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Original Article

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Causal Relationship Between Anti-TPO IgE and Chronic Urticaria by *In Vitro* and *In Vivo* Tests

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Disclosure
There are no financial or other issues that might lead to conflict of interest.

ABSTRACT

Purpose: Immunoglobulin (Ig) E autoantibodies against thyroid antigens such as thyroid peroxidase (TPO) have been demonstrated in chronic spontaneous urticaria (CSU) patients in higher frequency than healthy subjects. However, if these IgE autoantibodies can trigger urticaria is still a matter of study. The aim of this study was to investigate the relationship between concomitant IgE autoantibodies against thyroid antigens in CSU.
Methods: Patients with CSU, healthy subjects and patients with autoimmune thyroid disease (ATD) were recruited. Total IgE and specific anti-TPO IgE and IgG were assessed in all subjects. The basophil activation test and skin tests with TPO were performed to demonstrate whether this antigen could selectively induce urticaria reaction in subjects with positive anti-TPO IgE.
Results: Anti-TPO IgE was present in all 3 groups (CSU: 34.0%, ATD: 16.6%, healthy subjects: 8.1%). Anti-TPO IgE levels were higher in CSU patients, whereas anti-TPO IgG were higher in ATD patients. After exposure to TPO, CD203c expression from patients with CSU and anti-TPO IgE significantly increased in comparison to the other groups; 33.0% vs. 14.0% in ATD patients and 9.0% in control subjects ($P < 0.05$). Skin reactions with TPO were higher in patients with CSU according to the intradermal (CSU: 18.0%, ATD: 3.3%, control: 8.0%) and skin prick tests (12.0%, 0%, 0%, respectively). Passive transfer of anti-TPO IgE from a CSU patient to the skin of control subjects without anti-TPO IgE induced a positive skin reaction.
Conclusions: Anti-TPO IgE is not a specific biomarker for CSU. However, IgE against TPO plays a pathogenic role in inducing effector cell activation and skin exacerbation in some patients with CSU.

Keywords: Autoantigen; autoimmunity; hypothyroidism; IgE; thyroid peroxidase; urticaria; basophils

INTRODUCTION

Different mechanisms are involved in the pathogenesis of chronic spontaneous urticaria (CSU).[1,2] In the early 90s, Grattan *et al.*[3] demonstrated the presence of immunoglobulin (Ig) G with anti-IgE function in chronic urticaria. Two years later, the same research group found IgG against the high affinity IgE receptor and also demonstrated that these IgG autoantibodies could induce basophil activation.[4]

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After demonstrating IgG autoantibodies in CSU, Bar-Sela *et al.*[5] suggested that IgE may have an auto-reactivity role in urticaria by demonstrating IgE against thyroid peroxidase (TPO). However, while reproducing the results with IgG autoantibodies in multiple studies has been possible, the study of auto-reactive IgE has been more difficult. Studies from Concha *et al.*[6] indicate that the detection of IgE specific for TPO was an occasional finding, and the results suggest that these antibodies were unlikely to play an important pathogenic role in urticaria. With the depletion of IgG prior to the measure of IgE, or with new innovations in IgE detection techniques, some recent studies[7-9] have shown that anti-TPO IgE in patients with CSU is more frequent than previously described. However, if these IgE autoantibodies can trigger urticaria or not in patients with CSU has not been proven and the clinical role of this IgE has been little studied.

In this study, we investigated the clinical impact of IgE autoantibodies against TPO in CSU. For this purpose, we followed 3 steps:

1. Estimated the prevalence of anti-TPO IgE in our population.
2. Evaluated the interaction of anti-TPO IgE with basophil cells for the induction of CD203c expression.
3. Evaluated if TPO can induce urticaria on the skin of patients with anti-TPO IgE and if this response could be transferred to healthy subjects.

MATERIALS AND METHODS

Study population

This is a cross-sectional study with 3 groups based on their clinical status: the CSU, healthy control and autoimmune thyroid disease (ATD) groups. The CSU population was recruited from the Urticaria Research of Tropical Impact and Control Assessment (URTICA) cohort (ClinicalTrials.gov number: NCT01940393).[10,11] The CSU group included patients older than 12 years with CSU, defined as the recurrence of hives, with or without angioedema, for at least 6 weeks.[12] Patients could be receiving their treatment with antihistamines but not with anti-IgE antibodies. The exclusion criteria were the following: systemic diseases or pharmacologic treatment that could cause hives. The impact of CSU on the quality of life was assessed with the dermatology life quality index (DLQI) and the urticaria activity score (UAS) was used for the disease activity.[11] For skin tests, patients suspend antihistamines 7 days before the autologous serum skin test (ASST), intradermal, skin prick test (SPT) or passive transfer of serum.

The healthy control group consisted of people older than 12 years without historical autoimmune diseases, chronic urticaria or history of acute urticaria in the previous 2 years.

The ATD group included patients with Hashimoto's thyroiditis according to the American thyroid association; [13] the presence of thyroid autoantibody above the normal range (anti-TPO IgG ≥ 5.0 IU/mL and/or anti-thyroglobulin ≤ 100.0 IU/mL) in addition to thyroid dysfunction (free thyroxine ≤ 0.1 mUI/L and/or thyroid-stimulating hormone ≥ 5 mIU/L) was considered as ATD.[7]

TPO recombinant

The rTPO was obtained using *Escherichia coli* strains BL21 (DE3) as an expression vector (details are presented in the **Supplementary Data S1**).

Determination of total IgE, anti-TPO IgE and anti-TPO IgG

Total IgE levels in serum samples were determined using a flouroenzyme immunoassay (ImmunoCap System, Uppsala, Sweden).

The sera used for the quantification of sIgE were previously depleted of IgG by immunoaffinity depletion. The cut-off value for serum specific IgE level to TPO was defined as the mean and 3 standard deviations of absorbance values from 40 healthy controls without urticaria or autoimmune diseases. Subsequently, this cut-off point was confirmed with the complete control population (70 additional patients). Results were expressed in optical density (OD). Absorbance at 405 nm was determined using spectrophotometer (**Supplementary Data S1**).

The same protocol was followed to determine anti-TPO IgG, except for the IgG depletion and that the human sera adsorbed with *E. coli* lysate were diluted 1:50 and the secondary antibody was alkaline phosphatase-conjugated anti-IgG (Pharmigen, San Diego, CA, USA).

CD203c expression

Peripheral blood basophils were obtained from subjects from each group. We used the CD203c expression protocol previously reported by Shin *et al.*[9] (**Supplementary Data S1**).

The percentage of CD203c expression was defined as the percentage of basophils expressing more CD203c than the critical point, which was ≥10.0% of the basophils incubated with buffer only, similar to previous studies.[14] To evaluate the response of the basophils of each group to the stimulus with serum of subjects from another group, we performed incubations at room temperature for 4 hours of the mixed samples and subsequently performed the BAT according to what was described above.

ASST

The evaluation of ASST was performed according to international guidelines; [15] 0.05 mL of serum were injected via intradermal and a wheal larger than 1.5 mm over the negative control after 30 minutes was considered as a positive result.[15]

Intradermal and SPT

Recombinant TPO was used for serum and skin tests. The protein solutions were treated with LPS-Free (Toxin eraser Kit, Genscript Cat. No. L00338) and were prepared at 25.0 ng/μL in 50.0% glycerol, 0.4% phenol, and filtered using a 0.22-μm filter. Result interpretations were made according to international recommendations for skin tests and intradermal tests:[15-17] a 3-mm wheal over the negative control was considered positive. As a control, saline was incubated with sepharose-protein G to exclude the release of skin irritants from sepharose-protein G.[18,19] Atopic sensitization to the principal allergenic sources in this population[20] was evaluated with the SPT.

Passive transfer of serum

In order to prevent infections or other complications, before taking the test sample, donors were screened and evaluated for different infections (HIV 1-2, Hepatitis B, Hepatitis C, syphilis, Chagas), the determination of the ABO group, antigen D (Rh) and, screened for anti-erythrocytic antibodies and their identification.

The protocol for serum skin testing was similar to that used for ASST.[15] Venous blood was collected into sterile glass tubes (BD Vacutainer, Franklin Lakes, NJ, USA) without

anticoagulant or other additives. After collection, blood was allowed to clot at room temperature for 30 minutes before separation of serum by centrifugation at 500 g at 16°C for 15 minutes. The volar forearm skin was used after cleansing with antiseptic, leaving 3–5 cm gaps between each of the injections. A palpable bleb of fluid within the papillary dermis was performed with the injection. Three superficial intradermal injections were given using 0.05 mL of fresh undiluted serum from donor to minimize risk of sample contamination. After 24 hours, the SPT and the intradermal test with TPO (10 μg/50 μL and 1 μg/50 μL, respectively) were carried out in 2 of the 3 points where the passive transference had been done before. In the third point, saline solution was injected as negative control. SPT with histamine (10 mg/mL) was used as positive control. A test was considered positive when a wheal larger than 3 mm over the negative control after 30 appeared.

Ethical considerations

The Ethics review committee of the Universidad de Antioquia and the "IPS Universitaria" (university health care services provider and hospital), approved the study (Code number CCEI-5311-2016) according to the principles of the declaration of Helsinki. Written informed consent was obtained from all the participants or in the case of children, it was obtained from their parents. For the passive transfer tests, the same informed consent was made but with an addendum explaining the need to perform the previous control tests to avoid the infection risk.

Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics for Windows, version 21.0 (IBM, Armonk, NY, USA). The mean was reported for descriptive variables. The Mann-Whitney *U* test was used to compare anti-TPO IgE levels. Pearson's χ^2 test was used to evaluate the differences among groups. Differences between proportions were analyzed using Pearson's χ^2 test. Correlations were assessed with the Spearman coefficient (r).

Given the results of previous studies evaluating the presence of anti-TPO IgE,[8,9] we considered that a sample of at least 70 patients with CSU, 30 patients with ATD, and 50 healthy subjects would be adequate to ensure a power of 90.0% and an alpha error of 0.05 for the primary outcome measure (presence of anti-TPO IgE). A P value of < 0.05 was considered statistically significant.

For comparisons among the 3 study groups (CSU, ATD, and control group), we use the Kruskal Wallis test, for quantitative variables (*e.g.*, IgE levels, IgG levels); when this test showed a significant difference ($P \leq 0.05$), a multiple comparison test, using the same analysis, can be performed to compare the differences between each possible pair of groups.

Because experiments with the basophil activation test (BAT) were performed before and after exposure to TPO, a generalized linear model of repeated measures was used to compare intra- and inter-group measures and the analysis of covariates.

RESULTS

Population characteristics

One hundred patients with CSU, 30 with ATD and 110 healthy subjects were recruited. Age and sex have a similar distribution between the groups. The CSU group had a higher prevalence of atopy and total IgE than the ATD or healthy control groups ($P = 0.03$). Positive

Table 1. Population characteristics

Characteristics	CSU (n = 100)	ATD (n = 30)	Control (n = 110)	*P*
Age (yr)	28 (14–50)	32 (16–56)	29 (14–50)	0.2
Sex (female)	61 (61)	18 (60.0)	62 (56.3)	0.2
Atopy	42 (42)	10 (33.3)	26 (23.6)	**0.03**
Total IgE (IU/mL)	248 ± 113	178 ± 79	194 ± 109	**0.03**
Years with CSU	2 ± 3	N/A	N/A	N/A
Age of onset (yr)	25 (4–49)	N/A	N/A	N/A
Positive ASST	48 (48.0)	9 (30.0)	30 (27.2)	**0.04**
DLQI score	8 ± 6	N/A	N/A	N/A
UAS score	2 ± 3	N/A	N/A	N/A
Anti-TPO IgG	28 (28.0)	28 (93.3)	20 (18.1)	**0.01**
Mean IgG (IU/mL)	23.2 ± 52.0	63.5 ± 52.0	13.8 ± 33.0	**0.01**
(+)anti-TPO IgG and (+)anti-TPO IgE	24 (24)	5 (16.6)	7 (6.3)	**≤0.05**
Correlation among anti-TPO IgG and (+) IgE	r = 0.643; P = 0.03	r = 0.548; P = 0.05	r = 0.402; P = 0.05	N/A

Data are shown as number (range), number (%), or mean ± standard deviation. A $P \leq 0.05$ (bold numbers) was considered statistically significant in sociodemographic and clinical parameters among 3 groups according to Kruskal Wallis test.
CSU, chronic spontaneous urticaria; ATD, autoimmune thyroid disease; Ig, immunoglobulin; TPO, thyroid peroxidase; N/A, no apply; ASST, autologous serum skin test; DLQI, dermatology life quality index; UAS, urticaria activity score.

ASST was higher in CSU than the other 2 groups (P = 0.04). The CSU group had a higher atopy prevalence (42.0%) than the ATD (33.3%) or control groups (23.6%). Levels of total IgE are presented in **Table 1**.

Prevalence of anti-TPO IgE

Anti-TPO IgE was detected in all 3 groups (**Fig. 1**). More patients with CSU had positive anti-TPO IgE compared to the other groups and concentration levels were also higher in the CSU group (CSU O.D.: 0.294 ± 0.147; ATD O.D.: 0.244 ± 0.253; control O.D.: 0.163 ± 0.117). Total IgE level was higher among CSU patients with sIgE to mites (273.0 ± 108.0) compared to CSU patients without it (229.0 ± 125.0), but the difference was not statistically significant. Taking into consideration that atopy and total IgE levels were higher in CSU patients, we adjusted this analysis for these 2 variables. Although patients with atopy and total IgE levels over 300 IU/mL had a higher frequency of anti-TPO IgE (P = 0.05), no direct correlation was observed between total IgE and anti-TPO IgE. Patients with anti-TPO IgE had a mean UAS (3.0 ± 2.0 vs.

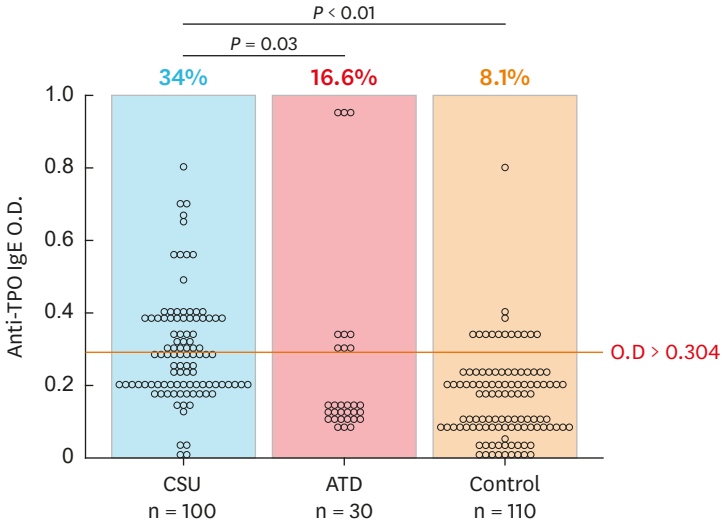


Fig. 1. Anti-TPO IgE levels in each group.
TPO, thyroid peroxidase; Ig, immunoglobulin; O.D., optical density; CSU, chronic spontaneous urticaria; ATD, autoimmune thyroid disease.

Table 2. Comparisons of CSU with anti-TPO IgE and anti-TPO IgE

Characteristics	CSU anti-TPO IgE (n = 34)	CSU (−) anti-TPO IgE (n = 66)	*P*
Age (yr)	27 (15–50)	28 (14–50)	0.2
Sex (female)	21 (61.7)	40 (60.6)	0.2
Atopy	16 (47.0)	26 (39.3)	0.1
Total IgE (IU/mL)	278 ± 119	228 ± 103	0.1
Years with CSU	2 ± 3	2 ± 3	0.1
Age of onset (yr)	25 (4–46)	25 (4–49)	> 0.05
Positive ASST	17 (50.0)	31 (46.9)	> 0.05
DLQI score	10 ± 6	7 ± 7	0.09
UAS score	3 ± 2	2 ± 1	0.09
Anti-TPO IgG	10 (35.7)	18 (27.2)	0.1
Mean IgG (IU/mL)	28.2 ± 42.3	20.7 ± 62.0	**0.03**
Baseline CD203c expression (%)	8 ± 4 (n = 10)	5 ± 3 (n = 5)	**0.05**
Positive SPT with TPO	6/20 (30)	0/30 (0)	**0.02**
Positive IDT with TPO	3/20 (15)	1/30 (3.3)	**0.05**

Data are shown as number (range), number (%), or mean ± standard deviation. Comparisons of clinical characteristics of CSU patients with positive and negative anti-TPO IgE. A $P \leq 0.05$ (bold numbers) was considered statistically significant.
CSU, chronic spontaneous urticaria; Ig, immunoglobulin; TPO, thyroid peroxidase; ASST, autologous serum skin test; DLQI, dermatology life quality index; UAS, urticaria activity score; SPT, skin prick test; IDT, intradermal test.

2.0 ± 1.0) and DLQI (10.0 ± 6.0 vs 7.0 ± 7.0) higher than patients with (−)anti-TPO IgE, but the difference was not statically significant ($P = 0.09$) (**Table 2**).

Among CSU patients and healthy subjects, there were some subjects with anti-TPO IgG and no clinical diagnosis of autoimmune thyroiditis. An additional subanalysis was done after excluding these patients and comparing these groups with the ATD group, but no significant statistical differences were found.

BAT

We observed a similar concentration of basophils in total blood in the 3 groups, being slightly lower in the CSU group (23 cells/mL) compared to the ATD group (32 cells/mL) on the healthy control group (36 cells/mL), without statistical differences.

For the BAT, basophils were obtained from 4 groups of subjects; CSU (+)anti-TPO IgE, ATD (+)anti-TPO IgE, control (−)anti-TPO IgE and control (+)anti-TPO IgE.

With the different dilutions of TPO used (dilution 0.01, $P = 0.04$; dilution 0.1, $P = 0.01$; dilution 1, $P < 0.01$) (**Fig. 2A**), CD203c expression from patients with CSU and anti-TPO IgE significantly increased compared to the other groups.

Basophils of control subjects with (+)anti-TPO or (−)anti-TPO IgE and no urticaria or ATD did not have a significant CD203c expression when stimulated with TPO. With 0.1 and 1 μg/mL dilutions, the ATD group had a CD203c expression, which was significantly lower than the CSU group and was not significantly different from control subjects with (+)anti-TPO or (−) anti-TPO IgE (**Fig. 2**). When the basal levels of CD203c were compared among patients of the CSU group, a higher expression of basal CD203c was observed in the group with anti-TPO IgE ($P = 0.05$) (**Table 2**).

When we mixed a pool of serum from CSU patients with anti-TPO IgE and basophils from a control subject with (−)anti-TPO IgE (**Fig. 2B**), CD203c expression was significantly increased ($P = 0.04$) after stimulation with TPO (1 μg/mL). A similar result was observed when basophils from ATD patients were used ($P = 0.05$).

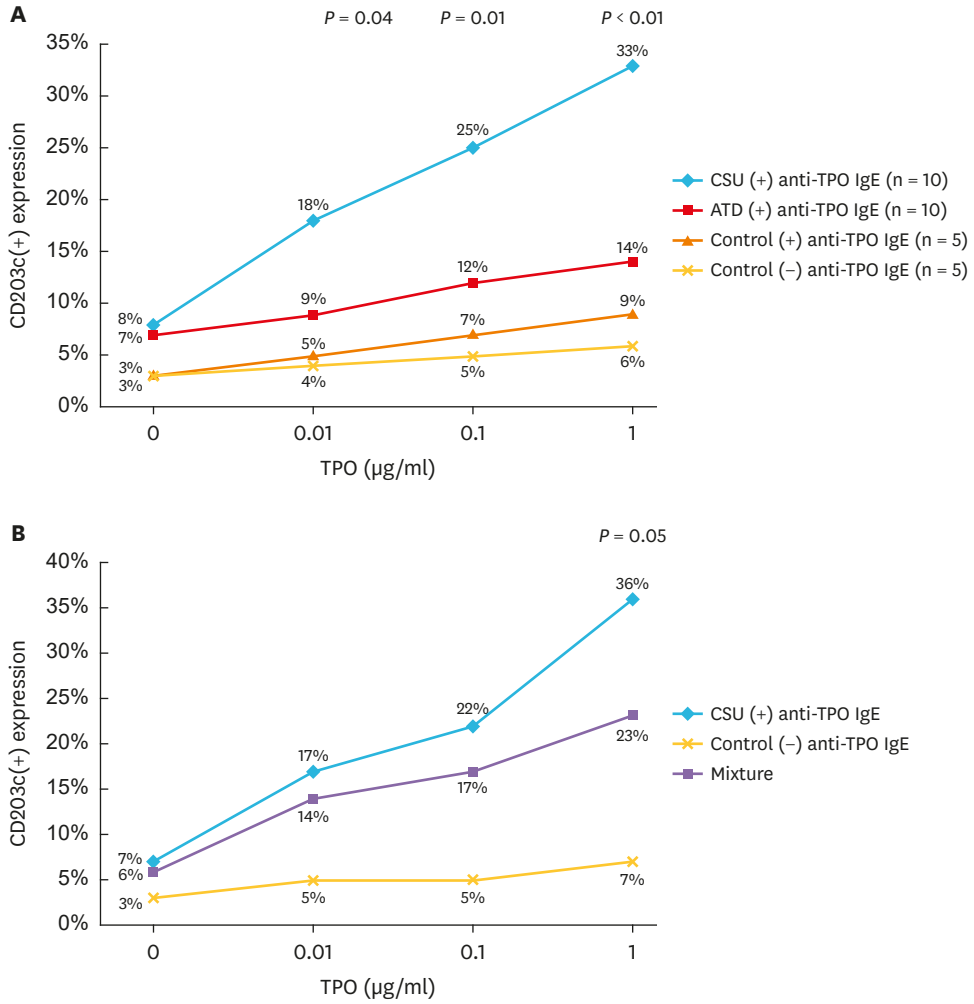


Fig. 2. Basophil activation test with TPO.
TPO, thyroid peroxidase; Ig, immunoglobulin; CSU, chronic spontaneous urticaria; ATD, autoimmune thyroid disease.

For the following experiments combining serum and basophils from different subjects, only the dilution of 1 μg/mL TPO was used: CSU basophils with sera from healthy control subjects with anti-TPO IgE induced the expression of CD203c after stimulation with TPO (1 μg/mL) (15.5%), but it was lower than with their own serum or with anti-TPO IgE serum from another CSU patient (23%). CSU basophils with sera from healthy control subjects with (−)anti-TPO IgE did not induce the expression of CD203c after TPO stimulation (5.0%).

Prevalence of anti-TPO IgG

The CSU group had a higher frequency of anti-TPO IgG than the control subjects (28.0% vs. 18.1%, $P = 0.04$), but it was lower than the ATD group (93.3%). CSU patients with anti-TPO IgE exhibited significantly higher anti-TPO IgG levels than those with (−)anti-TPO IgE (28.2 ± 42.3 vs. 20.7 ± 62.0, $P = 0.03$) (**Table 2**).

Levels of anti-TPO IgE and IgG had a significant correlation in the CSU and healthy control groups. Taking into consideration that this correlation could occur because most of these subjects had a negative result for IgG and IgE, we evaluated the IgG and IgE correlation using

only subjects with anti-TPO IgE, and there was a significant correlation in ATD ($r = 0.548$) and CSU ($r = 0.643$) for IgE and IgG (**Table 1**).

Twelve patients in the CSU group had Hashimoto's thyroiditis; 10 had anti-TPO IgG and 3 of these patients also had anti-TPO IgE. All subjects in the healthy control group had normal thyroid function, even those with positive IgG or IgE against TPO. We did not find that any clinical parameter related with active thyroid disease, like the presence of anti-TPO IgG, could predict the presence of anti-TPO IgE.

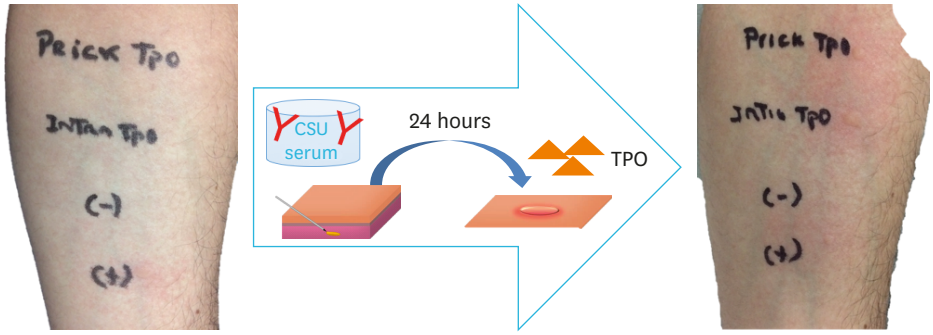
Skin response to TPO

The CSU group alone showed positive SPT to TPO (**Fig. 3A**), and intradermal skin reaction to TPO in this group was higher than the other groups ($P = 0.01$) (**Fig. 3A**). All subjects with positive SPT had positive serum anti-TPO IgE and positive intradermal test. Six of the 9 patients with CSU and positive intradermal test also had anti-TPO IgE (**Table 2**). Three of the 4 control subjects and the ATD patients with positive intradermal test had anti-TPO IgE. Among the subjects with positive skin tests in the 3 groups, there was a moderate

A

TPO	CSU (n = 50)	ATD (n = 30)	Control (n = 50)
SPT	6 (12.0%)	0	0
Intradermal	9 (18.0%)	1 (3.0%)	4 (8.0%)

B



Control (−)

Control (with CSU serum)

Fig. 3. Skin tests with TPO.
TPO, thyroid peroxidase; CSU, chronic spontaneous urticaria; ATD, autoimmune thyroid disease; SPT, skin prick test.

correlation ($r = 408$; $P = 0.05$) between the wheal size in the skin tests and the levels of sIgE against TPO.

We did passive intradermal transfer of anti-TPO IgE serum from 1 CSU patient to 2 healthy subjects with negative anti-TPO IgE in serum as well as negative SPT, intradermal test and ASST (**Fig. 3B**). The CSU serum used was negative for anti-TPO IgG. After 24 hours, we repeated intradermal test and SPT with TPO, and subjects presented positive reactions. In these 2 subjectss, after 2 and 4 weeks we repeated skin tests with TPO at the same point previously injected with anti-TPO IgE serum from a CSU patient; the skin test remained positive after 2 weeks, but it was negative after 4 weeks.

We did not observe clinical changes or alterations in thyroid function among patients with ATD who underwent skin tests with TPO, and no changes were observed either among the 2 subjects who participated in the passive transfer after 2 months of follow-up.

DISCUSSION

Different mechanisms of self-reactivity are involved in the development of CSU[8,21-25] and some studies described a strong association between self-reactivity and autoimmune diseases, especially thyroid diseases.[26-29] It is necessary to identify auto-reactive proteins in CSU.[30] The presence of anti-TPO IgE in CSU has led us to assume that this auto-reactive IgE may play a role in the development of CSU or can, at least, be an indicator of inflammation. To evaluate the causal role of anti-TPO IgE in CSU, we first calculated the prevalence of anti-TPO IgE in our population. We observed that 34% of patients with CSU had anti-TPO IgE and it was higher than in ATD patients (16.6%) or healthy subjects (8.1%). Nevertheless, anti-TPO IgE seems to be important only in some patients with CSU; the presence of this autoantibody in subjects without urticaria and the presence of urticaria in subjects without this autoantibody indicate that anti-TPO IgE is not the only factor influencing the development of the disease and that different inflammatory cells or other substances of the serum could have a relevant role.[31]

The direct detection of anti-TPO IgE in a wheal performing a biopsy in the acute phase of urticaria could help clarify the clinical relevance of anti-TPO IgE in urticaria. However, the technique for detecting this low serum antibody in fresh skin can be complex. Although BAT has limitations because it is an "*in vitro*" technique, it allows us to evaluate the capacity of anti-TPO IgE to activate basophils, which shares many characteristics with mast cells in terms of IgE activation. Mast cells play a prominent role in urticaria,[32] but recent studies using basophils have provided useful information to understand this disease. Rauber *et al.*[33] demonstrated that in CSU, different basophil phenotypes can be categorized after stimulating basophils through FcεRI. BATs using CD63 or CD203c showed that different allergens have similar sensitivity, with a slightly higher sensitivity to CD203c.[34-36] We used the protocol with CD203c, which has previously been tested with TPO; Shin *et al.*[9] suggested that anti-TPO IgE plays a pathogenic role in aspirin-exacerbated cutaneous urticaria in terms of the expression of CD203c. Some studies suggested that basophils from 10.0% to 20.0% of healthy individuals are non-releasers to FcεRI-dependent stimuli[37] and that basophils from chronic urticaria patients seem to have some changes in the FcεRI-signaling pathway.[38] Taking into account these results and that we observed an expression of CD203c among patients with ATD but without having urticaria, it is necessary to evaluate if basophils from patients with autoimmune thyroiditis have their particular characteristic phenotypes.

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These cell phenotypes are associated with different clinical characteristics, pointing to basophils as important players in CSU's physiopathology. We observed that patients with CSU and anti-TPO IgE had a higher CD203c expression than the 2 other groups. The lack of activation through anti-TPO IgE in ATD and healthy control groups suggested that in patients without urticaria, some factors can antagonize the effect of anti-TPO IgE, considering the results of Rauber *et al.*[33] that these groups may be have a high proportion of hypo-reactive basophils. Similar to Shin *et al.*[9] in aspirin urticaria, we also observed that among patients with CSU, despite having anti-TPO IgE, not all samples had CD203c. To clarify whether this lack of basophil response was due to the basophil type or perhaps to another factor present in the serum, we evaluated the basophil auto-reactive capacity by mixing basophils and serum from the 3 groups. We observed that when we use CSU anti-TPO serum IgE, basophils from ATD or control subjects express CD203c, but at a lower intensity than patients with CSU and only at the highest concentration of TPO. In addition, when we incubated basophils of CSU patients with anti-TPO IgE from control subjects, we observed expression of CD203c, but lower than what observed with its own anti-TPO IgE serum or with anti-TPO IgE serum from another CSU patient. Based on these observations, it seems that the different types of basophils may play a role in the type of response to TPO or that other factors from sera can prevent this activation at least partially. Inhibition experiments are needed in order to answer these questions.

As expected, IgG levels were higher in the ATD group, but the anti-TPO IgG levels were higher in the CSU group than in the healthy control group. Although previous studies have established the coincidental occurrence of autoimmune diseases and CSU,[39] there are no current data to determine the basis for this link. Due to the design of the study, we cannot state with certainty if thyroid diseases in the CSU group were more or less frequent than in the healthy control group. However, we observed that within the CSU group, patients with anti-TPO IgE had higher levels of anti-TPO IgG than those with (−)anti-TPO IgE, although the CD203 expression baseline was higher in the anti-TPO IgE group. Our results suggest that anti-TPO IgG could interfere with anti-TPO IgE. This hypothesis is supported by 2 results: (1) In patients with ATD and positive anti-TPO IgE, anti-TPO IgG levels were higher compared to ATD patients who did not have IgE and the activation of basophils was lower compared to the CSU group. (2) In CSU patients with positive IgE anti-TPO but negative skin test results, anti-TPO IgG levels were higher than in CSU patients with negative IgE (data not shown). As suggested by Mozena *et al.*,[32] it is plausible that urticaria and ATD are pathogenically linked, possibly reflecting a shared epitope targeted by different autoantibodies. It is possible that in patients with ATD and high levels of anti-TPO IgG and anti-TPO IgE, this IgE could play a role in worsening Hashimoto's thyroiditis. However, in patients with ATD, there must be some differences in the effector mechanism of anti-TPO IgE regarding what happens in patients with CSU because a smaller number of patients presented skin exacerbation with TPO and had less reactivity in BAT. Additional experiments are required with a larger number of patients to confirm this hypothesis.

The SPT and intradermal tests are excellent tools to evaluate the clinical impact of allergens on the skin.[11,40] The usefulness of skin tests in CSU has been limited in part by the lack of knowledge of mechanisms associated with the disease, which does not allow scientists to choose the right protein to be tested. The ASST has been the most commonly used diagnostic tool in urticaria, although it has excellent sensitivity but quite low specificity.[15] With the growing evidence of the possible role of TPO in a group of patients with CSU, we tested whether the stimulation of skin with TPO could induce the formation of wheals in patients

with CSU. We observed a strong correlation between *in vitro* and *in vivo* results. SPT had better specificity than the intradermal test, but the sensitivity of the intradermal test was better. With the passive transfer of serum from a CSU patient to the skin of 2 control subjects, we demonstrated that anti-TPO IgE plays a central role in the pathogenesis of wheal in CSU, at least in a group of patients. The standardization of skin tests with TPO will provide a easy and simple tool to identify patients in whom urticaria is due to this IgE auto-reactivity. Although at the moment anti-TPO IgE measurement is conducted only in some specialized centers, it will be generalized to all the clinical laboratories. However, according to our results, the advantages of the skin test (rapid implementation, avoids puncture, almost immediate results), can be supported by anti-TPO IgE measurement in serum.

Our study has some limitations. As mentioned earlier, we did not directly evaluate different phenotypes of basophils that could answer some of new questions arising from these results. In addition, we did not evaluate how other serum elements, such as complement factors, could play a role in auto-IgE pathogenesis. Although the case-control design of the study was appropriate for the main objective of the study, the lack of follow-up did not allow for evaluating whether IgE or IgG levels are dependent on the exacerbation of urticaria. Despite these limitations, the study has some strengths. First, the different experiments carried out show that anti-TPO IgE can induce the disease in some patients with CSU.Secondly,, this study could be considered a preliminary effort for future research in the technological development of new diagnostic tools for urticaria.

In conclusion, since anti-TPO IgE is present in patients with CSU and ATD as well as in healthy subjects, it is not a specific biomarker for CSU. However, IgE to TPO plays a pathogenic role in cellular effector activation and skin exacerbation in a subgroup of CSU patients, and this effect can be transferred to healthy subjects.

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SUPPLEMENTARY MATERIAL

Supplementary Data S1

Methods details for Thyroid peroxidase (TPO) recombinant production and clinical tests.

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SUPPLEMENTARY MATERIAL

Supplementary Data S1. Methods details for Thyroid peroxidase (TPO) recombinant production and clinical tests.

Thyroid peroxidase (TPO) recombinant

Escherichia coli strains BL21 (DE3) were transformed with the expression plasmid pET45b+/TPO and cultured in Luria-Bertani medium containing 100 mg/L ampicillin at 37°C to 0.4 O D at 600 nm. Protein expression was induced by the addition of 1 mM (Millimolar) isopropyl-β-D–thio-galacto-pyranoside to the medium and subsequent incubation for 4 hours. Cells were then harvested by centrifugation at 6,500 r p m. at 4°C for 15 minutes. The cell pellets were solubilized in 8 mol/L urea, 0.1 mol/L phosphate-buffered saline (PBS; NaH_2PO_4), 0.01 mol/L Tris-HCl, pH 8.0, and the lysate was pulsed by ultrasound several times on ice and then incubated by continuous rotation for 3 hours. Insoluble material was removed by centrifugation. The supernatant containing the recombinant was applied to nickel-nitrilotriacetic acid-Agarose and purified under hybrid conditions as indicated by the supplier. The proteins were eluted with native elution buffer (50 mM NaH_2PO_4, 0.5M NaCl, 250 mM Imidazole pH 8.0). Fractions containing eluted proteins were pooled and dialyzed against 50 mM NaH_2PO_4, 0.5 M NaCl (pH 8.0). The protein concentration was determined by Bradford assay. The purity and integrity of the protein was assessed by sodium dodecyl sulphate-polyacrilamide gel electrophoresis (SDS-PAGE) and subsequent staining with Coomassie Brilliant Blue R-250 (Bio-Rad Laboratories, Hercules, CA, USA).

Determination of total IgE, anti-TPO IgE and anti-TPO IgG

Total IgE levels in serum samples were determined using a flouroenzyme immunoassay (Phadia; ImmunoCap System, Uppsala, Sweden). Sera yielding total IgE levels above 100 IU/mL were diluted (1:10) to keep the test within the dynamic range.

The sera used for the quantification of sIgE were previously depleted of IgG by immunoaffinity depletion: for each 500 L of serum and 1X PBS (1:5) were added 80 L of Protein G (Sigma-P3296, Protein G Sepharose®). After 60 minutes of incubation at room temperature (RT) on a rotating

platform, the serum was recovered by centrifugation and stored at −20°C until use. Residual IgG contents in protein G-adsorbed sera were measured by enzyme-linked immunosorbent assay (ELISA) assays and sera were accepted for anti-TPO IgE measure when IgG were found to be less than 10 µg/mL.

For specific IgE, micro-plates were incubated with 100 µL of 1 µg/100 µL TPO per well over night (12 hours). After 5 washes with PBS, the wells were blocked with 100 µL of blocking buffer (1% BSA) and 0.02% sodium azide in PBS-T for 3 hours at RT in a wet chamber. After washing again, 100 µL of human sera (diluted 1:2 in PBS-10% BSA) was added to each well with further overnight incubation at RT. The wells were then washed 4 times, and 100 µL of alkaline phosphatase-conjugated anti-IgE, diluted 1:500 in 0.05 M buffer Tris pH 8.0, 1% BSA, 1mM MgCl 2 and 0.02% sodium azide were added and incubated for 2 hours. After the final wash, paranitrophenyl phosphate was added for a colorimetric reaction, and the wells were then incubated for 30 minutes. Results are expressed in optical density (O D). Absorbance at 405 nm was determined using spectrophotometer.

Taking into account a previous study,[8] the cutoff value for serum specific IgE level to TPO was defined as the mean and 3-fold standard deviation of absorbance values from 40 healthy controls without urticaria or autoimmune diseases.

For the determination of anti-TPO IgG, the same protocol was followed, except for the IgG depletion and that the human sera adsorbed with *E. coli* lysate were diluted 1:50 and the secondary antibody was alkaline phosphatase-conjugated anti-IgG (Pharmigen).

CD203c expression

Peripheral blood basophils were obtained from subjects from each group. We used the CD203c expression protocol previously reported by Shin *et al*.[8] Briefly, basophil activation test (BATs) were performed with the whole blood collected in EDTA tubes and red blood cells were lysed with a lysis buffer. The 100 µL of the leukocyte suspension were added along with calcium ionophore, anti-human goat IgE antibodies and anti-IgG4. Basophil activation was stimulated with different concentrations of TPO antigens (0.01, 0.1 and 1 µg/mL). After washing with PBS, the cells were

stained with phycoerythrin (PE)-conjugated antihuman CD203c (Beckman Coulter, Villepinte, France) and fluorescein isothiocyanate (FITC)-conjugated antihuman CD123. CD203c expression was measured by flow cytometry.

The percentage of CD203c expression was defined as the proportion of basophils expressing more CD203c than the critical point, which was > 10% of the basophils incubated with buffer alone, similar to previous studies.[13] To evaluate the response of the basophils of each group to the stimulus with serum of subjects from another group, we performed incubations at RT for 4 hours of the mixed samples and subsequently performed the BAT according to what was described above.

Passive transfer of serum

In order to prevent infections or other complications, prior to taking the sample for the test, the donors had been evaluated for different infections (HIV 1-2, Hepatitis B, Hepatitis C, syphilis and Chagas), determination of the ABO group, antigen D (Rh) and screened for anti-erithrocytic antibodies and their identification.

The protocol for serum skin testing was similar to that used for ASST.[14] Venous blood was collected into sterile glass tubes (BD Vacutainer) without anticoagulant or other additives. After the collection, blood was allowed to clot at RT for 30 minutes before separation of serum by centrifugation at 500 g at 16°C for 15 minutes. The volar forearm skin was used after cleansing with antiseptic, leaving 3-5 cm gaps between each of the injections. A palpable bleb of fluid within the papillary dermis was generated with the injection. Three superficial intradermal injections were done using 0.05 mL of fresh undiluted serum from donor to minimize the risk of sample contamination. After 24 hours, skin prick test (SPT) and intradermal test with TPO (10 μg/50 μL and 1μg/50 μL, respectively) were performed in 2 of the 3 points where passive transference was made. In the third point, saline solution as a negative control was injected. The SPT with histamine (10 mg/mL) was used as a positive control. A test was considered positive when a wheal larger than 3 mm over the negative control after 30 minutes was considered a positive result.