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The CD63 basophil activation test as a diagnostic tool for assessing autoimmunity in patients with chronic spontaneous urticaria

Background: Over the past years, it has become widely recognized that a proportion of chronic spontaneous urticaria (CSU) cases is of autoimmune origin, however, the search for reliable diagnostic tools to confirm underlying autoimmune pathophysiology is ongoing. The CD63 basophil activation test (CD63 BAT) has recently become useful for diagnosing autoimmune CSU. **Objectives:** To analyse the correlation between positive CD63 BAT results, total IgE antibody levels, and the presence of autoimmune thyroiditis as a comorbidity in patients diagnosed with CSU. **Materials and Methods:** We performed a retrospective analysis of CD63 BAT results obtained from 87 CSU and 20 non-CSU patients. The information extracted from the patients' records included age, gender, total IgE levels, clinical history of autoimmune thyroiditis (AT), and the presence of anti-thyroid autoantibodies. **Results:** Positive CD63 BAT results were significantly more frequent in CSU patients compared with non-CSU subjects ($p=0.045$). Furthermore, we found a strong significant negative correlation between the stimulation index (SI) value for CD63 BAT and total IgE levels in CD63 BAT-positive CSU patients ($p=0.004$; Spearman's rank correlation coefficient $\rho=-0.322$), meaning that higher SI values corresponded to lower total IgE values, and vice versa. **Conclusion:** The current standard set of diagnostic tools cannot be reliably used to determine when CSU is caused by autoimmune mechanisms. There is evidence that CD63 BAT represents a helpful diagnostic tool for detecting underlying autoimmunity. We show that high SI values in CD63 BAT-positive CSU patients correlate negatively with their total IgE levels. The clinical relevance of this effect needs to be investigated further.

Key words: chronic spontaneous urticaria, CD63 basophil activation test, autoimmune thyroiditis, autoimmunity, autoimmune urticaria

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Chronic spontaneous urticaria (CSU) is defined as a spontaneous occurrence of hives and/or angioedema for a duration of over six weeks [1]. It is often not possible to pinpoint its aetiology, however, a proportion of CSU cases is considered to be of autoimmune nature, based on experimental and clinical evidence [2]. Recent studies describe the presence of functional IgG autoantibodies against IgE and the high-affinity IgE receptor (FcεRI) in the sera of some CSU patients [3-6]. Because of its ability to detect the presence of autoreactivity in CSU patients, the autologous serum skin test (ASST) is a widely used *in vivo* diagnostic tool, however, a positive ASST result alone is not enough for a diagnosis of autoimmune urticaria [7]. In recent decades, *in vitro* assays - the basophil histamine release assay (BHRA) and the basophil activation test (BAT) - have also been shown to be highly effective for detecting autoimmunity [8, 9]. Furthermore, it has been suggested that positive results of these two *in vitro* tests might not only be linked to high disease activity in both adults and children, but may also serve as predictors for how rapidly the patient will respond to treatment [10-12].

In this investigation, we analysed the correlation between positive CD63 BAT results, total IgE antibody levels, and the presence of autoimmune thyroiditis as a comorbidity in patients diagnosed with CSU.

Patients and methods

Study design

We conducted a retrospective analysis of patient records with the agreement of the local ethics committee (Ethics Committee of the University Hospital of RWTH Aachen in Aachen, Germany, internal file number: EK 043/17).

Control subjects and patients with CSU

We performed a retrospective investigation of all the available CD63 BAT results of CSU patients who were diagnosed and/or treated at the Department of Dermatology and Allergology of the University Hospital of Aachen,

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Germany between 2007 and 2013. The data collected from the patients' files included such parameters as age, gender, total IgE levels (in kU/L), clinical history of autoimmune thyroiditis, and the presence of anti-thyroid autoantibodies (in U/L).

The study population included 87 patients diagnosed with CSU and 20 non-CSU patients (used as control subjects) who underwent diagnostic evaluation at the outpatient clinic for other, non-CSU, conditions, such as drug hypersensitivity reactions or food allergies, and received the CD63 BAT as a part of the diagnostic procedure.

The basophil activation test

Commercially available BAT kits were purchased from BÜHLMANN LABORATORIES AG, Schönenbuch, Switzerland (Flow Cast®). In each case, the BAT was performed on the same day when the patients' blood samples were collected; the sera were not frozen for storage. Prior to the testing, the sera were heat-inactivated for 30 minutes at 56°C in order to prevent non-specific basophil activation. All further steps were carried out in accordance with the manufacturer's instructions: 50 µL of each patient's serum was mixed with 50 µL of the donor's blood, then 100 µL of stimulation buffer and 20 µL of staining reagent (a mixture of monoclonal antibodies to human CD63 labelled with fluorescein isothiocyanate [anti-CD63-FITC] and to human chemokine receptor CCR3 labelled with phycoerythrin [anti-CCR3-PE]), both from BÜHLMANN LABORATORIES AG (Schönenbuch, Switzerland), were added. The samples were incubated in a water bath for 30 minutes at 37°C. A healthy, non-atopic volunteer (a 40-year-old female) served as a basophil donor for the entire study. To determine the reactivity of the donor's basophils, positive and negative control tests were performed. For the negative control - the donor's individual background - 50 µL of stimulation buffer was used instead of patient serum. For the two positive control tests, the incubation of the basophils was performed with highly specific monoclonal antibody to the high-affinity IgE binding receptor (anti-FcεRI mAb) and the non-specific cell activator, N-formyl-L-methionyl-L-leucyl-phenylalanine (fMLP); 50 µL each (both purchased from BÜHLMANN LABORATORIES AG, Schönenbuch, Switzerland).

After this step, 2 mL of prewarmed lysing solution was added and incubated for 10 minutes at room temperature (18-28°C) in order to remove the erythrocytes by a lysis reaction. After centrifugation for five minutes at 500g, the cells were resuspended in 300 µL of wash buffer and then analysed for CD63 expression in a BD FACScan™ flow cytometer using Flow Cast®. There were at least 500 to 600 basophilic cells acquired per sample.

Stimulation index (SI), defined as the ratio between the percentage of degranulated donor basophils (CD63⁺ cells) after stimulation with a patient's serum and the percentage of activated basophils without stimulation (negative control), was calculated for each patient. The BAT result was considered to be positive if the SI was ≥ 2.5 .

Total IgE

The total IgE levels in the patients' sera were measured with the ImmunoCAP™ 250 system (Phadia, Uppsala,

Sweden). The results were expressed in kU/L. The total IgE level was considered elevated if the value exceeded 100 kU/L.

Statistical analysis

Statistical analysis was performed using SPSS (version 16.0, SPSS, Inc., Chicago, IL). Descriptive statistics of metric-scaled data were presented by median and interquartile range (IQR = 1st to 3rd quartile). Pairwise group comparisons were performed using the Mann-Whitney U test. The distribution of categorical data was presented by absolute and relative frequencies and compared between the groups using the Chi-squared test (χ^2 test). Spearman's rank correlation was applied to analyse the association between the variables. Statistical hypothesis testing was conducted at 5% significance level.

Results

Epidemiological data

The median age of the 87 CSU patients (31 male and 56 female) was 41 (28-55) years. The age difference between the male and the female CSU patients was not statistically significant ($p=0.1$). The average age of the 20 non-CSU subjects (the control group) was 53 (41-76) years. There was also no statistically significant age difference between the male and the female non-CSU patients (six male and 14 female subjects; $p=0.13$). There was no statistically significant difference in gender distribution between the CSU and the non-CSU groups ($p=0.617$). However, the non-CSU subjects were significantly older compared to the CSU patients ($p<0.001$).

Total IgE levels

Measurement of total IgE was performed in 80 CSU patients and 18 control subjects (non-CSU patients). The median total IgE level in the patients of the CSU group was 72.0 (27.5-202.5) kU/L, and 15.2 (7.9-19.3) kU/L in the control group, qualifying as a statistically significant difference ($p<0.001$). In the CSU group, the male patients had significantly higher total IgE levels than the female patients ($p=0.008$). There were no statistically significant gender-related differences in total IgE levels in the control group ($p=0.289$).

The basophil activation test (CD63 BAT)

The CD63 BAT was performed in all 87 patients of the CSU group and was positive in 56 of the cases (64.4%). To investigate the clinical relevance of this finding, the CD63 BAT was also performed in all 20 of the control subjects, with eight positive results (40%). Compared with the control population, the proportion of positive CD63 BAT results was significantly higher in the CSU patients ($p=0.045$). In the CD63 BAT-positive CSU patients and control subjects, the median SI was 8.9 (4.4-16.0) and 6.5 (5.3-10.5),

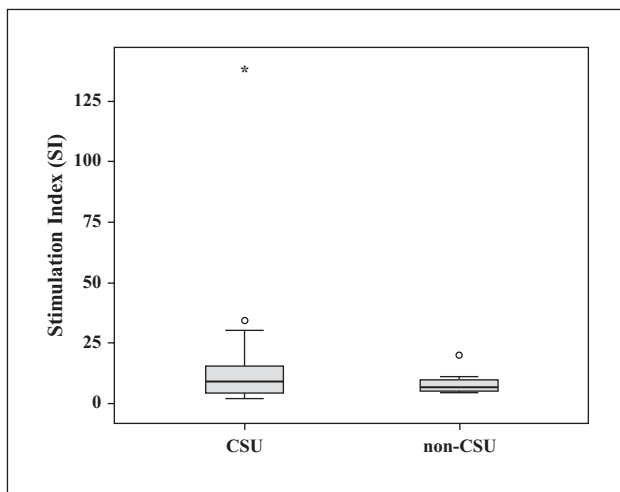


Figure 1. Stimulation index (SI) of CD63 BAT-positive patients in the CSU and non-CSU groups ($p=0.745$), showing greater variability of the SI values in CSU patients compared to control subjects.

respectively. There was a higher variability in the SI values of the BAT-positive CSU patients compared to CD63 BAT-positive control subjects (interquartile range: 11.6 vs 5.2) (figure 1). However, the SI values showed no statistically significant difference between the CD63 BAT-positive individuals of the CSU and the control groups ($p=0.745$). There were no significant differences in age between the CD63 BAT-positive and CD63 BAT-negative CSU patients, with a median age of 40 (27-55) and 42 (30-54), respectively ($p=0.670$). However, a statistically significant gender-related difference was found: 41 of the CD63 BAT-positive patients were female and only 15 were male ($p=0.021$), whereas in the CD63 BAT-negative subjects, the gender distribution was almost equal (15 female and 16 male). The average total IgE level in those CSU patients who had positive CD63 BAT results was 68.5 (22.8-178.0) kU/L. In the CSU patients with negative CD63 BAT results, this was 87.7 (62.0-238.0) kU/L. Therefore, there was no statistically significant difference in total IgE levels between these two subgroups of CSU patients ($p=0.052$). However, there was a statistically significant negative correlation between the SI values and total IgE levels in the CD63 BAT-positive CSU subjects ($p=0.004$; Spearman's rank correlation coefficient $\rho=-0.322$), which means that higher SI values corresponded to lower total IgE values, and vice versa (figure 2).

Autoimmune thyroiditis

Sixty-six of the 87 CSU patients were screened for clinical history or present clinical manifestations of autoimmune thyroiditis (AT). Of these 66 CSU patients, 37 (56.1%) had positive CD63 BAT results. Ten CSU patients had elevated serum levels of anti-thyroid autoantibodies: anti-thyroid peroxidase (TPO) antibodies and/or anti-thyroglobulin antibodies (TgAb); 13 reported a history of physician-diagnosed autoimmune thyroiditis. A total of 15 patients had elevated serum levels of anti-thyroid autoantibodies and/or reported a history of AT. The mean age of these patients was 40 (20-51) years. There was no significant difference in age compared to the CSU patients without a

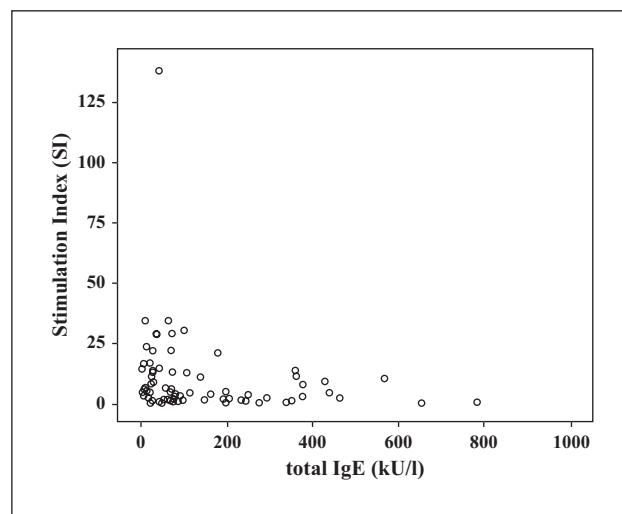


Figure 2. Negative correlation between stimulation index (SI) values and total IgE levels in CD63 BAT-positive CSU patients ($p=0.004$; Spearman's rank correlation coefficient $\rho=-0.322$).

clinical history of AT and with normal serum levels of anti-thyroid autoantibodies ($p=0.251$). Of the 15 patients with elevated serum levels of anti-thyroid autoantibodies and/or a known history of AT, 12 (80.0%) tested positive in the CD63 BAT. Additionally, almost a third (12/37; 32.4%) of the CSU patients with positive CD63 BAT results had a clinical history or presented with clinical manifestations of AT. However, statistically, there was no correlation between positive CD63 BAT results and a clinical diagnosis of AT ($p=0.188$), possibly due to the small sample size.

Discussion

Based on experimental and clinical evidence, it is being increasingly recognized internationally that a proportion of CSU cases has autoimmune aetiology [2, 13]. Currently, there is very limited evidence of an association between CSU and IgE autoantibodies, such as IgE anti-double-stranded DNA (dsDNA) or IgE anti-thyroperoxidase (TPO) antibodies, as only a few independent studies have shown that serum levels of IgE autoantibodies in CSU patients are significantly higher than in healthy controls [14-16]. Moreover, these results could not be reproduced in smaller cohorts [17, 18]. However, there are numerous investigations suggesting that IgG anti-IgE and/or IgG anti-FcεRI autoantibodies may play an important role in the pathophysiology of CSU in some patients [3-6, 19-21]. One of the screening tools most frequently used to detect the presence of pathogenic autoantibodies in the sera of CSU patients is the autologous serum skin test (ASST). It has been shown that positive ASST results occur more frequently in CSU patients than in healthy controls or non-CSU patients [22-24]. However, the EAACI/GA(2)LEN task force released a consensus statement that a positive ASST result alone is not sufficient for a diagnosis of autoimmune urticaria [2, 7]. Recently, the CD63 basophil activation test has gained prominence as a useful *in vitro* diagnostic tool [2, 25-27].

In the present study we investigated CD63 BAT results in CSU patients as a potential marker of autoimmunity. We also looked for a possible correlation between the test results and the patients' age, gender, and total IgE levels. Similar to previous investigations [23, 28], we found statistically significantly higher total IgE levels in CSU patients compared with the non-CSU control subjects. As hypothesized previously, elevated IgE levels in patients' sera may contribute to the upregulation of high-affinity IgE receptor FcεRI on mast cells and basophils and, therefore, lead to augmentation of activity and render the cells more susceptible to degranulation [29]. It has been shown that binding of the monomeric IgE to the FcεRI, even in the absence of an antigen, enhances cell surface expression of the FcεRI and leads to cytokine production and mast cell survival [30-32]. These findings provide a rationale for anti-IgE treatment in patients with chronic spontaneous urticaria.

Another finding, consistent with reports of other authors [9, 10, 25], is that positive CD63 BAT results are more frequent in CSU patients than in non-CSU controls. Although in our study the non-CSU controls were not healthy subjects, but patients with other, non-CSU, conditions, there was still a statistically significant predominance of positive CD63 BAT results in the CSU patients ($p=0.045$). Based on our investigation, the CD63 BAT was positive in 64% patients of the CSU group, which is comparable to the 40-57% rate reported in the current literature [8, 33]. There was no statistically significant difference either in age or total IgE levels between the CSU patients with positive and negative CD63 BAT results. However, we found that higher SI values corresponded to lower total IgE values in the CD63 BAT-positive CSU patients, and vice versa ($p=0.004$; Spearman's rank correlation coefficient $\rho = -0.322$) (figure 2). Current studies link lower total IgE levels to lower omalizumab treatment response in CSU patients [34-36]. However, a number of complete and partial responders to omalizumab therapy were also found to have low total IgE levels [34]. Therefore, total IgE level cannot be used as a sole predictor of omalizumab efficacy in CSU patients. A positive CD63 BAT result in CSU patients, on the other hand, has been shown to be predictive of a more severe course of the disease, which might also be resistant to therapy [10-12]. Recent research revealed that patients with autoimmune CSU also have significantly lower total IgE levels than those with non-autoimmune CSU [37]. We were not able to reproduce the latter finding in our patient collective. However, the correlation we found might represent a possible link between these two above-mentioned findings: patients with a positive CD63 BAT result and a high SI and low total IgE levels might not only be suffering from the autoimmune type of CSU and exhibit more pronounced and severe symptoms, but also have a lower response to omalizumab therapy. Further prospective studies are needed to verify this hypothesis.

Another phenomenon we were able to observe was a statistically significant higher prevalence of female gender in the CD63 BAT-positive group ($p=0.021$). If the assumption is valid that a positive CD63 BAT result is a sign of an existing autoimmune condition, the gender distribution might be explained by the fact that female gender is generally associated with a higher incidence of autoimmune disorders [38, 39]. It has also been observed that female patients were significantly more likely to have clinical manifestations of the autoimmune type of CSU [40].

A further aspect that we investigated was the connection between CSU and autoimmune thyroiditis. It has been shown by other researchers that CSU patients who test positive for IgG-autoantibodies tend to have autoimmune comorbidities, such as AT [2, 41, 42]. Of the CSU patients in our study, 23% (15/66) had elevated serum levels of anti-thyroid autoantibodies and/or a history of physician-diagnosed AT, a proportion similar to those reported in previous studies [23, 28]. Herein, we found that almost a third (12/37, 32.4%) of the CSU patients with positive CD63 BAT results had AT. This finding was not statistically significant in our study, presumably due to relatively small sample size and consequent limited statistical power. To summarize, a positive CD63 BAT result was more frequent in the CSU patient group compared with the non-CSU controls. We have also found that the percentage of CD63 BAT-positive patients in our CSU population was similar to that reported in other studies. A higher proportion of female subjects in the CD63 BAT-positive group in our cohort may suggest that female CSU patients tend to develop the autoimmune type more frequently. Additionally, we found that almost a third of the CSU patients with positive CD63 BAT results had AT as an autoimmune comorbidity. There was also a statistically relevant negative correlation between the SI values in the CD63 BAT-positive CSU patients and their total IgE levels. We hypothesize that a combination of high SI values for the CD63 BAT and low total IgE in patients with autoimmune CSU might be clinically significant and possibly predictive of an unfavourable treatment prognosis, however, this should be confirmed by further investigations.

The limitation of our study is its retrospective design, which resulted in some non-uniformity in the data (e.g. not all of the CSU patients were screened for elevated anti-thyroid autoantibody levels in the serum). In addition, the severity of the patients' symptoms and their response to treatment was not investigated. Therefore, further prospective studies with a larger number of patients are needed to reproduce the results and to investigate the relationship between positive CD63 BAT results, SI values, total IgE levels, and anti-thyroid autoantibodies in CSU patients and how these diagnostic tools can help to predict treatment efficacy. ■

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