

Reliability of basophil activation test using CD203c expression in diagnosis of pollen allergy

Seçil Kepil Özdemir, M.D.,¹ Deniz Güloğlu,² Betül A. Sin, M.D.,¹ Atilla H. Elhan, Ph.D.,³ Aydan İkinciogulları, M.D.,² and Zeynep Mısırlıgil, M.D.¹

ABSTRACT

Background: CD203c is a basophil surface marker and its expression is rapidly up-regulated after cross-linking of high-affinity immunoglobulin E (IgE) receptor (FcεR1) by an allergen. CD203c basophil activation tests have been studied for the *in vitro* diagnosis of several allergic conditions. However, there is limited data about its diagnostic usefulness. The optimum allergen concentrations for stimulation and allergen specific cutoff values remain unknown for a number of allergens. This study was designed to investigate the efficacy of basophil activation test via CD203c in the diagnosis of pollen allergy.

Methods: The CD203c basophil activation was determined in 31 allergic rhinitis patients with pollen allergy and 9 healthy nonatopic controls during the off-season. CD203c expression was evaluated using three-color staining protocol by flow cytometry.

Results: After an *in vitro* stimulation with grass pollen extract, the CD203c assay clearly discriminated pollen-allergic patients from controls ($p < 0.001$). A dose-dependent increase in the percentages of CD203c-activated basophils was shown in rhinitis patients with pollen allergy ($p < 0.001$). The sensitivity and specificity was 100% and optimal cutoff values were 14.05 and 10.05% with 45.1 and 4.5 $\mu\text{g/mL}$ Phl p 5 stimulation, respectively. Although the specificity was also 100%, the sensitivity was 93 and 87% and the cutoff values were 5.40 and 5.35% with 4.5×10^{-4} and 4.5×10^{-5} micrograms/mL Phl p 5 stimulation, respectively.

Conclusion: The CD203c basophil activation test seems to be a reliable tool in the diagnosis of grass pollen allergy. It could be used when conventional diagnostic tests fail or can not be performed.

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The diagnosis of allergy is commonly based on clinical history, skin tests, *in vitro*-specific immunoglobulin E (IgE) measurements, and, infrequently, allergen-specific provocation tests. However, skin testing and specific IgE antibody levels in the sera may sometimes be incompatible with clinical history. In addition, in cases of extensive dermatitis, a history of dermographism, or anaphylaxis, it may be difficult to perform the skin tests.¹ Therefore, more sensitive and specific methods are needed in the diagnosis of an allergy.

Basophils are multifunctional effector cells involved in allergic and inflammatory reactions.² The phenotypic hallmark of basophils is the expression of both high-affinity IgE receptors (FcεR1) on their cell surface and the activation-linked molecule CD203c.³ After allergen stimulation, allergen-IgE complexes bound to FcεR1 cause IgE receptors to cross-link. Cross-linking of FcεR1 by an allergen results in a rapid up-regulation of CD203c expression on the cell membrane and is accompanied by mediator release.^{2,3} Cellular basophil activation tests, such as histamine, sulfidoleukotriene, and cytokine release tests, have been used in the diagnosis of allergy for many years. However, the expression of cell surface markers such as CD63 and CD203c, detectable by flow cytometry, has attracted more recent attention.⁴ CD203c has been identified as a novel basophil-specific activation antigen.^{2,5} CD203c is up-regulated very quickly (<5 minutes) in response to specific allergen, anti-IgE, and anti-FcεR1 stimulation.^{3,6,7} Flow cytometry-based the CD203c basophil activation test has been studied for the diagnosis of several allergic conditions, such as inhalant, food, drug, and insect venom sensitivity.^{6,8–13} However, there are limited data about its diagnostic usefulness. The optimum allergen

concentrations for stimulation and allergen-specific cutoff values remain unknown for a number of allergens.

The aim of this study was to investigate the effectiveness of the basophil activation test *via* CD203c in the diagnosis of pollen allergy.

METHODS

Study Subjects

A total of 31 patients suffering from seasonal allergic rhinitis were recruited from the outpatient clinic between the years 2009 and 2010. At screening, a diagnosis of allergic rhinitis was made based on personal history according to the presence of typical symptoms (rhinorrhea, sneezing, nasal obstruction, and nasal pruritus) during the pollen season, clinical examination, and relevant positive skin test reactivity, in accordance with international guidelines.¹⁴ Skin-prick tests with grass pollen extract were positive in all allergic patients with compatible clinical history. All of the allergic patients had a provocative concentration of methacholine causing a 20% decrease in forced expiratory volume in 1 second level of $>8 \text{ mg/mL}$ and no symptoms suggestive of asthma. Nine healthy nonatopic individuals were selected as the control group according to the following criteria: no history of pollen allergy, a negative result on skin-prick tests to common inhalant allergens, and not having grass pollen-specific IgE in the sera. This study was performed during the off-season. Study subjects had not received any treatment with nasal topical or oral corticosteroids in the 4 weeks before testing and they had not received any antihistamines or antileukotriene drugs for at least 7 days. None of the patients were given allergen immunotherapy before the study. All subjects gave their informed consent and the Institutional Ethics Committee of Ankara University School of Medicine approved the protocol.

Skin Tests and Determination of Specific IgE Levels

All of the participants underwent skin-prick tests to a standard panel of 12 common aeroallergens including grass mix (*Dactylis glomerata*, *Lolium perenne*, *Phleum pratense*, *Poa pratensis*, and *Festuca pratensis*), cereal mix (*Hordeum vulgare*, *Avena sativa*, and *Triticum sativa*), *Secale cereale*, weeds (*Artemisia vulgaris*, *Urtica dioica*, *Taraxacum vulgare*, *Plantago lanceolata*, and *Chenopodium album*), trees (*Betula ver-*

From the Divisions of ¹Immunology and Allergy Diseases and ²Pediatric Immunology and Allergy, ³Department of Biostatistics, Ankara University School of Medicine, Ankara, Turkey

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Address correspondence and reprint requests to Seçil Kepil Özdemir, M.D., Ankara Üniversitesi Tıp Fakültesi, Göğüs Hastalıkları ABD, İmmünoloji ve Allerji Hastalıkları BD, 06100, Cebeçi, Ankara, Turkey

E-mail address: secilkepil@gmail.com

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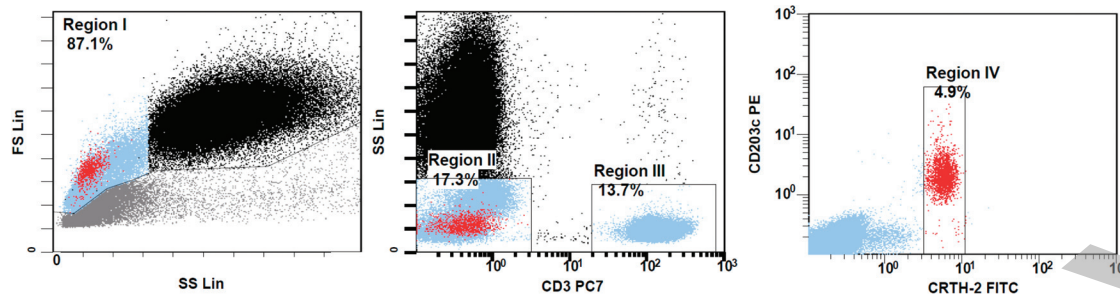


Figure 1. Optimal basophil gating in the CD203c protocol. "Region I" represents all leukocytes while excluding debris. "Region II" represents mononuclear cells that do not express CD3 (non-T cells). CD3+ T cells are being seen in "Region III." "Region IV" represents basophils expressing both CRTH-2 and CD203c molecules.

rucosa, *Fagus silvatica*, *Quercus robur*, *Platanus orientalis*, *Salix caprea*, *Populus alba*, *Ulmus scabra*, *Alnus glutinosa*, and *Coryllus avellana*), mold mix 1 (*Alternaria alternata*, *Cladosporium herbarum*, *Botrytis cinerea*, *Curvularia lunata*, *Fusarium moniliforme*, and *Helminthosporium halodes*), mold mix 2 (*Aspergillus fumigatus*, *Mucor mucedo*, *Penicillium notatum*, *Pullularia pullulans*, *Rhizopus nigricans*, and *Serpula lacrymans*), feather mix, cat and dog dander, house-dust mites (*Dermatophagoides pteronyssinus* and *Dermatophagoides farinae*), and *Blattella germanica* (Allergopharma, Reinbek, Germany). Histamine dihydrochloride (10 mg/mL) and phenolated saline glycerol diluents were used as positive and negative controls, respectively. After 20 minutes, the skin reaction was considered positive if any allergen caused a wheal of 3 mm or greater in diameter than that of the negative control. The levels of IgE antibodies specific for grass pollen mix were quantified by means of CAP fluoroenzyme immune assay (Pharmacia, Uppsala, Sweden); values of >0.35 kU/L were considered positive.

Dilution of Grass Pollen Extracts

Grass pollen extracts were purchased from Allergopharma. The extract was comprised of a mix of the six grasses, *P. pratense*, *L. perenne*, *Holcus lanatus*, *D. glomerata*, *P. pratensis*, *F. pratensis*, prepared from raw material. The concentrations of allergen extracts were expressed as the content of the major allergen *Phl p 5*. The extract was diluted to final concentrations of 45.1, 4.5, 4.5×10^{-4} , and 4.5×10^{-5} $\mu\text{g/mL}$ of *Phl p 5*, in phosphate-buffered saline (Sigma Diagnostics, St. Louis, MO). Previously, we performed dose-finding experiments in two pollen-allergic patients and two nonatopic controls with 10-fold dilutions of the extract (45.1 , 4.5 , 4.5×10^{-1} , 4.5×10^{-2} , 4.5×10^{-3} , 4.5×10^{-4} , and 4.5×10^{-5} $\mu\text{g/mL}$) to establish the allergen stimulation concentrations that induces maximal and submaximal cell stimulation. The four allergen concentrations that evoke maximal (*i.e.*, 45.1 and 4.5 $\mu\text{g/mL}$) and submaximal (*i.e.*, 4.5×10^{-4} and 4.5×10^{-5} $\mu\text{g/mL}$) cell stimulation were chosen for the study to investigate the optimal allergen concentrations for the diagnosis of pollen allergy.

Basophil Activation and Measurement of CD203c Expression by Flow Cytometry

Whole venous blood samples (4 mL) from all subjects were drawn into EDTA-containing tubes (Vacutainer; Becton Dickinson, Franklin Lakes, NJ) at the same time of day. All tests were performed within 2 hours of blood sampling. CD203c expression was evaluated using an Allergen Kit (Cellular Analysis of Allergy; Beckman Coulter, Marseille, France) according to the manufacturer's instructions. Briefly, 100 μL of the venous blood was stimulated with four different concentrations of grass pollen extract (45.1, 4.5, 4.5×10^{-4} , and 4.5×10^{-5} $\mu\text{g/mL}$) in polypropylene tubes. To evaluate background basal values with no stimulation (negative control), 20 μL of phosphate-buffered saline was added to whole blood in another tube. Twenty microliters of monoclonal anti-IgE antibody was used as positive

control. Thereafter, 20 μL of CRTH-2 (clone: BM16)-fluorescein isothiocyanate (FITC)/CD203c (clone: 97A6)-phycoerythrin (PE)/CD3 (clone: UCHT-1)-phycoerythrin-cyanin 7 (PC7) monoclonal antibodies and 100 μL of activation solution containing calcium were added to each tube and incubated at 37°C in a water bath for 15 minutes. The reaction was halted by adding 100 μL of stop solution. Tubes were then incubated for another 10 minutes for lysis at room temperature. A three-color staining method (CRTH-2-FITC/CD203c-PE/CD3-PC7) was performed by Flow Cytometer (FC500 Cytomics; Beckman Coulter, Miami, FL) equipped with an argon-ion laser and the data were analyzed with CXP2.2 software (Beckman Coulter). Cells expressing both CRTH-2-FITC and CD203c-PE were identified as basophils. At least 500 basophils were counted in each assay. Gating strategy is shown in Fig. 1. Placement of the statistical marker separating positive from negative staining adjusted to a level to include CRTH-2⁺CD203c⁺CD3⁻ cluster of cells (95–98% of cells) on the lower quadrant D4 from the nonactivated whole blood sample according to the manufacturer's instructions (negative control tube: histograms A1 and B1 in Fig. 2). So, lower quadrant (D4 region) defines the region of nonactivated basophils and upper quadrant (D2 region) defines the region of activated basophils in negative control tube (Fig. 2). The x-axis of the D4 region was carefully kept the same as the negative control in all events in a given individual, both in positive control (histogram A2/B2) and activated samples with different concentrations of allergen (histograms A3a/B3a, A3b/B3b, A3c/B3c, A3d/B3d in Fig. 2). Results were expressed as the stimulation index (SI) and the percentage of activated basophils according to the up-regulation of CD203c expression. The SI was calculated by allergen stimulation divided by negative control.

Statistical Analysis

Data are presented as median values with ranges or mean values with SDs. Comparisons were made by using the Student's *t*-test or the Mann-Whitney test where applicable. Receiver-operating characteristic (ROC) curves were used to assess the optimal cutoff values. Correlations were evaluated by the Spearman's correlation test. Values of $p < 0.05$ were considered significant differences.

RESULTS

Study Populations

Thirty-one patients with seasonal allergic rhinitis (aged 30.9 ± 9.4 years; women/men, 16/15) and nine nonallergic healthy controls (aged 31.1 ± 7.6 years; women/men, 4/5) were included in this study. The groups were similar with regard to age and gender ($p = 0.96$ and $p = 1.00$, respectively). Of the 31 confirmed allergic patients, 30 showed elevated specific IgE levels against grass pollens. The remaining one patient's specific IgE antibody titer was below the threshold of 0.35 kU/L, despite a positive result to the CD203c baso-

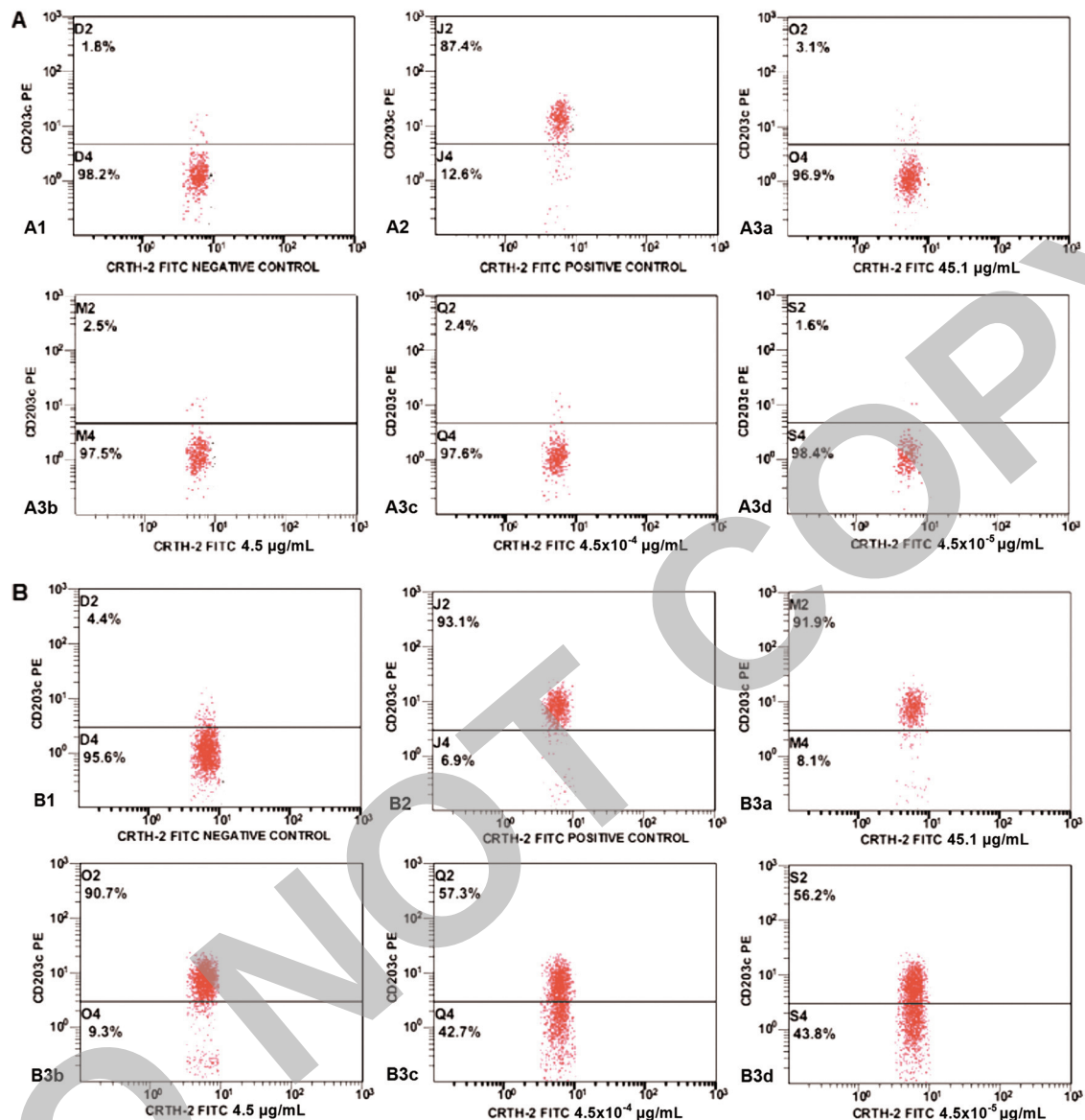


Figure 2. Expression of CD203c on basophils incubated with four different concentrations of grass pollen allergen extract (45.1, 4.5, 4.5×10^{-4} , and 4.5×10^{-5} $\mu\text{g/mL}$ of Phl p 5) in (A) a healthy control and (B) a pollen allergic patient (A1, B1, incubation with negative control; A2, B2, stimulation with positive control; A3a, B3a, stimulation with 45.1 $\mu\text{g/mL}$; A3b, B3b, stimulation with 4.5 $\mu\text{g/mL}$; A3c, B3c, stimulation with 4.5×10^{-4} $\mu\text{g/mL}$; A3d, B3d, stimulation with 4.5×10^{-5} $\mu\text{g/mL}$).

phil activation test. All of the healthy controls had negative grass pollen-specific IgE levels. Consequently, the sensitivity and specificity of the specific IgE tests were 96.8 and 100%, respectively, for the diagnosis of grass pollen allergy. We found median (minimum–maximum) specific IgE concentrations of 12.45 kU/L (0.25–381 kU/L) in allergic patients and 0.03 kU/L (0.02–0.06 kU/L) in controls.

Flow Cytometry

The median (minimum–maximum) numbers of basophils recovered were 972 (500–2260) in pollen-allergic patients and 881 (562–2006) in healthy controls. The percentages of CD203c-activated basophils after incubation with negative control ranged between 0.6 and 7.2% for the patients and 1.2 and 3.0% for the controls. The percentages of CD203c-activated basophils after stimulation with positive control varied between 28.5 and 95.4% for the patient group and 26.8 and 93.2% for the control group. No significant differences in basophil

numbers and the percentages of CD203c-activated basophils after incubation with negative and positive controls were seen between the allergic patient and healthy control groups ($p = 0.72$, $p = 0.07$, and $p = 0.63$, respectively).

Subsequent to an *in vitro* stimulation with grass pollen extract, the CD203c assay clearly discriminated pollen-allergic patients from non-allergic healthy controls ($p < 0.001$). Furthermore, a dose-dependent, statistically significant increase in percentages of CD203c-activated basophils was shown in patients with pollen allergy ($p < 0.001$). The histograms in Fig. 2 are representative of an allergic patient's and an individual healthy control's basophil activation test results. Figure 3 shows the allergen-induced, dose-dependent up-regulation in the CD203c expression of basophils.

ROC analysis between pollen-allergic patients and nonallergic controls generated threshold percentages of CD203c-activated basophils. The cutoff values, according to ROC analysis, were 14.05, 10.05, 5.40,

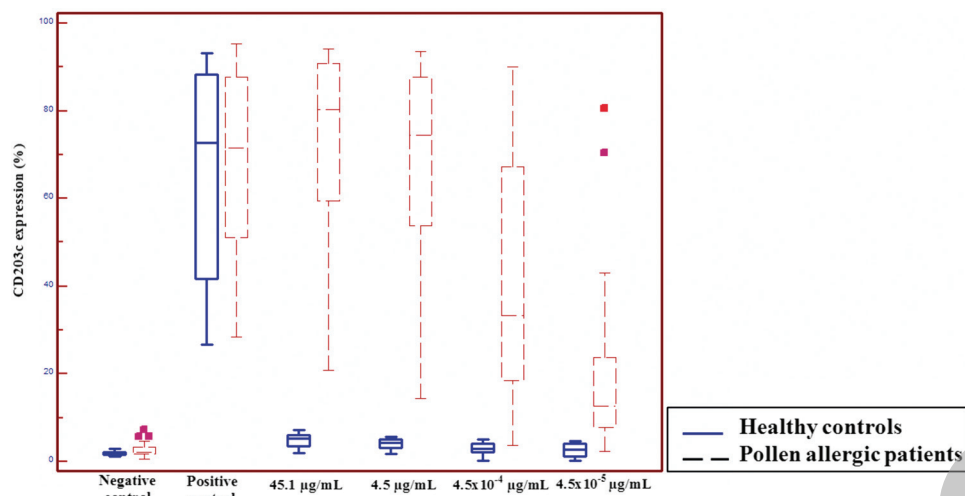


Figure 3. Percentages of CD203c-activated basophils after stimulation with serial dilutions of grass pollen extract (45.1, 4.5, 4.5×10^{-4} , and 4.5×10^{-5} µg/mL of Phl p 5) in patients with grass pollen allergy and healthy controls. The central box represents the values from the lower to the upper quartile (25th and 75th percentile), the line within the box indicates the median, and the whiskers the 5th and 95th percentile. More extreme values are plotted individually.

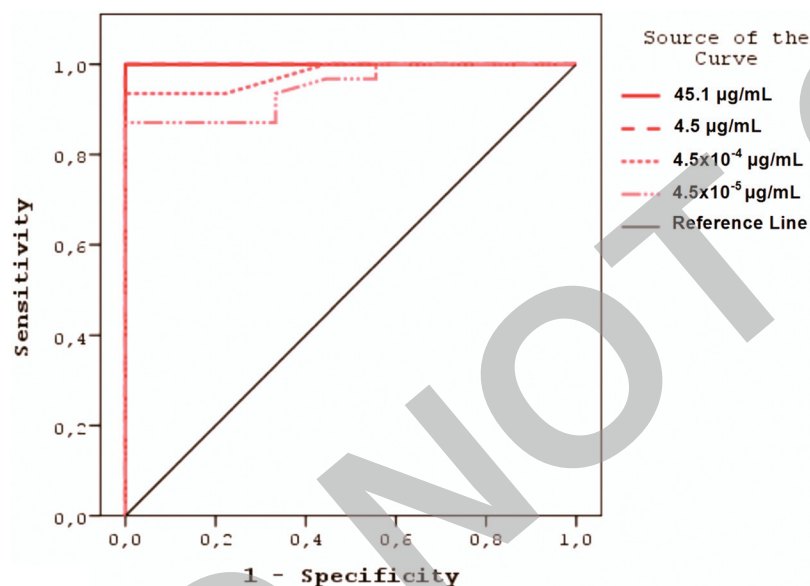


Figure 4. Receiver-operating characteristic (ROC) curves for basophil activation (percentages of CD203c-activated basophils) with 45.1, 4.5, 4.5×10^{-4} , and 4.5×10^{-5} µg/mL of Phl p 5.

and 5.35% for 45.1, 4.5, 4.5×10^{-4} , and 4.5×10^{-5} µg/mL of Phl p 5 concentrations, respectively. Sensitivity was 100% with 45.1 and 4.5 µg/mL, and 93 and 87% with 4.5×10^{-4} and 4.5×10^{-5} µg/mL of Phl p 5 stimulation, respectively. Specificity was 100% for all allergen concentrations used. Figure 4 shows the results of ROC analysis at 45.1, 4.5, 4.5×10^{-4} , and 4.5×10^{-5} µg/mL of Phl p 5 concentrations.

The cutoff values of CD203c basophil SI generated by ROC analysis were as follows: 4.25, 4.05, 2.73, and 2.80 for the respective allergen doses of 45.1, 4.5, 4.5×10^{-4} , and 4.5×10^{-5} µg/mL, respectively. The ratios of sensitivity were 100, 96, 93, and 77% for 45.1, 4.5, 4.5×10^{-4} , and 4.5×10^{-5} µg/mL of allergen concentrations, respectively. Specificity was 100% at all concentrations. The diagnostic values and specific cutoff values of the CD203c basophil activation test are shown in Table 1.

Relationship between CD203c Up-Regulation and Specific IgE Levels

We found a moderate positive correlation between grass pollen-specific IgE levels and the percentages of CD203c-activated basophils after *in vitro* stimulation with 4.5×10^{-4} and 4.5×10^{-5} µg/mL of allergen extract ($r = 0.666$ and 0.623 , respectively; $p < 0.001$; Fig. 5). However, a weak positive correlation was detected at 45.1 and 4.5

µg/mL concentrations ($r = 0.427$ and $r = 0.397$; $p = 0.006$ and $p = 0.011$, respectively; Fig. 5).

DISCUSSION

The present study indicates that the basophil activation test via CD203c expression is a sensitive and reliable tool in the *in vitro* diagnosis of grass pollen allergy. Our results indicate that 14 and 10% at 45.1 and 4.5 µg/mL of Phl p 5 concentrations are optimal cutoff values for basophil stimulation with grass pollen allergen. These cutoffs provided a sensitivity and specificity level of 100%. The study population consisted of patients with suggestive history of seasonal allergic rhinitis and positive skin-prick tests to grass pollens and nonatopic healthy controls. A limitation of our study is the lack of a group of patients in whom the diagnosis of pollen allergy is in doubt. Therefore, these sensitivities and specificities represent the clearly different populations tested and will not apply to all patients.

A number of previous studies have shown that flow cytometric quantitation of basophil activation can be used to measure IgE-dependent responses to allergens in sensitized individuals.^{6,8,13,15-18} However, most of the studies evaluating sensitization to aeroallergens have used antibodies against CD63, with limited data available regarding CD203c. The optimum allergen concentrations for basophil

Table 1 Diagnostic values of CD203c expression on basophils according to percentages of CD203c-activated basophils (%) and SI

	Major Allergen (<i>Phl p 5</i>) Concentrations			
	45.1 $\mu\text{g/mL}$	4.5 $\mu\text{g/mL}$	$4.5 \times 10^{-4} \mu\text{g/mL}$	$4.5 \times 10^{-5} \mu\text{g/mL}$
CD203c molecule expression (%)				
Cutoff	14.05	10.05	5.40	5.35
Sensitivity (%)	100	100	93	87
Specificity (%)	100	100	100	100
AUC	1.000	1.000	0.978	0.948
<i>p</i>	<0.001	<0.001	<0.001	<0.001
CD203c molecule expression (SI)				
Cutoff	4.25	4.05	2.73	2.80
Sensitivity (%)	100	96	93	77
Specificity (%)	100	100	100	100
AUC	1.000	0.996	0.982	0.907
<i>p</i>	<0.001	<0.001	<0.001	<0.001

AUC = Area under the curve; SI = stimulation index.

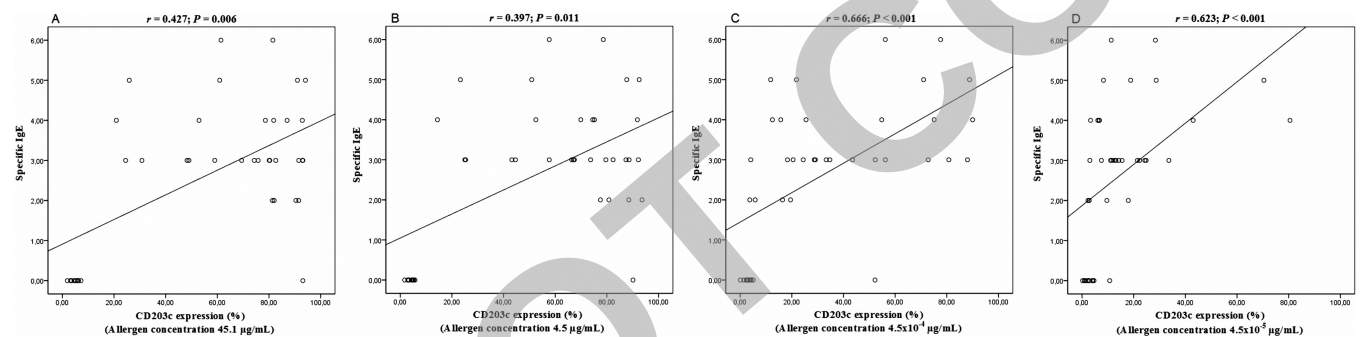


Figure 5. Correlations between results obtained with CD203c basophil activation test [A] 45.1 $\mu\text{g/mL}$; [B] 4.5 $\mu\text{g/mL}$; [C] $4.5 \times 10^{-4} \mu\text{g/mL}$; [D] $4.5 \times 10^{-5} \mu\text{g/mL}$ of *Phl p 5* and grass pollen mix-specific immunoglobulin E (IgE) measurement.

stimulation and allergen-specific cutoff values are not known for a number of allergens. Furthermore, ROC curves are also important for describing the accuracy of tests as well as in achieving optimal sensitivity and specificity. To the best of our knowledge, there has been no publication concerning the use of ROC curves to select appropriate cutoff points and allergen concentrations for the allergen-induced basophil activation test quantifying CD203c expression with pollen allergens. In a study by Hauswirth *et al.*, the stimulation of basophils with recombinant pollen allergens resulted in a significant up-regulation of CD203c in sensitized patients, with few false negative results.⁶ In another study, the CD203c basophil activation test for pollen allergy was investigated in 11 grass pollen-allergic and 9 nonatopic children.⁸ They found 100% in both the sensitivity and the specificity of the test, but ROC analysis was not performed in their study.⁸ Several studies have evaluated the application of the CD63 basophil activation test (Basotest) in the diagnosis of pollen allergy. In some of these studies, 85–100% sensitivity and 100% specificity have been reported.^{15,16,19,20} In a study by Hauswirth *et al.*, a significant correlation between CD63 and CD203c expression was observed.⁶

Nonresponders that are individuals whose cells are unresponsive to FcεR1 cross-linking are an important issue in basophil activation tests, and they influence sensitivity and specificity of the assay. Previously, it was shown that 13–28% of patients have basophils that do not release histamine with activation.^{21,22} For basophil activation tests the percentage of nonresponders reported is usually lower, <10%.^{23–25} In our study group there were no nonresponders; however, 1 subject among 9 (11%) in the control group and 2 subjects among 31 (6.5%) in the patient group have shown low CD203c up-regulation (ranging between 26 and 34%) with positive control. Sev-

eral groups have documented properties that are the same between releaser and nonreleaser basophils, including the numbers and composition of membrane FcεR1, their signaling responses to other stimuli, including calcium ionophores and formyl peptide receptor.^{21,22,26} Defects in early intracellular signal transduction involving the tyrosine kinases syk and lyn have been identified in nonresponders.^{27,28} CD203c up-regulation can also be mediated by IL-3 and inhibited by cerivastatin and atorvastatin.^{29,30} The reason for the lack of real nonresponders in our study group could be the highly restricted selection of the subjects (those with a clear history and monosensitized to pollens and those who do not use any medication such as statins).

We found large shifts between unactivated and activated samples in our study group. This was also the case for some other studies evaluating CD203c basophil activation tests with inhalant allergens.^{8,31} The properties of the allergens used in basophil activation tests might be an important factor. Some allergens such as drugs usually cause lower responses than those obtained with protein allergens.²³ Additionally, natural exposure *in vivo* to the allergen tested may cause high basal activation.²³ High basal values have been observed when a food-allergic patient has suffered a recent reaction or presumably was continuously exposed.³² Shreffler has found that CD203c is expressed at very high levels in patients with atopic dermatitis and food allergy.³² The large shifts between unactivated and activated samples found in our study might be related to the properties of the allergen tested and performing basophil activation experiments outside the pollen season.

In the present study, we found the rates for sensitivity and specificity of grass pollen-specific IgE to be 96.8 and 100%, respectively, and the sensitivity of the CD203c basophil activation test was ob-

served to be greater than that of the grass pollen-specific IgE. We showed a moderate positive correlation between grass pollen-specific IgE levels and the percentages of CD203c-activated basophils after *in vitro* stimulation with 4.5×10^{-4} and 4.5×10^{-5} $\mu\text{g/mL}$ allergen. However, a weak positive correlation was detected at 45.1 and 4.5 $\mu\text{g/mL}$ concentrations. The explanation for the weaker correlations between basophil activation with higher concentrations of allergen and specific IgE levels might be the fact that basophils reaching a maximal plateau and showing no more response to grass pollen-specific IgE, but basophils might be more responsive to allergen-specific IgE at lower allergen concentrations. Furthermore, only a limited number of aggregated IgE receptors are required to induce basophil activation³³; it is understandable that, above a certain threshold, basophil activation becomes independent of the extracellular IgE concentration.²³ Some authors reported a positive correlation between the basophil activation test and *in vitro*-specific IgE determination in the case of allergy to *D. pteronyssinus* and *L. perenne*¹⁷ as well as a mixed grass pollen allergen.¹⁵ However, no significant correlation between the results of the basophil activation test and serum-specific IgE levels was found in another study based on cypress pollen allergy.¹⁶

Without doubt, skin tests with inhalant allergens represent the most relevant and the least time-consuming method to diagnose respiratory allergy. However, basophil activation tests are becoming an important addition to the tools available for the diagnosis of allergy.²³ Flow cytometric methods, although expensive and skill dependent, could be used in patients with questionable test results or when conventional diagnostic tests fail or *in vivo* diagnostic tests can not be performed. In our opinion, basophil activation tests should be performed in appropriate clinical contexts in reference allergy centers because this method's clinical usefulness is not restricted to the diagnosis of respiratory allergy. There are also data that support its usefulness in food, venom, drug allergy, and autoimmune chronic urticaria.^{9,10,13,18,34–37} In addition, our findings strongly encourages its diagnostic usefulness for the patients with seasonal allergic rhinitis.

This study included patients presenting positive skin test reactivity to grass pollens. Nevertheless, there are a minor number of patients who have a clinical history compatible with pollen allergy while showing negative skin tests and *in vitro*-specific IgE measurements. Despite having negative skin tests and no detectable serum-specific IgE antibodies, some individuals may develop nasal symptoms on nasal provocation with various inhalant allergens.^{38–40} Whether or not the basophil CD203c activation test has a diagnostic value in cases with ambiguous clinical data (e.g., patients with compatible clinical history of seasonal allergic rhinitis, negative skin-prick tests, and *in vitro*-specific IgE measurements but positive nasal challenge tests with pollens) needs further investigation.

In conclusion, our study indicates that the basophil activation test detected by CD203c is a sensitive and reliable tool in the diagnosis of grass pollen allergy. The rates of 14 and 10% at 45.1 and 4.5 $\mu\text{g/mL}$ of *Phl p 5* concentrations, respectively, are the optimal threshold values for basophil stimulation with grass pollen allergen.

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