

Original Article

Evaluation of Basophil Activation Test in Suspected Food Hypersensitivity

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Background: Food hypersensitivity is characterized by a wide range of symptoms. The relationship between symptoms and food is more frequently suspected than objectively proven. Basophil activation test (BAT) is based on the evaluation of activation markers on blood basophils *in vitro* stimulated with drugs or allergens.

The aim of the study was to evaluate the usefulness of BAT when introduced in the routine work-up of suspected food hypersensitivity.

Methods: BAT was requested in subjects with food adverse reactions when a discrepancy existed among history and skin prick test (SPT) and/or specific IgE. Data from 150 subjects were analysed using CD63 as basophil activation marker. Thirty controls were evaluated for cut-offs. Immunoblots was performed with the sera of representative subjects positive for BAT and negative for SPT and sIgE.

Results: 1,024 BAT were carried out, the agreement (positive/positive and negative/negative) was 78.5% for BAT vs. SPT and 78.3% for BAT vs. IgE. Atopic patients, but not atopic controls, more frequently had a positive BAT than non-atopic patients ($P < 0.0001$). Among subjects with positive BAT, those with negative sIgE had lower total IgE, $P = 0.001$. Nearly 23.3% of all subjects had positive BAT (for at least one tested food) and both negative sIgE and SPT. Immunoblots revealed the presence of sIgE for the tested foods in representative patients with positive BAT, negative SPT and sIgE.

Conclusion: Introduction of BAT in routine of food hypersensitivity, limited to subjects with a discrepancy between history and traditional tests, might be useful particularly when total IgE are low. © 2015 International Clinical Cytometry Society

Key terms: basophil activation test; CD63; food hypersensitivity

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Three to four percent of adults (1) and 6–8% of young children present adverse responses to foods in westernized countries (2). Many recent papers reported different data on the epidemiology of food allergy (3,4).

Food induced allergic reactions are characterized by a large variety of symptoms involving the skin, the gastrointestinal tract and the respiratory system. The relationship between symptoms and food is more frequently suspected than objectively proven (5). As stated in the recently presented EAACI Food Allergy and Anaphylaxis Guidelines (3), the self-reported food allergy is six times higher than the diagnosed food allergy. The gold stand-

ard for the diagnosis of food allergy remains the double-blind, placebo-controlled food challenge (DBFC) (3). The severity of food adverse reactions varies from mild symptoms to anaphylaxis, and it is really difficult to

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foresee the extent of the reaction. Basophil activation test (BAT) is based on the evaluation of basophil reactivity after their incubation in vitro with food extracts (6,7). The evaluation of basophil activation occurs through the detection of membrane markers expressed only after activation, such as CD63, or through the increase of the expression of constitutional markers such as CD203c (8–10). Different works have dealt with the usefulness of this test in food allergy (11–15) but, to the best of our knowledge, none of them presented the results of the application of this test in the diagnostic routine of food hypersensitivity. The aim of our study was to evaluate the usefulness of BAT in this context, particularly when discordance exists between reported symptoms, skin prick test (SPT) and/or sIgE.

MATERIAL AND METHODS

Subjects

Clinicians from the Allergy Unit of Fondazione Salvatore Maugeri in Pavia and of the Medicine Unit of San Raffaele Hospital in Milan requested BAT in subjects with food hypersensitivity, when a discrepancy existed among history and skin prick test (SPT) and/or specific IgE. We evaluated data from 150 subjects (mean age 36.6 years; range 6–74) who reported symptoms related to food hypersensitivity: gastrointestinal symptoms (regurgitations, retrosternal and/or gastric pain, vomiting, diarrhea, gut cramps and pain, constipation), urticaria and/or angioedema, anaphylactic reactions (3), exacerbation of atopic dermatitis associated to food intake and overlap of symptoms (2). Anamnesis was collected and subjects underwent SPT, serum total and specific IgE (sIgE). Subjects with at least one positive SPT or sIgE were considered atopic. Subjects with oral intake of corticosteroids in the previous 10 days were excluded from the study, while antihistamines (cetirizine, desloratadine) were allowed as reported not to interfere with basophil activation (16,17). All the enrolled patients went to Salvatore Maugeri Foundation to perform the test.

Thirty controls were tested for the foods which they tolerated in order to define the technical cut-off for BAT. This study conformed to the declaration of Helsinki and was approved by the IRB of Fondazione Salvatore Maugeri.

Skin Prick Tests

SPT were performed with commercially available food extracts (Lofarma, Milan, Italy or Stallergenes, Paris, France, or ALK, Hørsholm, Denmark) following International guidelines (18).

Total and Specific IgE

Total and specific serum IgE were evaluated with ImmunoCap (ThermoScientific, Uppsala, Sweden). Sensitivity for total and sIgE was 0.1 kU l^{-1} .

Basophil Activation Test

Immediately after blood sampling, $50 \mu\text{l}$ of peripheral heparinized blood was pre-incubated with 20 ng ml^{-1} of IL-3 at 37°C for 10 min and then with the same food

extracts used for SPT (1:100 final dilution of the commercially available standardized extracts). The standardized commercial extracts of foods contain different amounts of total proteins but with approximately the same reactivity power and consistency from batch to batch. Optimal experimental conditions were set in previous experiments, data not shown. Fifty microliters of wash buffer (Basotest, Glycotope, Heidelberg, Germany) was used as negative control and $50 \mu\text{l}$ of monoclonal anti-IgE (200 ng ml^{-1} , clone G7-18, BD Biosciences, San Jose, CA) as positive control for IgE induced basophil activation. We did not use formyl-methionyl-leucyl-phenylalanine (fMLP) as control of in vitro cell reactivity, since the test was performed immediately after blood sampling. The reaction was blocked 8 min on ice. Then, basophils were stained with $20 \mu\text{l}$ of a mixture of purified anti-IgE PE (Glycotope) and with anti CD63-FITC (Glycotope) for 30 min on ice. Erythrocytes were lysed with NH_4Cl solution (DAKO, Glostrup, Denmark). Negative SPT extracts were used to exclude possible interference of prick solution in BAT. An inhibition of CD63 by the SPT buffer was excluded by diluting the positive control (Anti-IgE) in SPT buffer. A range of 400–1,000 basophils was considered suitable for the analysis.

Stimulation index (SI) was calculated as the ratio: % CD63 expression with food extract/% CD63 with wash buffer and it was used to allow the normalization of CD63 expression in different subjects with their basal CD63 expression (without any stimulation).

SDS PAGE and Immunoblot

Sera of patients with positive BAT and negative SPT and sIgE were used to evaluate their IgE reactivity to food extracts. Thirty μl of the same food extract used for SPT and BAT were run onto 12.5 or 20% SDS PAGE and proteins were then blotted on nitrocellulose (Whatman Dassel, Germany) using Mini-PROTEAN 3 Cell (Bio-Rad Laboratories, CA). Then the membrane was cut into strips, one for each run, blocked with 5% milk in Tris-buffer (10 mmol L^{-1} Tris, 150 mmol L^{-1} NaCl, 0.1% Tween 20, $\text{pH} = 7.4$) for 1 h, and incubated overnight at 4°C with patient's serum diluted 1:6 with 1% milk/Tris-buffer. sIgE were detected with a goat anti human IgE-HRP conjugated (Sigma-Aldrich, St. Louis, MO), diluted 1:1000 in 1% milk/Tris-buffer (Sigma-Aldrich, St. Louis, CA). After 1 h incubation, SuperSignal West-Pico chemiluminescent substrate (ThermoScientific, Rockford, IL) was used as a substrate and the signal produced impressed a X-ray film (Eastman Kodak, Rochester, NY).

Statistical Analysis

Data were expressed as median (25th–75th percentile). χ^2 was used to evaluate dichotomic variables and ANOVA to evaluate the variability of CD63 expression and SI among groups of subjects with different symptoms. Post hoc analysis was carried out with Bonferroni test. Comparison of continuous variables between two groups was performed with Mann-Whitney *U* test. Wilcoxon test was used to evaluate the effect of skin prick

Table 1
Characteristics of the Subjects Enrolled

	All subjects; <i>n</i> = 150	Gastroint symptoms; <i>n</i> = 39	Urticaria/ Angioed; <i>n</i> = 44	Mix symptoms; <i>n</i> = 34	Anaphylaxis; <i>n</i> = 20	Other symptoms; <i>n</i> = 13	<i>P</i>
Age (range)	36.6 (6–74)	36.6 (10–62)	37.2 (9–74)	36.5 (15–65)	42.2 (20–71)	26.3 (6–52)	ns
Sex F/M	118/32	35/4	35/9	26/8	14/6	8/5	ns
Atopy yes/no	110/40	20/19	32/12	29/5	20/0	10/3	0,001
Total IgE; (25th–75th perc.)	68.8 (26.7–1630.0)	60.8 (11.0–94.8)	142.0 (41.9–308.5)	50.0 (25.9–253.5)	94.3 (40.0–499.0)	64.1 (29.8–1395.0)	ns

The subjects were classified according to their reported symptoms as gastrointestinal, urticaria and/or angioedema, mixed symptoms (gastrointestinal + urticaria/angioedema) and anaphylactic shock. Subjects with oral allergic syndrome, exacerbation of atopic dermatitis related to foods and headache related to food ingestion were classified as “other symptoms.”

ns = not statistically significant ($P > 0.05$).

test buffer on basophil activation. P values < 0.05 (two-tailed) were considered as significant (Statistica for Windows software).

RESULTS

Basophil Activation Test in Controls

Thirty subjects, 15 atopic and 15 non-atopic, were included as controls for the food they tolerated (9 male, 21 female, mean age 36.7 years, range 13–62). 243 single BAT determinations were performed with food extracts in all the control subjects. The 95th percentile of CD63% and SI distribution in controls were respectively 4.70% and 1.68. No difference between atopic and non atopic controls was detected. The use of diluted SPT extracts, neither determined nonspecific activation of basophils nor inhibition of activation as demonstrated by CD63 expression in basophils activated with anti IgE (median CD63% = 47.7; 25th and 75th percentile 22.2–74.8%) and with anti IgE diluted in SPT extracts (median CD63% = 44.2; 25th and 75th percentile 22.8–83.8%, $P = 0.68$, Wilcoxon test).

We chose as positive technical cut-off, CD63 $> 5\%$ and SI > 2 , which is the same used in routine tests for drugs.

Patients

The 150 subjects studied were divided according to their symptoms: gastrointestinal symptoms ($n = 39$), urticaria and/or angioedema ($n = 44$), mixed symptoms (gastrointestinal + urticaria/angioedema, $n = 34$) and anaphylactic shock ($n = 20$). Subjects with oral allergic syndrome (OAS), with exacerbation of atopic dermatitis related to foods and headache related to food ingestion were collected in the group “other symptoms” ($n = 13$) (19). The characteristics of the subjects are shown in Table 1.

Basophil Activation Test in Subjects with Suspected Food Hypersensitivity

A total of 1024 BAT tests with food extracts were carried out on the enrolled patients, 947 sIgE determinations and 1011 SPTs. The expression of CD63 on basophils incubated with wash buffer (negative control) in

all the subjects was 2.6% median, (25th and 75th percentile 1.9–3.5).

When we compared the single tests, BAT vs. IgE and BAT vs. SPT, 27% of BAT and 17.9% of sIgE were positive ($\chi^2 < 0.0001$), while 29.3% of BAT and 14.3% of SPT resulted positive ($\chi^2 < 0.0001$).

The agreement (positive/positive and negative/negative) between single BAT and SPT was 78.5% and between single BAT and sIgE 78.3%. The subjects without activation with the positive control anti IgE, were 7/150 (4.6%), defined as “non releaser,” and had both negative SPT and sIgE.

When a discrepancy between SPT and sIgE was detected (17.6% of the tests), BAT agreed with sIgE in 46.7% and with SPT in 53.3% of the cases.

Considering the subjects enrolled, 110/150 (73.3%) had a positive BAT for at least one food. Atopic subjects (110/150), more frequently had a positive BAT (81.8%) than non atopic subject (45.0%, $\chi^2 P < 0.0001$). Among the subjects with at least one positive BAT, those with negative sIgE had lower total IgE ($P = 0.001$), Figure 1, panel A. No difference in age was found between these subjects ($P > 0.05$). Basophils of subjects with low total IgE were able to express as high an amount of CD63 as basophils of subjects with high total IgE, Figure 1, panel B and C. Evaluating the data according to the reported symptoms, no difference was found in BAT positivity among the groups with different symptoms ($\chi^2 P = 0.18$ all, and $P = 0.74$ only atopics), Figure 2.

Part of all the enrolled subjects, 23.3% (35/150) had positive BAT (for at least one tested food) and both negative sIgE and SPT, particularly, 33.3% of the subjects with gastrointestinal symptoms (13/39), 22.7% of those with urticaria/angioedema (10/44), 23.5% mixed symptoms (8/34), 15.0% anaphylaxis (3/20), 7.7% of those with other symptoms (1/13) ($\chi^2 P = 0.29$); 1.3% of the subjects only had positive sIgE for at least one tested food (negative BAT and SPT); 4.7% of the subjects only had positive SPT.

A significant variation was found in the expression of CD63 on basophils and in the SI when subjects were divided according to the reported symptoms (one way ANOVA $P < 0.001$). Post hoc analysis revealed that while

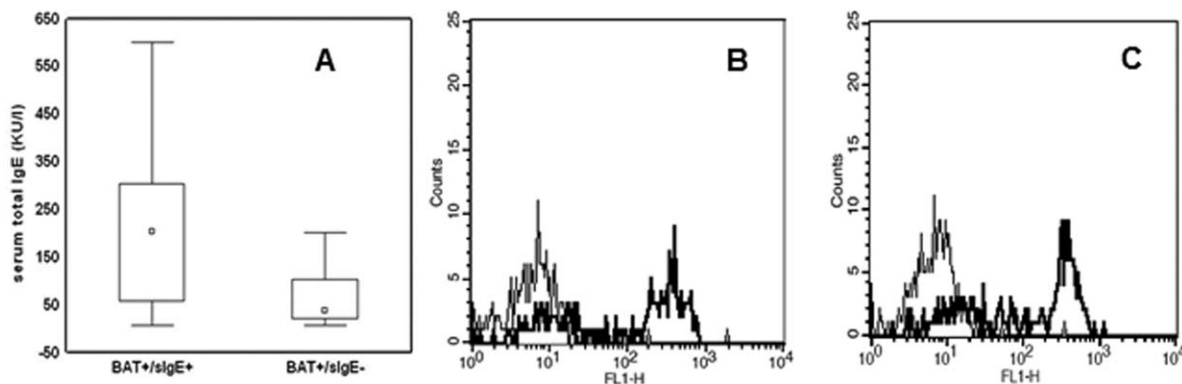


FIG. 1. Total IgE in patients with BAT+/sIgE+ and BAT+/sIgE-. The inner points represent the median values and the boxes the 25th and the 75th percentiles of the data, the whiskers min and max of non-outlier data, panel A. Overlay histograms of CD63 expression on basophils incubated with food extracts (bold line) and wash buffer (thin line) in a representative subject with high total IgE (691.0 kU L⁻¹) and concordance between positive BAT (CD63 = 71.9%, SI = 25.7) with fennel extract and positive specific IgE (4.99 kU L⁻¹), panel B, and in a subject with low total IgE (30.0 kU L⁻¹), positive BAT with peanuts extract (CD63 = 83.3%; SI = 43.8), and negative specific IgE (<0.1 kU L⁻¹), panel C.

CD63 expression was significantly lower in subjects with gastrointestinal symptoms, the SI was significantly higher in subjects with anaphylaxis symptoms, highlighting the importance of considering this index together with CD63 expression, Table 2.

Among the subjects with the anaphylactic reactions, 10% (2/20) had all negative tests, 55% (11/20) had all three positive tests (BAT, sIgE and SPT), 15% (3/20) had positive BAT and sIgE, 5% (1/20) positive BAT and SPT and 15% (3/20) only positive BAT. BAT resulted positive in 90% of these subjects, sIgE in 70% and SPT in 60%.

In Table 3, we reported the most frequently tested foods and the percentages of subjects who resulted positive for only one test. BAT and SPT were performed with the same food extracts. Shrimp, soy, peanuts were, among the tested foods, the ones which more frequently determined a positive BAT and negative sIgE and SPT.

Immunoblot of Patients with Positive BAT and Negative SPT and sIgE

We performed electrophoresis with the same food extracts used for BAT and SPT, and Immunoblot with the serum of subjects with positive BAT and negative SPT. In Figure 3, we showed results of four patients' sera: two with a positive BAT for peanuts, one with a positive BAT for soy and another for corn, all with negative SPT and sIgE. sIgE to proteins with different

molecular weights were detected in all the samples tested suggesting the presence in the serum of these patients of sIgE not detectable by immunoassays.

DISCUSSION

This is a "real-life" study, in which we analysed the results obtained by introducing BAT into food adverse reaction diagnostic work-up. We evaluated data obtained from 150 subjects with gastrointestinal symptoms, urticaria/angioedema, mixed symptoms, anaphylactic reactions and other symptoms related to food hypersensitivity. The number of subjects with a BAT positive for at least one tested food was higher than those with positive SPT or sIgE. About 20% of the analysed subjects had positive BAT and both negative sIgE and SPT (15% of patients with anaphylaxis). BAT was more frequently positive in atopic subjects with suspected food hypersensitivity, but not in atopic controls. CD63 expression after basophil activation was lower in subjects with gastrointestinal symptoms, and SI was higher in subjects with anaphylactic reactions. Among BAT positive subjects those with negative sIgE had low total IgE.

The aim of this study was to propose BAT as a useful test to select, among the subjects with suspected food hypersensitivity and negative sIgE and/or negative SPT, those suitable for food diet in order to evaluate symptoms disappearance and for DBFC with food when a

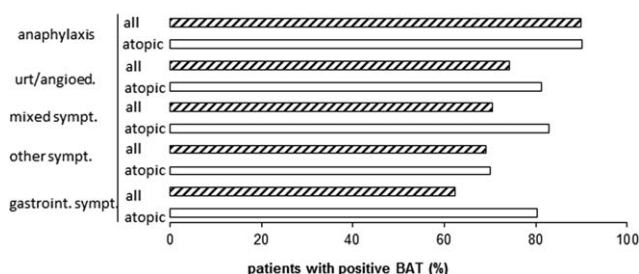


FIG. 2. Patients with a positive BAT for at least one tested food extract. Shaded bars: percentages of all subjects divided according to their reported symptoms. Open bars: percentages of atopic patients divided according to their reported symptoms.

Table 2
CD63 Expression and Stimulation Index (SI) in Positive BAT Divided According to Patients' Symptoms

Symptoms	CD63% ^a		<i>P</i> post hoc test	Stimulation index (SI) ^a		<i>P</i> post hoc test
	Median	25th–75th percentile		Median	25th–75th percentile	
Gastrointestinal	6.9	5.5–21.0	<0.02 vs. all the other groups	3.1	2.3–9.2	<0.02 vs. urticaria/angioedema and anaphylaxis
Urticaria/angioedema	21.6	8.1–87.3		8.0	2.8–37.2	
Mixed symptoms	18.8	9.3–62.2	<0.02 vs. anaphyl. and others	8.1	3.0–18.1	<0.02 vs. anaphylaxis
Anaphylaxis	45.6	14.1–82.3		20.0	4.8–33.1	
Other symptoms	81.3	22.3–90.9		6.2	5.1–10.0	

^aOne-way ANOVA $P < 0.001$ both for CD63 and SI.

standardized procedure is available. To define BAT as positive, we calculated technical cut-offs with data from atopic and non atopic controls with no reported food reactions. This cut-off should be the basis in order to identify other clinical cut-offs to predict positive in vivo challenges. The same happens for sIgE, with a technical cut-off usually $>0.1 \text{ kU L}^{-1}$, and different clinical cut-offs to predict a positive in vivo challenge (20,21). Most of the commercially available kits proposed CD63-cut-offs for foods around 10–15%, without taking into consideration the SI. We considered both basophil CD63 expression and the SI in order to reduce the possibility to have positive BAT in in vitro hyperreactive subjects.

When interpreting BAT positivity to foods, we have to consider that extracts used to activate basophils in vitro are a mixture of different proteins, some of them thermo-labile and/or pH sensitive (22,23).

As reported in Table 3, the foods which more frequently resulted positive for BAT and negative for sIgE and SPT are shrimps, peanuts and soy possibly due to the particular conformation or concentration of the allergens in the commercial extracts.

We found a positive BAT for at least one tested food in 23.3% of the subjects with negative SPT and sIgE. At least in some of them, we showed, with Immunoblot, the presence of serum sIgE to some proteins present in the same food extract used for BAT. These subjects should be monitored, and after a diet period, if their symptoms disappear or decrease, they should be challenged with the food resulted positive with BAT if a standardized procedure is available (18,24–26). It is relevant to underline that in our study, BAT was the sole positive test in 15% of patients with anaphylaxis. Hence, in patients with idiopathic anaphylaxis (negative SPT and sIgE) BAT might be relevant in order to identify the causative agent. Patients with a history of anaphylaxis should undergo challenges only if the results of the laboratory tests are not conclusive, and the test must be performed in an intensive care unit (27).

Subjects with a positive BAT and negative sIgE had lower total IgE than subjects with both positive BAT and sIgE, suggesting that BAT is particularly useful in subjects with low total IgE. Furthermore, basophils of subjects with low total IgE maintain the same capacity to be

Table 3
Percentages of Subjects with Only One Positive Test (BAT or sIgE or SPT) for the Most Tested Food Extracts

Extract	Number of tests	BAT+/sIgE-/SPT-(%)	sIgE+/BAT-/SPT-(%)	SPT+/BAT-/sIgE-(%)
Shrimp	36	36.1	0.0	0.0
Peanuts	50	16.0	0.0	0.0
Soy	41	19.5	0.0	0.0
Oats	21	0.0	4.8	0.0
Casein	33	0.0	9.1	0.0
β -lactoglobulin	28	3.6	3.6	0.0
Lactalbumin	27	7.4	7.4	0.0
Crab	15	26.7	6.7	0.0
Barley	15	20.0	13.3	0.0
Peach	23	8.7	4.3	0.0
Hazelnut	33	3.0	3.0	0.0
Corn	27	7.0	0.0	3.7
Milk	52	13.5	5.8	1.9
Yeast	43	13.9	2.3	11.6
Yolk	25	8.0	4.0	4.0
Wheat	63	6.3	6.3	1.6
Albumen	36	5.5	13.9	2.8
Tomato	54	1.9	18.5	3.7

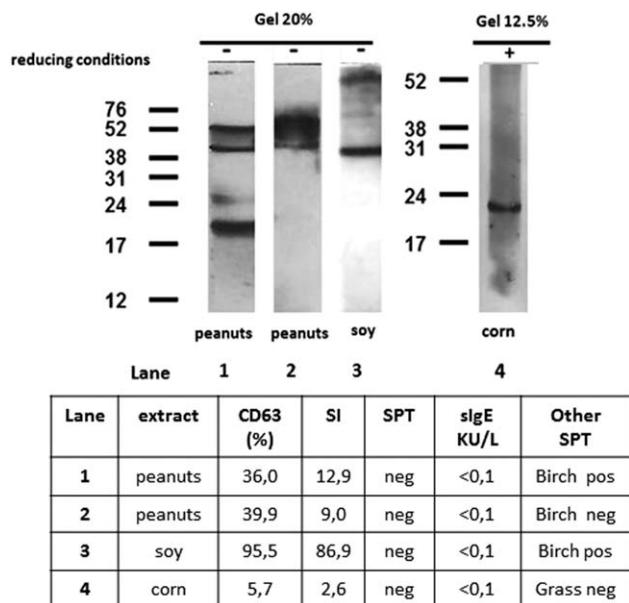


FIG. 3. Sera of 4 representative patients with positive BAT and negative slgE and SPT run in SDS PAGE, blotted onto nitrocellulose and incubated with anti IgE. The characteristics of the gels are indicated in the picture. Results of BAT as CD63 expression and stimulation index (SI), skin prick test (SPT) and serum specific IgE (slgE) are show in the table.

activated in vitro as subjects with high total IgE, as shown for two representative patients in Figure 1, panel B and C. These subjects might have very low circulating slgE for foods, but a quantity of slgE bound to the FCεRI on basophil membrane sufficient to activate basophils when incubated with food. As shown in Figure 3, BAT reactivity seems caused by IgE even when no circulating slgE can be detected with the commonly used immunoassays. A local production of IgE might be the cause of very low systemic specific IgE, as recently reported (28,29). The lower basophil CD63 expression found in subjects with gastrointestinal symptoms compared to the other groups could be explained considering that systemic slgE amounts can be low although the production of IgE can be high in affected tissues (e.g., intestines).

In our study the sensitivity of SPT and slgE is likely underestimated since BAT was performed particularly in subjects with a discrepancy between anamnesis and/or SPT and slgE. The positivity of SPT could be particularly low since, to more appropriately compare BAT and SPT, we used commercial food extracts, and not prick by prick with fresh food.

The use of BAT in oral allergy syndrome was previously published by Ebo et al. who demonstrated the utility of the test particularly in comparison with slgE which resulted in a really low specificity (30), since they can reflect primary sensitization rather than allergy.

In conclusion, a good correlation between BAT and the reported symptoms was shown in subjects with a history of food hypersensitivity. BAT is a functional test which should be limited to evaluate food reactions when a discrepancy between reported history and tradi-

tional tests occurs, particularly in subjects with low levels of total IgE. Other “in vivo” studies are needed to evaluate if BAT could be predictive of these reactions.

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