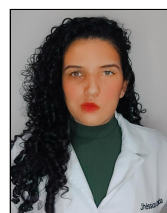
Ms. Pinto's *curriculum vitae* can be accessed at <http://lattes.cnpq.br/6423437970305610>. ORCID: 0000-0002-4464-6669.



A. P. M. Teixeira. Born in Brazil in 1996. Biomedic graduated from the Faculty of Americana (2018).

She is a researcher from the Americana's Alergoimuno Institute (Instituto Alergoimuno de Americana). She was trained in the field of laboratory Medicine, acting mainly on the following themes: preparation of allergens and allergoids for in vivo, ex vivo, and in vitro diagnosis of allergy, skin-allergic tests, leukocyte adherence inhibition tests, precipitins, and immunoblot.

Miss Teixeira's *curriculum vitae* can be accessed at <http://lattes.cnpq.br/9364659098398568>. ORCID: 0000-0001-5140-9285.



J. L. S. Santana. Born in Brazil in 1997. Biomedic graduated from the Faculty of Americana (2021). She is a researcher from the Americana's Alergoimuno Institute (Instituto Alergoimuno de Americana).

She was trained in the field of laboratory Medicine, acting mainly on the following themes: preparation of allergens and allergoids for in vivo, ex vivo, and in vitro diagnosis of allergy, skin-allergic tests, leukocyte adherence inhibition tests, precipitins, and immunoblot.

Miss Santana's *curriculum vitae* can be accessed at <http://lattes.cnpq.br/4811507530904182>. ORCID 000-0001-8519-127X.



R. A. P. G. dos Santos. Born in Brazil in 1976. Nurse graduated from the Anhanguera Santa Barbara Faculty (2016).

She is a researcher at the Americana's Alergoimuno Institute (Instituto Alergoimuno de Americana). She has experience in the field of diagnostic Medicine, acting mainly on in vivo diagnosis of allergies.

Ms. Santos's *curriculum vitae* can be accessed at <http://lattes.cnpq.br/2757001223214460>. ORCID: 0000-0001-6469-8207.



R. P. dos Santos Lima. Born in Brazil in 1985. Biomedic graduated from the Anhanguera Santa Barbara Faculty (2015). A former researcher in the Americana's Alergoimuno Institute (Instituto Alergoimuno de Americana), nowadays she is working as a staff manager of Lavoisier's laboratories in Brazil.

She has experience in the field of laboratory Medicine, acting mainly on the following themes: preparation of allergens and allergoids for in vivo, ex vivo, and in vitro diagnosis of allergy; skin-allergic tests, leukocyte adherence inhibition tests, precipitins, and immunoblot.

Miss Lima's *curriculum vitae* can be accessed at <http://lattes.cnpq.br/2757001223214460>. ORCID: 0000-0003-4845-8822.



E. S. Monteiro. Born in Brazil in 1989. Graduated in Pharmacy from Iguacu University (2010). Specialist in Pharmaceutical Engineering from Racine Institute (2013). MBA in Executive Management of Projects by "Getúlio Vargas" Foundation (2020). He has 10 years of experience in the pharmaceutical industry in the areas of Research and Development, Quality Assurance, Production, and Regulatory Affairs, working for companies such as Prati Donaduzzi, Eurofarma Allergan, Roche, and in international projects for Azbil-Telstar in Portugal and Spain.

He is currently a master's student in Allergy and Immunopathology at the Faculty of Medicine of the São Paulo University (under the orientation of Prof. Dr. Fábio Fernandes Morato Castro) and technical manager at CEMA-Center for Allergy Manipulation. Mr. Monteiro's *curriculum vitae* can be accessed at <http://lattes.cnpq.br/8006604665154155>. ORCID: 0000-0001-9664-9098.