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Research Article

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

Celso Eduardo Olivier*, Daiana Guedes Pinto, Regiane Patussi dos Santos Lima, Ana Paula Monezzi Teixeira, Jhéssica Letícia Santos Santana

Department of Allergy and Immunology, Instituto Alergoimuno de Americana, São Paulo, Brazil

Abstract

Background: The diagnosis of non-IgE mediated food allergy is done mainly by *in vivo* Oral Food Challenge (OFC) tests that depend on well-succeeded previous exclusion diets.

Objective: To evaluate the opportunity of the *in vitro* semi-quantitative research of specific precipitins to select food allergens to proceed with exclusion diets and further *in vivo* oral food challenge tests in food-allergic patients.

Methods: The tube titration of specific precipitins against food allergens in food allergic patients was compared with the *in vivo* oral food challenge tests performed after a well-succeeded exclusion diet. The probability of a positive or negative OFC was estimated according to the precipitin's titrimetry.

Results: The correlation coefficient between the precipitin's titrimetry and the probabilities of a positive OFC was 0,76 (p=0.017).

Conclusion: The semiquantitative research of specific precipitins against food allergens is a useful triage test to select food allergens to proceed with exclusion diets and oral food challenges to diagnose non-IgE mediated food allergy in adults with recurrent episodes of acute urticaria.

Keywords: Antigen-Antibody complex; Immune complex diseases; Immunoassay; Hypersensitivity; Precipitins; Precipitins tests; Urticaria

INTRODUCTION

Food allergies are immune reactions with the potential to produce disabling diseases and death, converting them into an increasing medical concern [1]. In most cases, an anamnesis done by the assistant physician can easily identify a suggestive or a convincing history, especially in monosensitized patients [2]. On the contrary, polysensitized patients are subject to particular difficulties in the identification of the specific nutritional ingredients related to their symptoms [3]. When the allergy is IgE-mediated, it is relatively easy to clarify the diagnosis with batteries of allergy skin tests and/or laboratory assays. However, the diagnosis of non-IgE mediated food allergies may depend mainly on *in vivo* Oal Food Challenge tests (OFC) [4]. Oral Food Challenges tests are laborious and expensive; they present potential health risks; they must be done when the patient is not using pharmaceutical drugs such as antihistamines, antacids, steroids, and beta-blockers; they depend on a dedicated team, and they may demand several days to come to a conclusion [5]. They are initiated by the dietary exclusion of the suspected foods, until the disappearance of the symptoms, and then, followed by a monitored progressive oral challenge done inside a secure environment with a medical staff prepared and equipped to attend any eventual allergic reaction [6]. When planning oral food challenge tests, it is essential to select a group of suspected allergens (candidates) to proceed with the exclusion diet before the OFC [7]. Oral food challenge tests are most feasible when there is a limited

Correspondence to: Celso Eduardo Olivier, Instituto Alergoimuno de Americana, Rua Cuba, 836-Bairro Frezzarin-Americana, São Paulo, Brazil, E-mail: celso@alergoimuno.med.br

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number of suspected foods, such as in the context of the infant's cow's milk allergy [8]. However, they are almost impracticable to perform when the patient is unable to discontinue the use of the medication or there are multiple suspects (or no suspect at all). Additionally, the food-allergic reactions may also depend on thresholds (eliciting doses) and concurrent situations to manifest, such as exercise, dual food intake, and/or the concomitant use of pharmaceutical drugs, like non-steroidal anti-inflammatories [9-14]. Motivated by these limitations, some scientists began to study a controversial field: the contribution of the food-specific IgG antibodies to the pathogenesis of food allergy [15]. Unrecommended by most specialists, the biggest criticism of this approach is that the clinically available laboratory methods to quantify food-specific IgG antibodies do not differentiate among their four functional subclasses [16]. The bivalent IgG antibodies from classes 1 to 3 can participate in Gell and Coombs' type 2 (antibody-dependent cell-mediated cytotoxicity) and type 3 (mediated by immune complexes and the complement system) hypersensitivity reactions [17]. However, the IgG4 is an ambivalent antibody unable to produce immune complexes or activate the complement system, being considered a tolerogenic physiologic blocker immune response [18-20]. Even among bivalent classes of IgG, there are different affinities to bind and activate the Complement system. The IgG1 subclass is the most effective to activate Complement at high antigen concentrations; the IgG3 subclass is the most effective to activate complement at different antigen concentrations; the IgG2 subclasses only activates complement at relatively high antigen concentrations [21]. Among IgG subclasses, it is IgG1, so far, the best candidate to produce, or, at least, to predate IgE sensitization to foods [22]. So, the big question is: How the clinical laboratory can differentiate the pathogenic effector antibodies from the physiological blocker antibodies? The response may be simpler than the question and is based on the pioneer laboratory procedure responsible for the creation of immunology as a science: the research of precipitins [23]. The research of precipitins to food proteins is historically used as a tool to study anaphylaxis, even before the understanding of the antibodies' structure [24]. Precipitins are serum antibodies able to produce *in vitro* precipitation when added to their specific soluble antigens and their concentrations correlate well with the concentrations of immune complexes found in the serum of non-IgE mediated food-allergic patients [25]. The ambivalent IgG4 blockers antibodies, however, do not precipitate, because they are unable to produce immune complexes. If an antibody can precipitate, it can produce immune complexes and, consequently, it can participate in Gell and Coombs type 3 hypersensitivity reactions. In the same way, ambivalent antibodies are also unable to establish dual antigen links when bounded to aggregable membrane Fc receptors to elicit cellular responses, as occurs, for instance in IgG and IgE antibody-dependent degranulation (Gell and Coombs types 1 and 2 hypersensitivity reactions) [26]. To maintain the integrity of the organism, the immune system must be able to distinguish constitutional self-proteins from foreign non-self-antigens, as well to differentiate harmful bacterial toxins from harmless nutritional proteins that inadvertently gain access to the internal environment [27,28]. Food proteins are supposed to be physiologically digested to amino acids residues in gut lumen to become useful to human metabolism; therefore, the presence of non-digested nutritional proteins in blood is a sign of an undesirable increase on intestinal permeability [29]. A physiological exception is the mammalian newborn which, during the first week of life, can absorb maternal antibodies delivered by breast milk, mainly through residual selective endocytosis mediated by FcRn epithelial expression [30]. The immune cells associated with the digestive system are supposed to keep contact with nutritional proteins captured from the digestive lumen through professional antigen-presenting cells (APCs), such as the Peyer's patch M cells, the submucosal Dendritic Cells, and even certain enterocytes [31]. These professional APCs did not allow the entry of whole proteins into the circulation; instead of this, they digest these antigens and their conformational epitopes and present their peptides and linear epitopes to naïve T cells inside a membrane-associated Major Histocompatibility Complex context, along with a set of cytokines that reflects the physiologic (noninflammatory) or the inflammatory context in which the proteins were collected [32-34]. The presence or absence of inflammatory signals near the interface with the external environment where the antigens were collected is capital to prime the APCs and determine the further nature of the immune response [35]. When the lumen antigens are collected in a non-inflammatory environment, the mucosal-associated APCs present the linear epitopes to the naïve T cells and direct them, *via* tolerogenic cytokines such as IL-10 or TGF-β, to differentiate to T regulatory cells [36,37]. When the lumen antigens are collected in an inflammatory environment, the APCs present to the naïve T cells, along with the processed antigens, stimulatory type-1 T helper cells cytokines, such as INF-γ; or type-2 T helper cells cytokines, such as IL-4, that directs them to a Th1 or a Th2 inflammatory profile, respectively [38,39]. Therefore, directed by the APCs, the immune system has two main pathways to process antigens: the tolerogenic and the inflammatory [40]. In the tolerogenic pathway the differentiation of T regulatory cells stimulates the production of tolerogenic cytokines that induce B cells to produce blocker antibodies, such as the mucosal Secretory-IgA (sIgA) or the ambivalent IgG4. In the inflammatory pathway the T helper cells produce pro-inflammatory cytokines that stimulate B cells to differentiate to plasma cells and produce effector antibodies such as IgM, IgE and the three bivalent subclasses of IgG. The complexity of these phenomena may be appreciated by the destination of the immune complexes assembled with the sIgA: When they follow the intestinal flow to be eliminated in the feces, or when they are direct to be captured by the M cells in Peyer's plaques, these phenomena define the sIgA as a tolerogenic antibody. Otherwise, when it backflows through the enterocyte's tight junctions into the lamina propria, it becomes an effector antibody, prone to precipitate and to participate in pathogenic immune reactions such as celiac disease. In health and physiologic conditions, whole proteins are supposed to be unable to gain systemic circulation through the cellular membranes of the luminal cells of the digestive mucosa or between the tight junctions between them. However, some proteins succeed to gain the "milieu intérieur" by the persorption phenomena. Persorption is the paracellular passage of large particles through loose tight junctions, predominantly found around intestinal goblet cells or damaged digestive epithelia. Alcohol is a particular dietary substance able to transiently increase the intercellular leak and the systemic absorption of macromolecules. Until a certain limit the persorption phenomena may be considered a physiologic process, but to a greater extent, it may become noxious, inducing deleterious immune reactions. When those food proteins that occasionally reach the internal environment are recognized in an inflammatory context, they may elicit innate and

adaptive responses that result in the production of specific antibodies able to assembly antigen-antibody complexes. While the free antigens circulating in serum usually are harmless, the antigen-antibody complexes may exert a biological activity producing symptoms such as vasculitis and skin reactions. With exception of the ambivalent IgG4, all the bivalent (IgG, IgE, and IgA), tetravalent (sIgA), and decavalent (IgM) antibodies can produce antigen-antibody complexes when finding an antigen with more than one similar epitope [20]. The immune complexes produced by the sIgA into the gut lumen are a physiologic way to exclude undesirable microorganisms and undigested proteins, blocking their persorption, leading to fecal elimination, or yet, directing them to be internalized and processed by the M cells in Peyer's patches. When the undigested proteins gain circulation by persorption, they can produce antigen-antibody complexes that may be small or large; soluble or precipitable, depending on their stability, the number of similar epitopes of the antigen, the valency of the antibody, and the proportion between antigens and antibodies. The larger the complexes, the less soluble and more prone to precipitate they are. Circulating immune complexes may deposit in tissues, producing immune complex diseases, however, their interaction with circulating immune cells, such as neutrophils and platelets can trigger non-IgE mediated anaphylactic reactions. Immune complexes assembled with IgM and IgG may activate the classical pathway of the Complement system through the binding of C1q, cleaving C3 and C5, liberating anaphylatoxins such as C3a and C5a that act directly on mast cells as histamine releasers. Through binding of their cognate G protein-coupled receptors these anaphylatoxins can also activate neutrophils, monocytes, macrophages, T cells, and B cells. Additionally, IgG immune complexes can induce Neutrophil Extracellular Traps (NET) release, producing non-IgE–dependent anaphylaxis. Immune complexes produced by food allergens were already described to produce acute recurrent urticaria, bronchospasm, and facial and airway angioedema. Fortunately, this is not the rule, but the exception. Usually, most immune complexes originated by food allergens are small and cleared from circulation by the reticuloendothelial system. The sole presence of serum antibodies able to precipitate when challenged *in vitro* with food antigens is not per se an indicator of disease, intolerance, hypersensitivity or allergy. However, the titration of food-specific precipitins into the serum of non-IgE mediated food-allergic patients is an easy and feasible laboratory procedure that, in our hypothesis, may provide some clues about the food allergens responsible for the clinical allergic symptoms and help to choose proper candidates to precede the OFC.

MATERIALS AND METHODS

Study design

The study was designed to obtain the diagnostic accuracy of the titration of food-specific Precipitins, as compared with the OFC after a well-succeeded food exclusion diet for diagnosis of non-IgE mediated food allergy.

Patients

To contribute to study the utility of the precipitin's titration in the diagnosis of non-IgE mediated food allergy, we invited a group of patients with these characteristics: A) recurrent episodes of acute urticaria associated with the ingestion of specific food allergens; B) self-imposed food restriction of these specific food allergens self-perceived as temporally related with the urticaria aggravation; C) at least one episode, appreciated by the investigators, of acute (non-vascular, not-pigmentated) urticaria discriminated by surface microscopy related with the ingestion of these food allergens;66 D) Non-reagent allergic-skin scrape tests done with the suspected food allergens. E) Non-detectable specific-IgE to these or any allergen. F) Previously recorded (negative or positive) OFC tests done with the food allergens suspected in the elicitation of the acute episodes of urticaria. After receiving institutional review board approval, 23 selected outpatients (9 male; 20-72 years; mean age 46 years; SD 14,9 years) from the Instituto Alergoimuno de Americana (Brazil) presenting with the above-mentioned characteristics were invited with informed consent formularies, to voluntarily provide blood samples to perform *in vitro* 109 food-specific precipitation titrations, according to the principles of the world medical association declaration of helsinki and the international committee of medical journals editors requirements of privacy.

Precipitins tests

The patient's blood was collected in a clot-activator collecting tube. After serum separation, the tube was centrifugated at 1,000 rpm for 20 minutes. The allergen extracts were allocated in sets of nine glass tubes at progressive duplicated dilutions. The progressive dilutions were combined with the same volume (1 mL) of serum resulting in the dilution ratios of 1:1; 1:2; 1:4; 1:8; 1:16; 1:32; 1:64 and 1:128. The ninth tube was a blank control done just with the serum. After 24 hours, the tubes were examined by one of us and the tubes with visible precipitation on the bottom were recorded.

Oral food challenges

After a successful elimination diet, the asymptomatic and unmedicated patients were oriented to proceed with unblinded supervised oral food challenges, with each suspected food allergen, with increasing doses until the appearance of allergic symptoms or the consumption of at least 100 g of the suspected food. The OFC tests were performed at least a week apart.

Statistical analysis

Statistical analyses were performed by calculating the probabilities of a positive or negative Oral Food Challenge test (as the gold standard reference diagnosis method) according to the precipitin's titration. The correlation coefficient between the tests was performed using GraphPad Prism software (version 5.0; GraphPad Software, Inc., San Diego, CA, USA).

RESULTS

The clinical characteristics of the patients and results of precipitins titration and oral challenge tests are distributed in Table 1. The probabilities of a positive or negative oral food challenge associated with each precipitin's positive or negative titration are distributed in Table 2. The correlation coefficient between the precipitin's titrimetry and the probability of a positive OFC was 0,76 (p=0.017). The negative or low precipitin's titles were well correlated with negative OFC tests. The high precipitin's titles were well correlated with the positive OFC tests Figure 1.

Table 1: Clinical characteristics and results of precipitins titrimetry related to 109 oral food challenge tests of 23 patients with acute episodes of urticaria associated with food ingestion.

Patient	Age	Sex	Food allergen	Positive dilutions	OFC
AF	49	F	Yeast	1:1 to 1:8	Negative
AF	49	F	Cocoa	1:1 to 1:16	Negative
AF	49	F	Swine meat	1:1 to 1:16	Positive
AF	49	F	Latex	1:1 to 1:32	Positive
AF	49	F	Peanut	1:1 to 1:32	Positive
AR	40	M	Latex	1:1 to 1:2	Negative
AR	40	M	Cocoa	1:1 to 1:8	Negative
AR	40	M	Yeast	1:1 to 1:16	Positive
AR	40	M	Peanut	1:1 to 1:64	Positive
AR	40	M	Swine meat	1:1 to 1:128	Positive
CA	53	M	Yeast	1:1 to 1:2	Negative
CA	53	M	Cocoa	1:1 to 1:4	Negative
CA	53	M	Peanut	1:1 to 1:64	Positive
CA	53	M	Cow's milk	1:1 to 1:128	Positive
CA	53	M	Latex	1:1 to 1:128	Positive
DC	32	F	Cocoa	1:1 to 1:128	Positive
DC	32	F	Cow's milk	1:1 to 1:128	Positive
DC	32	F	Gluten	1:1 to 1:128	Positive
DC	32	F	Latex	1:1 to 1:128	Positive
DC	32	F	Peanut	1:1 to 1:128	Positive
ED	72	M	Cocoa	1:1 to 1:4	Negative
ED	72	M	Cow's milk	1:1 to 1:4	Negative
ED	72	M	Latex	1:1 to 1:16	Positive
ED	72	M	Gluten	1:1 to 1:32	Positive
ED	72	M	Peanut	negative	Negative
ER	30	M	Cow's milk	1:1 to 1:8	Negative
ER	30	M	Peanut	1:1 to 1:8	Positive
ER	30	M	Swine meat	1:1 to 1:16	Positive
ER	30	M	Yeast	1:1 to 1:16	Positive
ER	30	M	Latex	1:1 to 1:32	Positive
IN	51	F	Swine meat	1:1 to 1:4	Positive
IN	51	F	Chicken's meat	1:1 to 1:16	Negative
IN	51	F	Latex	1:1 to 1:16	Positive
IN	51	F	Egg's white	1:1 to 1:32	Positive
IN	51	F	Egg's yolk	negative	Negative
JM	65	F	Bovine meat	1:1 to 1:4	Negative
JM	65	F	Egg's white	1:1 to 1:4	Negative
JM	65	F	Egg's youlk	1:1 to 1:8	Negative
JM	65	F	Latex	1:1 to 1:16	Positive
JM	65	F	Cow's milk	1:1 to 1:128	Positive
KA	43	F	Beans	1:1 to 1:2	Negative
KA	43	F	Chicken's meat	1:1 to 1:4	Negative
KA	43	F	Egg's white	1:1 to 1:16	Negative
KA	43	F	Rice	1:1 to 1:32	Positive
KA	43	F	Cow's milk	1:1 to 1:128	Positive
LA	61	M	Cocoa	1:1 to 1:4	Negative
LA	61	M	Cow's milk	1:1 to 1:128	Positive
LA	61	M	Swine meat	1:1 to 1:128	Positive
LA	61	M	Latex	negative	Negative
LE	20	F	Cocoa	1:1 to 1:4	Negative
LE	20	F	Cow's milk	1:1 to 1:16	Negative
LE	20	F	Swine meat	1:1 to 1:16	Positive
LE	20	F	Peanut	1:1 to 1:32	Positive

LE	20	F	Latex	negative	Negative
LM	51	M	Latex	1:1 to 1:16	Positive
LM	51	M	Swine meat	1:1 to 1:16	Positive
LM	51	M	Cow's milk	1:1 to 1:128	Positive
LM	51	M	Cocoa	negative	Negative
LU	37	F	Cocoa	1:1 to 1:4	Negative
LU	37	F	Yeast	1:1 to 1:8	Positive
LU	37	F	Latex	1:1 to 1:16	Positive
LU	37	F	Cow's milk	1:1 to 1:32	Positive
LU	37	F	Peanut	1:1 to 1:128	Positive
MA	63	F	Orange	1:1 to 1:4	Positive
MA	63	F	Beans	1:1 to 1:16	Negative
MA	63	F	Rice	1:1 to 1:16	Positive
MA	63	F	Latex	1:1 to 1:32	Positive
MA	63	F	Schrimps	1:1 to 1:128	Positive
MJ	57	F	Swine meat	1:1 to 1:64	Positive
MJ	57	F	Cochineal red	1:1 to 1:128	Positive
MJ	57	F	Latex	negative	Negative
MM	60	M	Swine meat	1:1 to 1:2	Negative
MM	60	M	Cocoa	1:1 to 1:8	Negative
MM	60	M	Peanut	1:1 to 1:16	Positive
MM	60	M	Yeast	1:1 to 1:16	Positive
MM	60	M	Latex	1:1 to 1:128	Positive
MO	53	F	Orange	1:1 to 1:8	Positive
MO	53	F	Cochineal red	1:1 to 1:16	Negative
MO	53	F	Peanut	1:1 to 1:16	Positive
MO	53	F	Swine meat	1:1 to 1:16	Positive
MO	53	F	Cocoa	1:1 to 1:32	Negative
MS	54	F	Swine meat	1:1	Negative
MS	54	F	Peanut	1:1 to 1:4	Positive
MS	54	F	Cocoa	1:1 to 1:128	Positive
MS	54	F	Latex	1:1 to 1:128	Positive
RE	20	F	Latex	1:1 to 1:2	Negative
RE	20	F	Cow's milk	1:1 to 1:16	Negative
RE	20	F	Peanut	1:1 to 1:16	Positive
RE	20	F	Yeast	1:1 to 1:16	Positive
RE	20	F	Swine meat	1:1 to 1:32	Positive
SI	39	F	Shrimp	1:1 to 1:4	Positive
SI	39	F	Latex	1:1 to 1:8	Positive
SI	39	F	Peanut	1:1 to 1:16	Positive
SI	39	F	Swine meat	1:1 to 1:32	Positive
TA	24	F	Peanut	1:1 to 1:32	Positive
TA	24	F	Swine meat	1:1 to 1:64	Positive
TA	24	F	Latex	1:1 to 1:128	Positive
TA	24	F	Cocoa	negative	Negative
TH	30	M	Cocoa	1:1 to 1:16	Negative
TH	30	M	Swine meat	1:1 to 1:16	Positive
TH	30	M	Latex	1:1 to 1:32	Positive
TH	30	M	Bovine meat	1:1 to 1:64	Positive
TH	30	M	Cow's milk	1:1 to 1:64	Positive
TH	30	M	Shrimp	1:1 to 1:64	Positive
WI	55	M	Orange	1:1 to 1:2	Negative
WI	55	M	Swine meat	1:1 to 1:4	Negative
WI	55	M	Gluten	1:1 to 1:128	Positive
WI	55	M	Cocoa	negative	Negative
WI	55	M	Latex	negative	Negative

Table 2: Clinical characteristics and results of precipitins titrimetry related to 109 oral food challenge tests of 23 patients with acute episodes of urticaria associated with food ingestion.

Precipitin's Titration	Probability of a Positive OFC	Probability of a Negative OFC
Negative	0% (0/9)	100% (9/9)
Positive from 1:1 to 1:1	0% (0/1)	100% (1/1)
Positive from 1:1 to 1:2	0% (0/6)	100% (6/6)
Positive from 1:1 to 1:4	28,6% (4/14)	71,4% (10/14)
Positive from 1:1 to 1:8	44,5% (4/9)	55,5% (5/9)
Positive from 1:1 to 1:16	71,4% (20/28)	28,6% (8/28)
Positive from 1:1 to 1:32	92,9% (13/14)	7,1% (1/14)
Positive from 1:1 to 1:64	100% (7/7)	0% (0/7)
Positive from 1:1 to 1:128	100% (21/21)	0% 0/21)

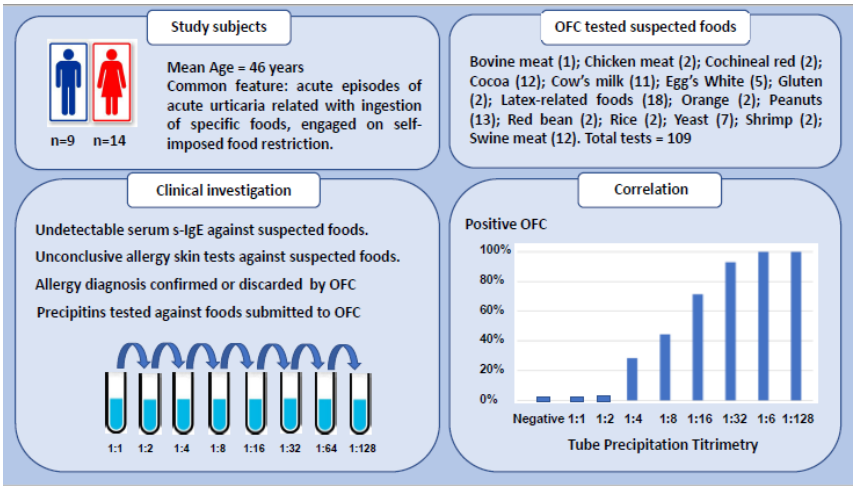

Figure 1: Correlation of the frequency of a positive oral challenge test according to precipitins titration in 109 pairs of tests performed on 23 food allergic patients with acute episodes of urticaria.

DISCUSSION

Non-IgE mediated food allergies syndromes are yet big challenges to the IgE-paradigmatic health community. The lack of specific immune assays to diagnose these syndromes has, unfortunately, produced, particularly in nonspecialists, an "IgE-dependent mentality", re-enforced when a blood test with a non-detectable specific-IgE erroneously suggests "absence of allergy", eclipsing the real diagnosis from the perspective of the health professional. However, the IgE-independent allergic reaction is a relatively common condition that must not be ignored by the assistant physician. A multicenter study done in Turkey identified a non-IgE mediated etiology in 28.2% of food-allergic children. Besides IgE quantification, there are some nonspecific laboratory assays, easily available to most physicians that may give a clue to the diagnosis of non-IgE mediated allergies, such as the complete blood count. The eosinophil cationic protein, the circulating immune complexes, the tryptase, the erythrocyte sedimentation rate, the C-reactive protein, and the Complement dosages. More specific non-IgE mediated assays exploring *ex vivo* challenge tests, such as the Lymphocyte Proliferation Tests, the Leukocyte Migration Inhibition Tests, or the Leukocyte Adherence Inhibition Tests (LAIT) are more elaborated assays that are not performed at most clinical laboratories. Besides the gastrointestinal hypersensitivity chronic syndromes, suspected mainly by the biopsy of endoscopic samples, in clinical practice we observe a lot of elusive clinical situations, manifested by food-related symptoms, that remain unproven by the lack of specific laboratory assays. Usually, these syndromes are managed after laborious clinical observations based on empirical exclusion diets and diagnosed through the OFC tests. The research of precipitins has historically in augured both Immunology and Allergology as correlated sciences but, unfortunately, was forgotten by most researchers and physicians. It is an easy and inexpensive assay that can be very useful when properly indicated and interpreted inside the clinical allergology investigation set. This is a preliminary report from a pilot study with clear limitations.

CONCLUSION

The study group is small and was selected retrospectively from a pool of patients which common characteristics that were already diagnosed by unblinded and non-uniform OFC methodology. As a proof-of-concept, there is also no control group, so we recognize that the study was not designed in a manner from which strong conclusions can be drawn. However, our experience indicates that the evaluation of precipitins may be a worthwhile avenue of investigation for the clinical study of non-IgE mediated allergy. Most

comprehensive studies designed prospectively with double-blinded active and control arms may bring more strong evidence of the utility of food-specific precipitins in the diagnostic routine of food-allergic patients. The research of precipitins is not the complete solution for the diagnosis of non-IgE mediated allergies, but may represent an easy and feasible start for the clinical investigation, helping in the screening of suspected allergens to planning exclusion diets and further proceed to more sophisticated and elaborated *in vivo* tests and *in vitro* and *ex vivo* assays.

CONFLICT OF INTEREST

Authors have no conflict of interest.

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