

Original article

Use of CD63 expression as a marker of *in vitro* basophil activation and leukotriene determination in metamizol allergic patients

Background: We assessed the reliability of basophil activation test (FAST) and sulphidoleukotriene production (CAST) in the *in vitro* diagnosis of allergy to metamizol, evaluating its sensitivity and specificity.

Methods: Twenty-six patients allergic to metamizol and 30 control individuals were studied. Skin tests with metamizol, FAST, and CAST were performed.

Results: FAST sensitivity was 42.3% and specificity 100%. The PPV of FAST is 100% and the NPV 99.4%. The likelihood ratio for a positive value cannot be calculated because the specificity is 100% and the likelihood ratio for a negative value is 0.58. CAST sensitivity was 52%, and specificity 90%. The PPV of the test is 5% and the NPV 99.5%. The likelihood ratio for a positive result was 5.2 and that for a negative result 0.53. FAST detects a larger number of cases when patients are studied within the first 6 months after the clinical reaction ($\chi = 4.2$, $P = 0.04$) than later. Together with skin tests, FAST allowed detection of 69.2% patients allergic to metamizol, the same as CAST 76%. The joint use of the three techniques allowed identification of 76.9% of cases.

Conclusions: FAST and CAST are useful for the diagnosis of allergy to pyrazolones. Its usefulness clearly increases when recent reactions are studied.

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In Spain, pyrazolones are the second cause of IgE-mediated allergic reactions to drugs, only preceded by betalactam antibiotics, which constitute 11% of drug allergies (1). Up to now, there are no commercialized *in vitro* diagnostic tests demonstrated to be effective in the diagnosis of allergy to pyrazolones. Therefore, we tested the usefulness for that purpose of two new *in vitro* diagnostic techniques: basophil activation test (FAST) and leukotriene release (CAST). FAST measures the expression of a CD63 protein present in the cytoplasmic granules of inactive basophils, and expressed on their surface once activated. CAST measures the amount of sulfidoleukotrienes released by these cells after activation. The diagnostic usefulness of both techniques has been demonstrated for inhalant allergens (2), foods (3), betalactam antibiotics (4), latex (5), and other drugs (6).

In this study, we evaluated the diagnostic reliability of flow-cytometric allergen stimulation test (FAST), also called Flow-CAST (Bühlmann laboratories, Allschwil, Switzerland), and based on CD63 detection in activated basophils, by flow cytometry after allergen (Ag)-specific stimulation *in vitro*. For that purpose, we use two monoclonal antibodies: an anti-IgE labelled with FITC as basophil marker, and an anti-CD63 labelled with

phycoerythrin (PE) as activation marker. We assessed the reliability of the technique in the *in vitro* diagnosis of allergy to metamizol, evaluating its sensitivity and specificity at an optimal given cut-off point.

Material and methods

Patients

Twenty-six patients allergic to metamizol, six men and 20 women (41.6 ± 13.6 years), who had presented anaphylaxis (14 cases) or urticaria/angioedema (12 cases) immediately (less than 1 h) after administration of metamizol exclusively were studied. The first 26 consecutive patients diagnosed of allergy to metamizol in the University Clinic of Navarra or in the Hospital de Basurto (Bilbao, Spain) are subject of this study. All the patients with previous history of intolerance to other analgesics such as aspirin or NSAIDs, or with chronic or recurrent urticaria were excluded from the study.

Patients had to be asymptomatic for a follow-up period of at least 1 year after avoiding administration of pyrazolones. Intolerance to analgesics such as NSAIDs was ruled out by means of oral provocation test with acetylsalicylic acid and diclofenac, which were performed in both patients and controls and found negative. Other possible causes of urticaria or anaphylaxis were also ruled out by means of clinical history and the corresponding complementary tests (skin tests to foods, specific IgE, etc.). Subjects with infectious,

cardiological, hepatic, and endocrinological diseases were excluded. Patients stopped the intake of antihistamines at least 5 days before blood sampling (6 weeks for astemizol) and corticosteroids at least 1 month before, when applicable.

Patients were diagnosed as allergic to pyrazolones in all cases with positive skin tests, and if they had presented clear-cut metamizol-induced anaphylaxis independently of the skin test results. Patients with urticaria were included if they had positive skin tests or several urticaria-angioedema episodes produced exclusively after administration of metamizol. In some patients with only one urticaria episode and negative skin tests, oral provocation test was performed if the patients gave their informed consent.

Thirty control individuals were selected among patients who attended the allergy consultation in both hospitals for diagnosis of any other allergic condition (13 men and 17 women) (38.9 ± 13.4 years); they had negative skin tests and oral provocation tests to metamizol and acetylsalicylic acid.

In order to avoid bias, three diagnostic tests were performed by different persons, none of whom knew the results of the other tests.

All subjects included in the study were informed orally about the study and signed the corresponding informed consent. The study obtained approval from the Ethics Committee.

Skin tests

Skin tests were performed according to the usual techniques (7). Prick tests were performed using ALK Abelló lancets, followed when negative by intradermal tests with 261/2-gauge hypodermic needles, injecting 0.05 ml in the volar surface of the forearm. Metamizol 0.1 mg/ml (Boehringer Ingelheim, Barcelona, Spain) was used. Histamine hydrochloride (10 mg/ml) as positive control and NaCl 0.9% as negative control were used. The results were read after 15 min. Wheals 3 mm greater than the negative control for prick testing and 5 mm greater than the negative control for intradermal testing were considered positive.

In vitro tests

Flow-cytometric cellular allergen stimulation test (FAST). The technique has been described previously (2). Blood was collected in 6 ml ACD tubes (Vacutainer, Becton Dickinson, Meylan Cedex, France) and stored at 4°C; the test was carried out within 24 h of blood sampling. For testing up to three allergens, one ACD tube was required. The tubes were centrifuged at 4°C, 10 min, at 550 *g*. Cells from the layer on top of the red blood cells were collected and centrifuged again at 4°C, 10 min, at 550 *g*. The supernatant was decanted and 500 µl of HEPES calcium buffer denominated stimulation buffer (HEPES 20 mM, NaCl 133 mM, KCl 5 mM, CaCl₂ 7 mM, MgCl₂ 3.5 mM, BSA 1 mg/ml, pH 7.4), containing IL3 (2 ng/ml) and 10 µl of heparin (5000 UI/ml; ROVI, Madrid, Spain) were added to each tube, mixed, and gently shaken.

In a polystyrene microtitre U plate (Greiner Microlon) we added 10 µl of 10^{-8} M C5a (Sigma) in all wells and 50 µl of metamizol (Boehringer Ingelheim, Barcelona, Spain), at two different final concentrations: 5 and 1 mg/ml, in the corresponding wells. These concentrations were chosen after being tested on a pool of control subjects, in order to verify the lack of unspecific activation. As a positive control, we used a monoclonal Le27 anti-IgE antibody (Bühlmann, Allschwil, Switzerland) at a final concentration of 1 µl/ml. In order to evaluate basal values without stimulation, we added merely 50 µl of stimulation buffer in another well.

We then placed 50 µl of the patient's cell suspension in each well and covered the plate with an adhesive plastic sheet. The plates were incubated at 37°C for 40 min.

The reaction was then stopped by adding 100 µl of HEPES buffer, pH 7.3, without calcium or magnesium but containing EDTA (HEPES 20 mM, NaCl 133 mM, KCl 5 mM, EDTA 0.27 mM) denominated as washing buffer. Soon afterwards, plates were centrifuged for 5 min at 4°C, 1000 *g*.

The basophils from the pellet were double labelled by adding 20 µl of anti-CD63 PE-labelled antibody (Caltag Laboratories, Burlingame, CA), diluted 1 : 80 in tubes Cytometer Falcon and anti-IgE FITC-labelled antibody (Caltag Laboratories, Burlingame, CA) diluted 1 : 60 in washing buffer. After incubation for 30 min at 4°C (protected from light exposure), we added 4 ml of erythrolytic reagent (Ortho-Munelytic reagent, Ortho Diagnostic System Inc., San Fernando de Henares, Madrid) in each tube and left them at room temperature for 5 min. The cell lysis was stopped with 1 ml of washing buffer. After centrifuging another 5 min at 1000 *g*, the supernatant was decanted and 500 µl of washing buffer added in each tube, which was gently shaken before flow-cytometric analysis.

Flow-cytometric analysis of surface markers was performed at 488 nm on a FACScan flow cytometer equipped with a 15 nW argon ion laser (Becton Dickinson) and analysed by CELLQUEST software.

On the histogram defined by forward scatter and side scatter, the initial cell gate was defined by a bitmap around lymphocytes. The second gate was defined around cells showing high-density anti-IgE-label, identifying them as basophils. In each assay, we assessed at least 500 basophils. The other parameter analysed on the identified basophils was CD63.

In order to rule out small unspecific activations, only activation percentages equal to or greater than 5% were considered as positive. We also considered the results positive when the stimulation index (percentage of activated basophils with metamizol/percentage of activated basophils in basal conditions) was equal to or greater than 5 at any of the two metamizol concentrations.

In order to evaluate the variability of the technique, 60 FAST determinations in 10 patients were done in duplicate at both metamizol concentrations used. Duplicates were performed on the same blood samples.

Antigen specific sulphidoleukotriene production (CAST). For allergen-induced sulphidoleukotriene (sLT) ELISA determination, we followed the instructions of the assay's manufacturer (CAST-ELISA, Bühlmann Laboratories, Allschwil, Switzerland), using the same metamizol concentrations as for FAST. As in FAST, these concentrations were selected after being previously tested on a pool of control subjects in order to exclude concentrations that cause unspecific leukotriene release.

In order to rule out small unspecific leukotriene releases, releases over 100 pg/ml were considered significant. A stimulation index (percentage of leukotrienes released after contacting with the antigen/percentage of leukotrienes released without stimulus) larger than 3 at any of the two concentrations of metamizol was considered positive.

Statistical analysis

The data were analysed through the statistical program SPSS 10.0. The data are expressed in mean $\pm$ SD for variables with normal distribution. Concordance between the different diagnostic tests was assessed by kappa test. Comparison between qualitative variables was done by square Ji test with Yates correlation when necessary or Fisher's exact test. All tests were performed two-sided. Reproducibility of FAST results was assessed by intraclass correlation coefficient. This test measures the reproducibility between two diagnostic tests with continuous results by the creation of two new

variables: the difference between the determinations and the means of both techniques. The results are considered acceptable when this coefficient shows values between 0.4 and 0.75, and excellent when the values are over 0.75. Values of $P < 0.05$ were considered statistically significant.

To obtain a specific cut-off point for the significant percentage of activated basophils, we used a receiver operating characteristic (ROC) curve. The optimal cut-off point calculated by ROC curves combines sensitivity and specificity and takes as coordinates the furthest point of the curve from the diagonal. The actual cut-off point chosen aimed at high specificity values instead of sensitivity since allergy to metamizol is a low-prevalence pathology.

Results

Clinical characteristics

Clinical data of the patients, together with their FAST and CAST results are shown in Table 1. Eleven patients had urticaria/angioedema and 15 had anaphylaxis. Skin tests were positive in 13 patients (50%), two in prick tests and 11 in intradermal tests. The lapse of time from the clinical reaction to the moment of the study was 16.9 ± 30.6 months.

FAST. The intraclass coefficient variation studied in 60 determinations in basal, anti-IgE, and Ag-specific response in duplicate was 0.99 ($P < 0.0001$). The mean and standard deviations of the activation percentage in both determinations were 7.46 ± 15.9 and 7.85 ± 16.1 , respectively. The mean of variation quotient (determination A/determination B) is equal to 0.09.

Eleven patients showed a positive FAST and the other 15 were negative. In Fig. 1 we show an example of a positive FAST in one of our patients. All controls showed a negative FAST. Sensitivity was therefore 42.3% and specificity 100%. In controls, the basal CD63 values were $7.3 \pm 13.2\%$, whereas after stimulation at both metamizol concentrations they were 7.2 ± 13.9 and $7.1 \pm 13.2\%$. Therefore, in controls the values after stimulation with metamizol are not statistically different from the basal values.

There are no recent epidemiological studies about the prevalence of allergy to metamizol, but if we assume a prevalence similar to allergy to betalactam antibiotics (1%), the positive predictive value is 100% and the negative predictive value 99.4%. The likelihood ratio for a positive value cannot be calculated because the specif-

Table 1. Data corresponding to the patients with urticaria or anaphylaxis after metamizol administration

No.	Clinical reaction	FAST*	CAST*	Skin tests	Age (years)	Months from the last reaction (number of reactions with metamizol)
1	Anaphylaxis	Basal 1.8; metamizol 45.9; SI 25.5	Negative	Negative	55	1 (1)
2	Urticaria	Negative	Negative	Negative	24	72 (3)
3	Urticaria	Negative	Basal 40 ; metamizol 150 ; SI 3.75	Positive	46	5 (2)
4	Anaphylaxis	Negative	Negative	Negative	50	4 (1)
5	Urticaria	Negative	Not done	Positive	26	72 (1)
6	Urticaria	Basal 6; metamizol 30; SI 5	Negative	Positive	35	1 (2)
7	Anaphylaxis	Basal 10; metamizol 53.1; SI 5.31	Basal 31.5 ; metamizol 157 ; SI 4.98	Negative	36	1 (1)
8	Anaphylaxis	Negative	Negative	Negative	35	22 (1)
9	Anaphylaxis	Negative	Negative	Negative	27	1 (1)
10	Anaphylaxis	Basal 2.5; metamizol 90; SI 36	Basal 193 ; metamizol 1427 ; SI 7.3	Positive	30	1 (1)
11	Urticaria	Negative	Basal 109; metamizol 481; SI 4.41	Positive	44	1 (2)
12	Anaphylaxis	Negative	Negative	Positive	29	36 (3)
13	Urticaria	Negative	Basal 53; metamizol 287; SI 5.41	Negative	50	1 (3)
14	Urticaria	Negative	Negative	Positive	57	6 (1)
15	Anaphylaxis	Negative	Negative	Positive	50	14 (3)
16	Anaphylaxis	Basal 1.2; metamizol 9; SI 7.2	Basal 0.1 ; metamizol 492 ; SI 4970	Negative	25	4 (4)
17	Anaphylaxis	Basal 1.9 ; metamizol 40.3 ; SI 21.2	Basal 0.1 ; metamizol 323 ; SI 3230	Positive	60	24 (2)
18	Urticaria	Negative	Basal 0.1; metamizol 182 ; SI 1870	Positive	39	18 (2)
19	Urticaria	Basal 2.8; metamizol 63.3; SI 22.6	Basal 0.1; metamizol 573 ; SI 5730	Positive	60	2 (2)
20	Anaphylaxis	Negative	Basal 0.1; metamizol 104; SI 1040	Negative	24	4 (2)
21	Urticaria	Negative	Negative	Negative	43	132 (4)
22	Urticaria	Negative	Negative	Negative	43	6 (1)
23	Anaphylaxis	Basal 5.9; metamizol 61; SI 10.3	Basal 23 ; metamizol 160 ; SI 6.95	Negative	29	2 (1)
24	Anaphylaxis	Basal 5.2 ; metamizol 79.4 ; SI 15.26	Basal 82 ; metamizol 2436 ; SI 29.7	Positive	37	6 (1)
25	Anaphylaxis	Basal 14.3; metamizol 63.6; SI 5.29	Basal 38 ; metamizol 553 ; SI 14.5	Negative	77	1 (1)
26	Anaphylaxis	Basal 0.7; metamizol 13.7; SI 19.5	Negative	Positive	50	5 (2)

* Only the highest positive value of the different concentrations used is included.

FAST: flow-cytometric cellular allergen stimulation test. Results given in % activated basophils.

CAST: antigen-specific sulphidoleukotriene (sLT) production test. Results given in picograms sLT per milliliter.

SI: metamizol value divided by the basal response.

Table 2. Summary of the clinical characteristics and *in vitro* results of patients allergic to metamizol in relation to the symptomatology

	CAST		FAST		Skin test		Symptoms	
	+	-	+	-	+	-	Anaphylaxis	Urticaria
Patients (number of cases)	13	12	11	15	13	13	15	11
CAST								
Positive			8	5	7	6	8	5
Negative			3	9	5	7	7	5
FAST								
Positive	8	3			6	5	9	2
Negative	5	9			7	8	6	9

+, positive result; -, negative result.

icity is 100% and the likelihood ratio for a negative value is 0.58.

Sulphidoleukotriene release test. Thirteen patients showed a positive CAST and 12 were negative. In controls, 27 had a negative CAST and three were positive. Sensitivity was therefore 52%, and specificity 90%. Assuming an approximate prevalence in the population of 1%, like in FAST, the positive predictive value of the test is 5% and the negative predictive value 99.5%. The likelihood ratio for a positive result was 5.2 and the likelihood ratio for a negative result 0.53.

Out of the 13 patients with positive skin tests, six (46.2%) had positive FAST and 7/12 in whom CAST was performed had positive CAST (58.3%) (Table 2), with no significant differences with the patients with negative skin tests, among whom five had positive FAST (38.5%) and six positive CAST (46.2%) (Ji 0.16 and 0.37, respectively; $P > 0.5$).

Concordance between FAST and CAST in the whole patient group (kappa index) is moderate with a value of 0.44. FAST and CAST are both positive in eight cases, FAST only in three cases and CAST only in five cases.

Together with skin tests, FAST allowed detection of 18/26 patients (69.2%) allergic to metamizol, the same as CAST 19/25 (76%). The joint use of the three techniques allowed identification of 20/26 patients (76.9%).

If we consider the results of the *in vitro* tests in relation with the time elapsed since the clinical reaction, we noticed that the patients studied within the first 6 months after the clinical reaction, FAST was positive in 10 out of 18 cases (55.6%), and CAST in 11 out of 18 cases (61.1%), while skin tests were positive in eight out of 16 patients (50%). In this group of patients, the joint use of the three techniques allowed diagnosis of 15 cases (83.3%).

When more than 6 months had elapsed since the clinical reaction only one out of eight cases was positive in FAST (12.5%), two out of the seven cases were positive in CAST (28.6%), and five out of eight cases had positive skin tests (62.5%). Therefore, FAST detects a larger number of cases when patients are studied within the first 6 months after the clinical reaction ($\chi = 4.2$, $P = 0.04$) than later.

Analysing the symptomatology presented by the patients, in those who had anaphylaxis, FAST detected 9/15 cases (60%), whereas in patients with urticaria, it detected only 2/11 (18.2%) ($P = 0.051$). On the other hand, CAST detects approximately 50% of the cases with both kinds of reactions (Table 2). Most important is that among eight cases of anaphylaxis with negative skin test (No. 1, 4, 7, 9, 16, 20, 23, 25), FAST and/or CAST detected six (75%). In the four cases of urticaria with negative skin test (No. 2, 13, 21, 22), CAST detected only one.

Discussion

IgE-mediated allergic reactions to pyrazolones are the second cause of drug allergy in our environment, being in half of the cases life-threatening. Up to now, the only test available for diagnosis is the skin test, since the

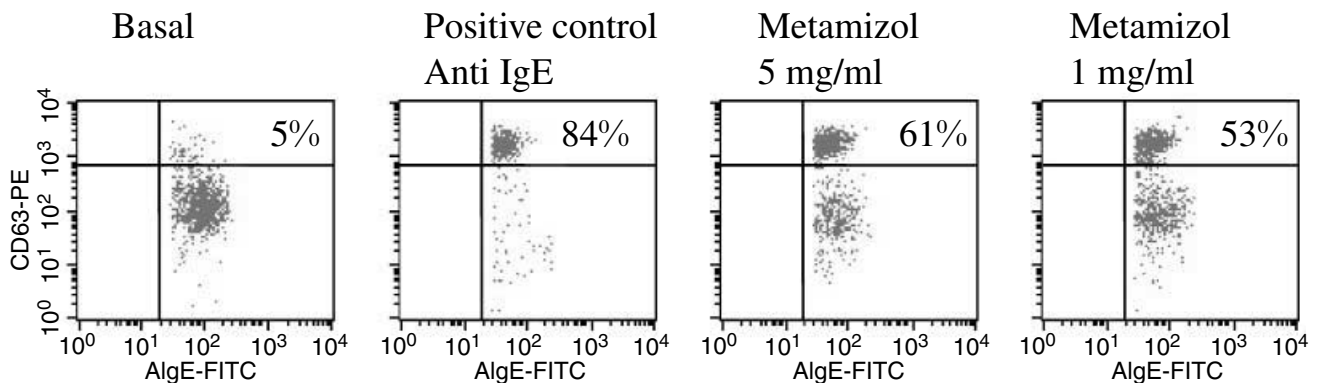


Figure 1. Example of positive case in FAST. In the plots, the expression CD63-PE against anti-IgE-FITC is observed. In the upper right square the activated basophils expressing CD63 in basal determination, in response to anti-IgE (positive control) and in response to the metamizol concentrations 5 and 1 mg/ml are presented.

provocation test is often too risky for such patients due to the severity of the clinical reactions. In our series, skin tests present a sensitivity equal to 50%, somewhat higher than reported previously (15/58; 15%, ref. 8 and 20/69; 29%, ref. 9) while others (10) indicated poor correlation between skin tests and a history of pyrazolone allergy.

Up to now, there were no established *in vitro* diagnostic techniques for this condition. RAST and ELISA techniques have been published previously for determination of specific IgE to pyrazolones that have been useful in experimental animal models (11). In humans, *in vitro* antigen-specific IgE determination results are contradictory, and Kowalski et al. (10) did not detect *in vitro* specific IgE in any of the 28 patients with immediate reaction to pyrazolones. On the other hand, Wüthrich et al. (8) found that 28% of their patients with IgE-mediated allergic reactions to pyrazolones showed a positive specific IgE test to 4-amino antipyrin. Other authors (12) found positive specific IgE to 1-phenyl-2,3-dimethyl-3-pyrazoline-5 in as many as 17 out of 19 cases of patients allergic to this drug.

At present, the usefulness of these IgE tests cannot be evaluated because there is no standardized commercialized *in vitro* IgE test with clinical studies assessing its reliability.

In this study, we present the validation assays of two new *in vitro* diagnostic tests for the diagnosis of IgE-mediated reactions to pyrazolones. The study was performed in patients with immediate reactions after administration of metamizol, for whom other causal factors had been ruled out, such as intolerance to non-steroidal anti-inflammatory drugs by provocation tests to ASA and diclofenac. Given that our patients presented reactions to metamizol, we only studied this drug, considering that, as is known, cross-reactivity between different pyrazolones is rare and only partial (9, 13). The usefulness of FAST and CAST has already been shown in the diagnosis of allergy to inhalant allergens (2), latex (5), foods (3), and betalactam antibiotics (4). FAST is a highly reproducible technique for the *in vitro* diagnosis of allergy to metamizol, as demonstrated by the intra-class variation coefficient obtained in our patients (0.99, $P < 0.0001$). FAST among our patients was highly specific (100%) with a global sensitivity of 42% and a high positive predictive value, which is very useful for the clinician since it shows that 100% of all subjects with positive FAST are sensitized to pyrazolones. These results are reflected also in their likelihood values, both positive and negative. CAST shows a high specificity

(90%) with a sensitivity of 54.2% and a likelihood ratio for a positive result of 5.2. Their joint use with skin tests allows identification of 76.9% of the patients allergic to metamizol with a specificity of 90%. When patients are studied strictly 1–6 months after the clinical reaction these values even increase, and allow diagnosis of 55.6 and 61.1% of the patients with FAST and CAST, respectively, and of 83.3% of the cases when combined with skin tests. Sensitivity and specificity may even in reality be higher if the patients negative to all tests (skin tests and *in vitro* tests) are not considered as undetectable IgE-mediated allergies but belong to a special category of ‘pyrazolone idiosyncrasy’ or intolerance, according to some authors (8, 9). Such patients may be similar but not identical to those possessing the classical ASA/NSAID intolerance. In all our cases, classical manifestations of NSAID intolerance were excluded by negative provocation tests.

It may be wondered why basophil activation (FAST) and sulphidoleukotriene release (CAST) are not always associated. In this study, they are associated in eight cases but in three cases FAST is positive alone while CAST is positive alone in five cases. A similar finding has been observed in immediate reactions to betalactams (14). This may merely reflect slight sensitivity differences among patients: the cases with associated FAST and CAST appear to be high reactors (Table 1). But, as is well known and observed in other circumstances (15), sulphidoleukotrienes production, histamine release, and basophil activation are related but not identical cellular processes, are not obligatorily linked, may occur separately and may reflect different pathways. In a study performed in 55 patients with proven immediate adverse reactions to betalactams, ASA, and acetaminophen, Lebel et al. (16) found that CAST was positive in 19 of the 55 patients and in nine of 64 controls studied. In six cases of allergy to acetaminophen, CAST was positive only in two (33% sensitivity but with a specificity of 100%).

The results presented demonstrate that we have now two validated new *in vitro* diagnostic techniques very useful for the diagnosis of allergy to pyrazolones. The tests are technically simple and yield reproducible results. Both techniques have a high specificity, a necessary condition for usefulness in the clinical diagnosis of low-prevalence clinical processes, with sensitivities over 40%. Its usefulness clearly increases when recent reactions are studied, which confirms the need for a quick study of the adverse reactions to drugs which would avoid in most patients the presentation of several urticaria and/or anaphylaxis before their diagnosis.

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