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Presence of IgE Autoantibodies Against Eosinophil Peroxidase and Eosinophil Cationic Protein in Severe Chronic Spontaneous Urticaria and Atopic Dermatitis

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ABSTRACT

Purpose: Eosinophils are frequently found in atopic dermatitis (AD) and chronic spontaneous urticaria (CSU) that release eosinophil peroxidase (EPX) and eosinophil cationic protein (ECP). Continuous exposure to these proteins could trigger an autoimmune response which may contribute to the pathogenesis and severity of skin inflammation. In this study, we investigate the immunoglobulin E (IgE) response against eosinophil proteins in CSU and AD.
Methods: We recruited patients with severe AD, severe CSU and healthy subjects to explore the presence of IgE autoantibodies and cross-reactivity against EPX, ECP and thyroid peroxidase (TPO). The potential cross-reactive epitopes among the peroxidase family were determined using *in silico* tools.
Results: The frequencies of anti-EPX IgE (28.8%) and anti-ECP IgE (26.6%) were higher in the AD group, and anti-TPO IgE was higher in the CSU group (27.2%). In the CSU group, there was a correlation between the anti-EPX IgE and anti-TPO IgE levels ($r = 0.542$, $P < 0.001$); TPO inhibited 42% of IgE binding to EPX, while EPX inhibited 59% of IgE binding to TPO, suggesting a cross-reactivity with EPX as a primary sensitizer. There was greater inhibition when we used a pool of sera CSU and AD, TPO inhibited 52% of IgE binding to EPX, while EPX inhibited 78% of IgE binding to TPO. *In silico* analysis showed a possible shared epitope in the peroxidase protein family.
Conclusions: IgE against eosinophil proteins may contribute to chronic inflammation in patients with AD and CSU. Cross-reactivity between EPX and TPO could explain thyroid problems in CSU patients.

Keywords: Allergy; atopic dermatitis; autoantibodies; eosinophil peroxidase; eosinophil cationic protein; immunoglobulin E; peroxidase; thyroperoxidase; chronic urticaria

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Disclosure

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

INTRODUCTION

Atopic dermatitis (AD) and chronic spontaneous urticaria (CSU) are common skin diseases with a high impact on quality of life.[1-4] Despite their clinical differences, they share some characteristics in the inflammatory process: production of Th1 and Th2 cytokines as well as immunoglobulin (Ig) E reactivity to several autoantigens has been reported,[5-8] and skin infiltration of eosinophils is frequent.[9,10]

The clinical implications of auto-IgE and auto-antigens in allergic and non-allergic disorders are not fully understood,[11,12] but multiple tests *in vitro* and a study *in vivo* suggested that these auto-antibodies induce the degranulation of mast cells in the skin and subsequent skin inflammation,[13-16] indicating that IgE reactivity to self-proteins may represent an important mechanism involved in the maintenance of chronic inflammation.[17] Why this reaction to self-proteins occurs is still unknown. A hypothesis is that, after chronic recognition and production of IgE to environmental allergens, these immunoglobulins by molecular mimicry could recognize similar epitopes present in human proteins and epitope spreading could enhance autoreactivity. Previous studies supported this hypothesis in dermatitis,[6,18,19] but it is less clear in urticaria .

Additionaly, CSU was associated with an increased frequency of autoimmune diseases, particularly hypothyroidism.[20] The bridge between this skin disease and the formation of IgE autoantibodies against thyroid proteins could be the result of cross-reactivity between thyroid peroxidase (TPO) and proteins exposed on the skin during the inflammation process.

In AD and CSU, cytokines are released, which results in the influx of inflammatory cells including basophils, neutrophils and eosinophils.[21,22] Eosinophils and neutrophils can release inflammatory mediators such as major basic protein (MBP), eosinophil peroxidase (EPX), eosinophil cationic protein (ECP) and neutrophil myeloperoxidase (MPO). IgG response to EPX was detected in asthma,[23] and this protein share common epitopes with TPO as they belong to the same family.[24-26]

Considering the eosinophil infiltrate in the skin in CSU and AD patients and their release of cytoplasmic granules in skin lesions, our hypothesis is that EPX and ECP may possibly be recognized by IgE autoantibodies generated during a primary immune response and then react later by cross-reactivity against proteins distant to the site of skin inflammation, such as TPO. The aim of this study was to explore the presence of IgE autoantibodies against eosinophil proteins and the possible IgE cross-reactivity of EPX and TPO.

MATERIALS AND METHODS

Study design and population

A cross-sectional study was performed using 3 groups based on their clinical status: patients with severe AD, patients with severe CSU and healthy subjects. The CSU population was recruited from the "Urticaria Research of Tropical Impact and Control Assessment" cohort,[27,28] and severe AD patients were recruited from the "Tropical Environment Control for Chronic Eczema and Molecular Assessment" cohort.[29,30] CSU was defined as the recurrence of hives, with or without angioedema, for at least 6 weeks.[31] CSU group included patients older than 12 years with a urticaria activity score for 7 days ≥ 28 points. Dermatitis group included

patients older than 12 years and diagnosed according to the international guidelines[32-34] with a severe scoring for AD > 40 points. Healthy control group consisted of subjects older than 12 years without a clinical history of autoimmune diseases, chronic urticaria or a history of acute urticaria in the previous 2 years.

Participants with a diagnosis of systemic or other skin diseases (*e.g.*, mastocytosis or nodular prurigo) or pharmacologic treatment that could cause hives or eczema were excluded. Patients had received treatment with antihistamines or topical steroids, but not with immunosuppressants or biological therapy.

Antigen production

For EPX, there are several abbreviations (EPO; EPP; EPXD; EPX-PEN); in this article, we will use EPX because it is encoded in the UniProt database that we used for *in silico* analyses. TPO, ECP and EPX were obtained as recombinant proteins according to a previous protocol[13] using *Escherichia coli* BL21 (DE3) as an expression vector (**Supplementary Data S1**). Commercial human EPX (Product Ref: SRP6187; Sigma-Aldrich, St Louis, MO, USA) was also used for performance comparison.

Determination of the total and specific IgE autoantibodies

Total IgE levels in the serum samples were determined using a flouroenzyme immunoassay (ImmunoCap System, Uppsala, Sweden). When total IgE levels were above the reading range of the equipment (> 100 KU_A/mL), the sample was diluted at 1:5 or 1:10, depending on the serum samples, and the total concentration was calculated by conversion.

We explored the presence of IgE autoantibodies against TPO, EPX and ECP using a previously tested enzyme-linked immunosorbent assay (ELISA) protocol.[13] The sera used for the quantification of IgE were previously depleted of IgG by immunoaffinity depletion. Considering a previous study,[13] the cutoff value for the serum specific IgE level to TPO was defined as the mean and 3-fold standard deviation of absorbance values from 60 healthy controls with neither urticaria nor autoimmune diseases. The same method was used for the the cutoff value for serum anti-EPX IgE and anti-ECP IgE. The absorbance at 405 nm was determined using a spectrophotometer (**Supplementary Data S1**).

IgE-binding inhibition assays

The cross-reactivity between thyroid and eosinophils proteins was evaluated using ELISA and immunoblotting IgE-binding inhibition assays according to a previous protocol with some modifications (**Supplementary Data S1**).[35]

Considering previous studies,[24,36] for our ELISA and immunoblotting experiments, antigens were reduced with SDS and 5% β-mercaptoethanol, respectively. For immunoblotting, the proteins were electro-transferred onto nitrocellulose membranes and incubated overnight with the serum pool; strips were incubated with 2 mL of each pool, previously adsorbed (6 hours at room temperature) with 100 μg/mL inhibitor. Bovine serum albumin (BSA) was used as a negative control.

In silico comparative analysis

We compared the amino acid sequences of TPO, EPX and ECP as well as included other peroxidases (MPO, lactoperoxidase [LPO], and peroxidasine-like) to determine the conserved regions (**Supplementary Tables S1** and **S2**).

The amino acid sequences of TPO, EPX, ECP, MPO, LPO and peroxidasine-like enzyme were obtained from the Uniprot database (https://www.uniprot.org/). Multiple and pairwise alignments were performed using the IBIVU server (http://www.ibi.vu.nl/programs/pralinewww/). Detailed methods are presented in **Supplementary Data S1**.

Ethical considerations

The Ethics Committees of the University of Antioquia and the Clinic "IPS Universitaria" approved this study (Code F-017-00 from University of Antioquia and Code number CCEI-5311-2016 from Clinic "IPS Universitaria"). Written informed consent was obtained from all participants or parents in cases of children.

Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics for Windows, version 21.0 (IBM, Inc, Chicago, IL, USA) and GraphPad Prism 8 (La Jolla, CA, USA). The Shapiro–Wilk test was used to check for normality, and our descriptive and statistical analyses were chosen according to the results. The data are presented as the means and standard deviation for variables with normal distribution and as the median and range for variables with non-normal distribution. The Mann-Whitney *U* test and the Kruskal–Wallis test were used to compare specific IgE levels. Pearson's χ^2 test was used to evaluate differences among groups and proportions. Correlations were assessed with the Pearson or Spearman coefficient (r).

Given the results of previous studies,[13,18,37] we considered that a sample of at least 40 patients with CSU, 40 patients with AD, and 40 healthy subjects would be adequate to ensure a power of 80% and an alpha error of 0.05 for the primary outcome (the presence of IgE autoantibodies). A *P* value of < 0.05 was considered statistically significant as long as it had correspondence with the dispersion measures (standard deviation or confidence interval). For comparisons among the 3 study groups (*e.g.*, the CSU, AD and control groups), we used the Kruskal-Wallis test for quantitative variables (*e.g.*, IgE level) and a multiple comparison test to compare differences between the groups.

RESULTS

Patients characteristics

Sixty control subjects, 45 AD patients, and 56 CSU patients were recruited (**Table 1**). Due to selection criteria for each group, some differences were observed: patients with AD were younger than those with CSU and experienced the disease for a longer time. The AD group had higher levels of total IgE ($P = 0.006$) and blood eosinophils ($P = 0.005$). These variables were similar between the CSU and control groups.

Atopy to house dust mites or pet dander was present in 56% of the CSU patients. According to selection criteria, all patients in the AD group had atopy, while subjects in the control group did not.

IgE against eosinophil and TPO autoantigens

IgE autoantibodies against EPX and ECP were observed in the 3 groups. The frequency of eosinophil autoantibodies was higher in the AD group (anti-EPX IgE 28.8% and anti-ECP IgE 26.6%) compared with the CSU group (10.9% and 5.4%, respectively) ($P = 0.008$) and the control subjects (3.3% and 1.6%, respectively) ($P < 0.001$) (**Fig. 1A**). The IgE autoantibody

Table 1. Sociodemographic characteristics

General characteristics	CSU (n = 55)	AD (n = 45)	Control (n = 60)
Age median (range)	33 (42)	16 (22)	25.5 (42)
Sex (female)	35 (63.6)	25 (55.5)	36 (60)
Atopy	31 (56.3)	45 (100)	0
Total IgE (IU/mL)	238.2 ± 282.8	688.0 ± 645.1*	152.6 ± 89.1
Eosinophil serum (cells/count)	115.7 ± 64.3	347.8 ± 47.7*	111.0 ± 44.7
Years with CSU/AD median (range)	2 (6)	13.2 (17)	NA
SCORAD median (range)	NA	50 (30)	NA
UAS7 median (range)	31 (14)	NA	NA
DLQI score median (range)	17.5 (10)	18 (12)	NA

Data are shown as mean ± standard deviation or number (%).
All subjects were over 12 years old. According to the selection criteria, all patients in the AD group had atopy, while the control group did not.
CSU, chronic spontaneous urticaria; AD, atopic dermatitis; IgE, immunoglobulin E; NA, not apply; SCORAD, severe score for atopic dermatitis; UAS7, urticaria activity score for 7 days; DLQI, dermatology life quality index.
*$P < 0.01$.

levels were higher in the AD and CSU groups compared with the control group. The eosinophil autoantibody levels were higher in the AD group compared with the CSU group; however, the TPO autoantibody levels were higher in the CSU group (**Fig. 1A**).

Only the CSU group showed a significant correlation among the anti-TPO IgE and anti-EPX IgE levels ($r = 0.542$; $P < 0.001$; confidence interval, 0.316–0.708). The other correlations in all groups were less than 0.300 and were not statistically significant (**Fig. 1B**).

In the CSU group, anti-TPO IgE was the most prevalent autoantibody, and all patients with anti-EPX IgE were reactive to TPO (**Fig. 1C**). For the AD group, 4 patients with anti-TPO IgE also had IgE to EPX (n = 2) and ECP (n = 2). In the control group, only 5 patients were IgE-sensitized to autoantigens: 1 patient was monosensitized to ECP; 2 patients were sensitized to EPX and also had anti-TPO IgE; and 4 patients were sensitized to TPO.

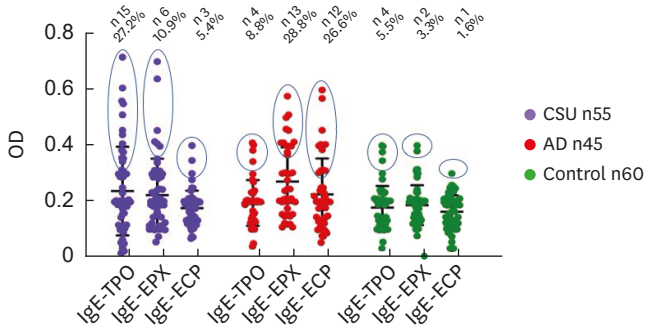
There was no association between autoantibody levels and blood eosinophils in terms of correlation or different cutoff stratification.

Cross-reactivity among eosinophil and thyroid proteins

The CSU serum pool (n = 16) and AD serum pool (n = 17) for the inhibition test were composed of sensitized patients with IgE autoantibodies defined according to the cutoff value (**Fig. 1C**). In CSU, TPO inhibited 42% of IgE binding to EPX, while EPX inhibited 59% of IgE binding to TPO (**Fig. 2A**), suggesting a cross-reactivity between these peroxidase proteins. We did not observe such inhibitions using an additional pool of CSU IgE-monosensitized patients (n = 7) to TPO (higher than 20%). We also analyzed the inhibition of IgE binding using a pool of only 3 CSU patients with anti-ECP IgE; however, ECP inhibited less than 20% of IgE binding to TPO or EPX (data not shown).

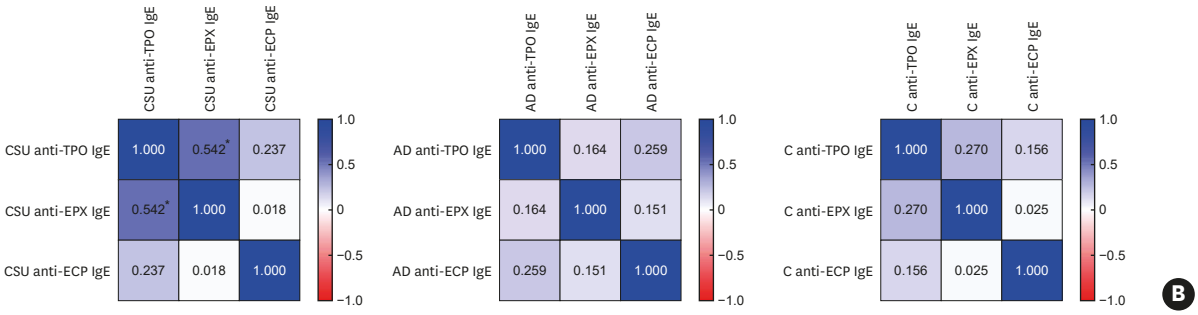
With the AD serum pool, TPO inhibited 32% of IgE binding to EPX, while EPX inhibited 39% of IgE binding to TPO. ECP inhibited less than 20% of IgE binding to TPO or EPX. Similar results were obtained with the pool of 17 AD patients with IgE against at least one of the autoantigens (data not shown).

An elevation in the concentration of the solid phase antigen (2,500 μg/mL) using the CSU pool and AD pool did not increase the percentage of inhibition observed at 1,000 μg/mL.



OD MEAN	CSU n55	AD n45	Control n60
Anti-TPO IgE	0.2385 SD 0.158	0.194 SD 0.081	0.179 SD 0.077
Anti-EPX IgE	0.224 SD 0.132	0.272 SD 0.122	0.187 SD 0.071
Anti-ECP IgE	0.177 SD 0.062	0.226 SD 0.2265	0.164 SD 0.058

A



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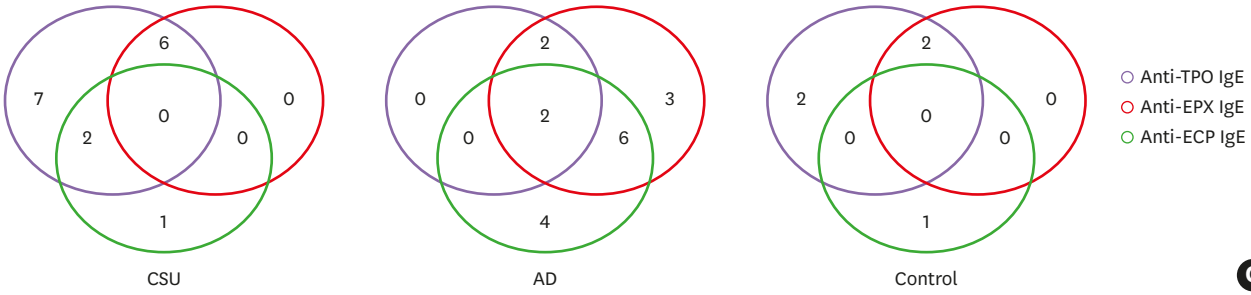

C

Fig. 1. IgE autoantibodies against TPO, EPX and ECP. (A) Patients with values above the cutpoint are grouped in circles. In the table, concentration levels are present according to the mean and standard deviation. (B) Correlation among IgE autoantibodies. (C) Interactions of autoantibodies in the CSU, AD and control groups. In the CSU group, the sera pool for inhibition test was composed of 6 patients with anti-TPO IgE/anti-EPX IgE and 2 patients with anti-ECP IgE alone (**Fig. 2A**).
OD, optical density; CSU, chronic spontaneous urticaria; AD, atopic dermatitis; TPO, thyroid peroxidase; EPX, eosinophil peroxidase; ECP, eosinophil cationic protein; IgE, immunoglobulin E.
*$P < 0.001$.

To explore whether the recognized epitopes in CSU patients were the same as those in AD patients, we performed a pool combining the 2 pools (CSU pool and AD pool, 1:1 ratio) (**Fig. 2C**). We observed a higher inhibition of IgE binding to TPO by EPX (78%) and IgE binding to EPX by TPO (52%) than in the AD or CSU pool alone. This suggests that, in both diseases, there is recognition of autoantigens; however, the increase in inhibition suggests that they may be attributed to different epitopes recognized as well as cross-reactivity.

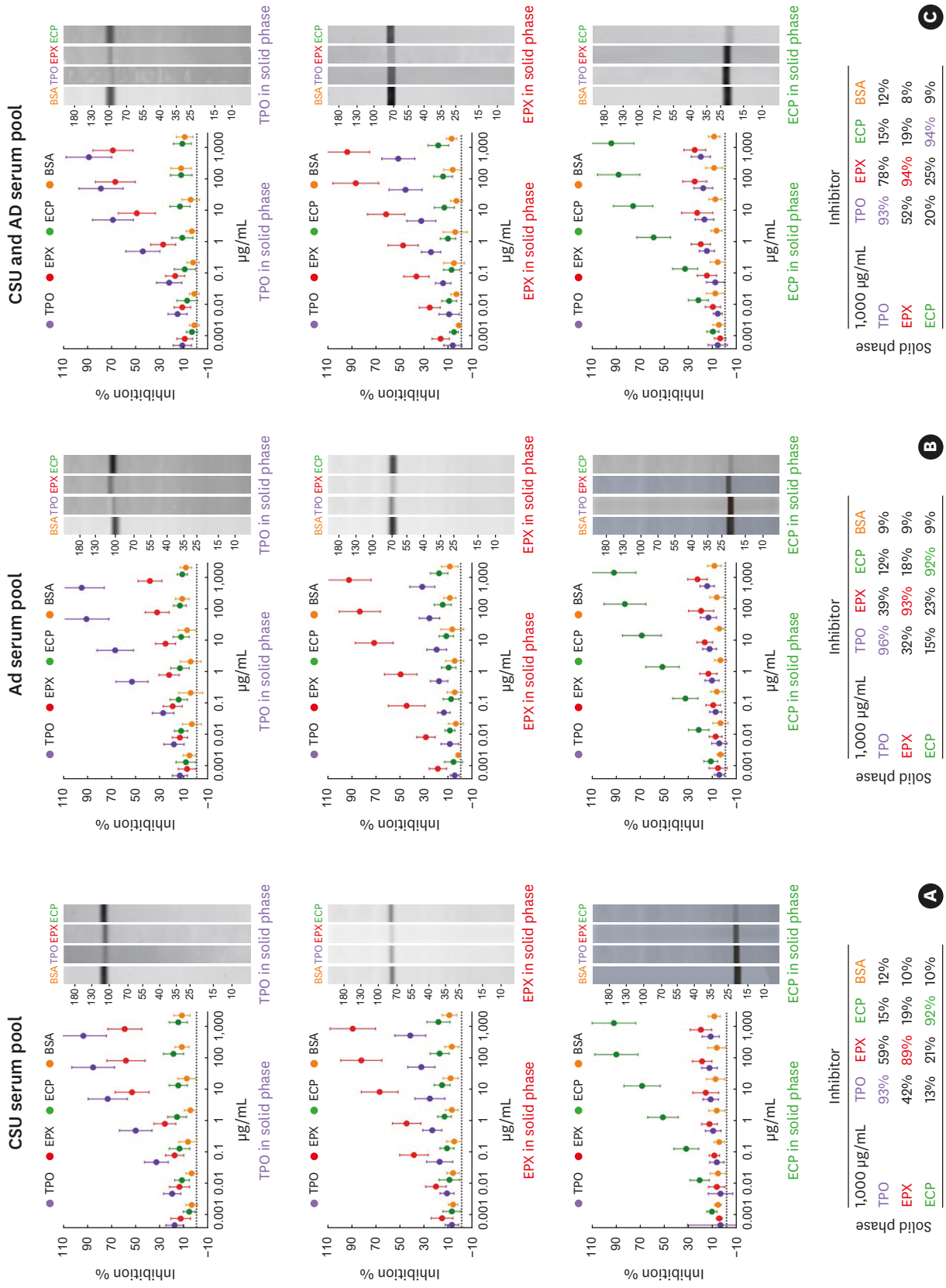

Fig. 2. Cross-reactivity among eosinophils and thyroid proteins. Enzyme-linked immunosorbent assay and immunoblotting test with CSU pool (A), AD pool (B) and CSU and AD pool (C).
CSU, chronic spontaneous urticaria; AD, atopic dermatitis; TPO, thyroid peroxidase; EPX, eosinophil peroxidase; ECP, eosinophil cationic protein.

Table 2. The matrix of identity among oxidases

Proteins		1	2	3	4	5
1	TPO					
2	MPO	0.44				
3	EPX	0.44	0.70			
4	LPO	0.42	0.53	0.53		
5	ECP	0.21	0.16	0.17	0.09	
6	Peroxidasin-like protein	0.33	0.37	0.38	0.35	0.14

Matrix of identity among oxidases from 0 (%) to 1 (100%). The percent sequence identity was obtain using the PRALINE server from IBIVU.
TPO, thyroid peroxidase; MPO, myeloperoxidase; EPX, eosinophil peroxidase; LPO, lactoperoxidase; ECP, eosinophil cationic protein.

The results of the immunoblotting inhibition tests were similar to those observed with the ELISA tests: ECP in the solid phase was almost completely inhibited with ECP, but remained virtually unchanged with TPO or EPX among the pool of sera from CSU and AD.

When EPX was in the solid phase, TPO decreased the reactivity to the EPX band, but when the TPO was in the solid phase, EPX more intensely decreased TPO. These results were observed in the CSU and AD groups; however, the decrease in intensity to TPO and EPX was greater in the CSU group.

Comparison of sequences by *in silico* analysis

The percent sequence identity according to the pairwise alignment was over 40% among EPX, TPO, LPO and MPO (**Table 2**). TPO shared an identity level of over 40% with MPO, EPX and LPO in their amino acid sequences. The lowest identity level was found with ECP (21%), as it does not belong to the peroxidase group. The highest sequence identity level was between MPO and EPX (70%).

Among the peroxidases, we observed several amino acids conserved and a sequence of 54 residues located in the TPO and EPX at positions between 381 to 441 and 369 to 422, respectively. The percent sequence identity of this patch among TPO and EPX was 59% (**Supplementary Fig. S1**).

TPO and EPX showed the best results with MPO (5mfa.1.A) as a template. The most conserved sequence was identified in the protein structure. We observed that this sequence forms a secondary structure of alpha helices on both proteins (2 for TPO and 3 for EPX), distributed inside and on the surface of the protein (**Fig. 3**). Within this antigenic patch, an epitope was identified using the ELIPRO server.

DISCUSSION

IgE responses to autoantigens have been demonstrated in several diseases, particularly in severe conditions.[38,39] Despite the fact that CSU and AD have a different pathogenesis,[38-42] multiple autoantigens have been reported in both diseases.[5,12,20,42,43] The release of EPX and ECP by eosinophils appears to play an important role in the inflammatory process,[44,45] and they have been used as *in vitro* parameters of inflammation in AD, urticaria and asthma.[45-49]

In this study, we described for the first time 1) IgE antibodies against eosinophil proteins (EPX and ECP) in patients with AD or CSU; 2) possible cross-reactivity between thyroid

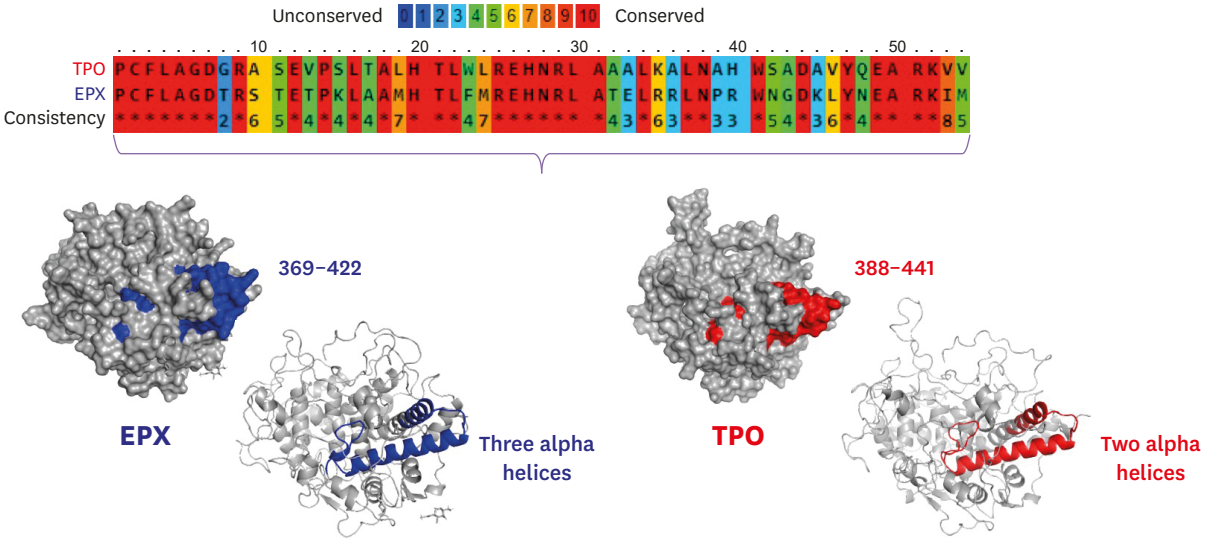

Fig. 3. Modeling of epitope distribution. Three alpha helices with 3 loops for EPX and 2 alpha helices with 2 loops for TPO were found. Sequence with the higher identity among EPX (369-422) and TPO (388-441). The percent sequence identity was 59%.
EPX, eosinophil peroxidase; TPO, thyroid peroxidase.

and EPX proteins (TPO and EPX, respectively); and 3) AD and CSU patients that identified different epitopes in TPO and EXP.

Unlike AD,[50] there have been few studies on CSU in which IgE to autoantigens has been detected.[51] Anti-TPO IgE has been most extensively studied[12]; however, IgE reactivity has also been described for cytokines, such as IL-24,[51] and even double-stranded DNA molecules.[52] Anti-TPO IgE has previously been described in patients with CSU, demonstrating that it could induce basophil activation.[14,37] The transfer of anti-TPO IgE was able to induce hives in healthy subjects.[13] In this study, some patients with AD and healthy subjects demonstrated anti-TPO IgE; however, a higher frequency of anti-TPO IgE was found in patients with urticaria, and it is unknown why TPO—an extracutaneous human protein—induced an IgE response in 20% to 40% of CSU patients.[12,13]

Allergens, such as plant profilins,[6] fungal manganese superoxide dismutase[7] and mites fatty acid binding proteins,[18] share IgE epitopes with human proteins in severe AD patients. A possible cross-reactivity between TPO and environmental proteins in CSU as in AD with certain autoantigens seems unlikely to occur. In a recent *in silico* study, the identity of TPO against 22 common allergens was evaluated. Although possible linear and conformational epitopes of TPO were identified, it appeared that these epitopes were not present among environmental allergens.[53] Studies by Bang *et al.*
[24] with autoimmune thyroid disease (ATD) patients demonstrated that anti-TPO IgG autoantibodies show discrete patterns of cross-reactivity to other peroxidases including MPO, which were later confirmed by Haapala, *et al.*[54] Epitopes recognized by auto-IgG in ATD and systemic vasculitis were different, suggesting a polyclonal anti-TPO response that varies according to diseases.[36,54]

According to the results of the inhibition tests, we found that CSU and AD patients generated autoantibodies with cross-reactivity to TPO and EPX. This was further supported by *in silico* analyses, where a large number of conserved amino acids were present among these peroxidases. Additionally, there was a correlation between the TPO and EPX levels. In CSU

patients, the TPO IgE-binding inhibition with EPX was higher than the EPX IgE-binding inhibition with TPO, suggesting that IgE sensitization to EPX precedes that to TPO. These results provide a possible explanation for the relationship between the thyroid and the skin in CSU patients. The following scenario seems to be likely from the immunological point of view: the high release of eosinophil granule proteins or neutrophil MPO during an inflammatory reaction[22,55,56] may induce a primary immune response involving stimulation of anti-EPX IgE production by plasma cells. The presence of EPX and TPO (and also MBP) might further boost the auto-IgE production by plasma cells due to their overlapping protein sequences promoting the development of epitope spreading among different proteins.[50,57]

The role of eosinophils in a type 2 classic model of allergic disease, such as AD, may not be expected. In contrast, little is known regarding this role of eosinophils in urticaria. Currently, researchers observed that eosinopenia in the blood was associated with the presentation of CSU and hypothesized that this is due to compartmental redistribution (recruitment into the skin during active disease).[10] However, this was only based on epidemiological observations. Here, we observed that eosinophils were maintained at the same levels as in healthy controls. The reason for this could be that although the level of eosinophils is high, the reactivity of IgE to eosinophil products leads us to suspect their possible fundamental role in the persistence of the disease.

Prospective investigations are necessary to identify which source induces the primary response; however, our results suggested that anti-peroxidase IgE response occurs initially through anti-EPX IgE sensitization and subsequently by cross-reactivity with TPO. We found 9 CSU patients with anti-TPO IgE and no anti-EPX IgE, which indicates that, in some cases, sensitization did not occur by cross-reactivity between peroxidases and that TPO could be a neoantigen. According to the *in silico* analysis, we observed that neutrophil MPO shared identity with TPO and EPX, which suggests that other peroxidases present in the skin could also have cross-reactivity with TPO and EPX.

In the AD group, all patients with anti-TPO IgE were co-sensitized to EPX, which supports that EPX is the primary sensitizer in this reaction. However, the percentages of TPO and EPX with IgE-binding inhibition were lower in the AD group than in the CSU group. This could be explained by a low concentration of the antibodies or a lower affinity for the antigen. Based on the detected optical density, the concentration of these antibodies in AD patients was similar to that in CSU patients; thus, the second hypothesis would be that a third protein (another peroxidase) could be the primary sensitizer (*e.g.*, MPO).

IgE against the 2 eosinophilic proteins was more frequent among patients with AD than those with CSU or the control group. This may be because although eosinophils also play an important role in CSU, skin infiltration by these cells and degranulation is higher in AD.[58] Despite EPX and ECP both being produced by eosinophils during the inflammatory response, there was no correlation between anti-ECP IgE and anti-EPX IgE levels, suggesting that although they are released by the same cell, the intensity of the IgE response is different. ECP did not inhibit IgE binding to EPX or TPO, possibly because it does not belong to the peroxidase family and cross-reactivity with these antigens is unlikely to occur.

In both AD and CSU, IgE response to self-antigens appears to be a process that does not occur at the beginning of the disease, but as a result of the inflammatory process. The majority of self-antigens in AD have an intracellular location, which is why some authors have

suggested that they are exposed to the extracellular medium where they can be recognized by autoantibodies as a consequence of chronic skin inflammation. In CSU, based on our hypothesis of cross-reactivity between TPOs and eosinophils, an initial inflammatory process would be necessary to induce the infiltration of eosinophils (and perhaps other cells), the release of peroxidases in the skin, and IgE antibody formation with subsequent cross-reactivity. However, in CSU, various autoimmune mechanisms have been elucidated, so the type of immunological response will vary among patients.

The *in silico* analysis showed that the proteins have a high degree of conservation. Certain epitopes have been reported in previous studies, and IgG-binding capacity has been evaluated in patients with thyroid diseases.[36,59-62] A section of 59 conserved residues was identified between TPO and EPX. In a previous study, we identified 3 possible antigenic patches located in the linear structure of TPO.[53] When comparing the sequence of the epitopes described with the most conserved sequence among the group of oxidases, we found that one of them is within it, suggesting that this epitope is possibly shared by this protein family. To our knowledge, this is the first study to report these associations with allergy diseases. Among the conserved sequence between TPO and EPX, an alpha helix fraction is in the intramolecular portion and is not exposed to the protein surface, suggesting that it could be a cryptoepitope and not normally exposed to the antibodies. Thus, performing inhibition assays of TPO and EPX using overlapping peptides to confirm that the role of these sequences is necessary within allergic and autoimmune diseases.

It was uncertain whether the recognized epitopes are the same or different between the 3 groups studied. However, in inhibition tests using a mixture of AD and CSU sera, we observed an increase in the IgE-binding inhibition of TPO and EPX, suggesting that there was likely recognition of different epitopes—if they had been the same, epitope saturation of the junction points would have occurred. When increasing the concentration of the solid phase proteins, there was no significant increase in the inhibition, indicating that the greater inhibition of the AD + CSU pool compared to the pool of each disease alone was not secondary to an increase in the antibodies or the availability of the binding points.

One weakness of the study was that we performed the inhibition tests under reduced conditions and with denatured proteins, which are better conditions for exploring linear epitopes. Despite the fact that anti-TPO IgE was detected under non-reduction conditions, *in silico* studies suggested that antigenic patches are linear among peroxidases with the highest probability of cross-reactivity.

In conclusion, we demonstrated that IgE autoantibodies against eosinophil proteins EPX and ECP were present in CSU and AD. The different frequencies of the anti-TPO IgE and anti-EPX IgE sensitization and inhibition between CSU and AD suggested that they did not share the same epitopes recognized by IgE autoantibodies. The IgE cross-reactivity between thyroid and EPX proteins could be a plausible explanation of the link between ATDs and CSU, but these could also be related to autoimmune comorbidities in patients with AD. In the future, evaluating the role of IgE antibodies against eosinophilic proteins in inducing the activation of basophils, mast cells and other cells of the inflammatory response would be of interest.

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SUPPLEMENTARY MATERIALS

Supplementary Data S1

Evaluated methods

Click here to view

Supplementary Table S1

Peroxidase used in the *in silico* analysis

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Supplementary Table S2

Quality parameters results of modeling by homology of TPO and EPX

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Supplementary Fig. S1

Sequence alignments among oxidases using the PRILINE server are shown. The total aligment score was 56,372 and the score per aligned residue pair was equal to 13.26. The percent sequence identity among the proteins was 50%. The thyroid peroxidase antigenic patches are marked with colored squares (blue, red and green).

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Supplementary Data S1. Evaluated methods

Thyroid peroxidase (TPO), eosinophil cationic protein (ECP) and eosinophil peroxidase (EPX) recombinant production

Escherichia coli strains BL21 (DE3) were transformed with the expression plasmid pET45b+/TPO, pET45b+/ECP and pET45b+/EPX, and cultured in Luria-Bertani medium containing 100 mg/L ampicillin at 37°C to 0.4 OD 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 rpm. 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 recombinants 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 were assessed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and subsequent staining with Coomassie Brilliant Blue R-250 (Bio-Rad Laboratories, Hercules, CA, USA).

Determination of total immunoglobulin E (IgE), specific IgE

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 anti-TPO IgE, anti-ECP IgE and anti-EPX IgE 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, anti-EPX IgE and anti-ECP IgE measure when IgG were found to be less than 10 μg/mL.

For specific IgE, micro-plates were incubated with 1 μg/100μL TPO, EPX or ECP per well over night (12 hours). After 5 washes with PBS, the wells were blocked with 100 μL of blocking buffer (1% bovine serum albumin [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 (OD). Absorbance at 405 nm was determined using spectrophotometer.

Considering a previous study, the cutoff value for serum specific IgE level to TPO was defined as the mean and 3-fold standard deviation of absorbance values from 60 healthy controls without urticaria or autoimmune diseases. [1] The same method was used for the the cutoff values for serum anti-EPX IgE and anti-ECP IgE.

ELISA inhibition assay

For ELISA, pools were adsorbed with increasing concentrations of inhibitors. After adsorption for 12 hours at 4°C, 100 μL was loaded into wells coated with the relevant antigen, and the plates were incubated overnight. Antibodies bound to the solid phase were measured by ELISA, and the results were expressed as the percentage of inhibition, calculated as follows: percent of inhibition = OD without inhibitor − OD with inhibitor/OD without inhibitor × 100. All assays included BSA as nonrelated inhibitors.

Immunoblotting

According to a previously published protocol with some modifications, recombinant proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (20 µg/lane). [2] Samples were loaded with 5%-b-mercaptoethanol for reducing conditions. Proteins were electro-transferred onto nitrocellulose membranes using buffer 25 mM Tris–HCl, 192 mM glycine, 20% MeOH (pH 8.3). Membranes were blocked with 3% BSA/PBS, washed with 0.1% Tween-PBS (TPBS) and incubated overnight with serum pool. Strips were washed and incubated with alkaline-phosphatase-labelled anti-IgE (1:400) in buffer 0.5% TPBS, 0.2% BSA and then washed with buffer 0.15 M NaCl 0.05 M Tris–HCl (pH 7.5). Allergenic components were revealed using freshly prepared p-nitro-blue-tetrazolium and 5-bromo-4-chloro-3-indoyl-phosphate dissolved in alkaline-phosphatase buffer (pH 9.5) during 5 min. For immunoblotting inhibition, strips were incubated with 2 ml of each pool, adsorbed (6 hours) with 100 µg/mL inhibitor. All assays included BSA as nonrelated control inhibitor and negative control serum. Estimation of molecular weight was done by calculating relative mobility according with the migration of known MW standards (ThermoFisher Scientific, Waltham, MA, USA).

***In silico* analysis**

The parameters to perform alignment were set up to use BLOSUM62 as the exchange matrix and conditional compositional score matrix adjustments. No filters or markings were used. The iterations used were 3 with an E-value of 0.01.[3] The scoring scheme worked from 0 for the least conserved alignment position, up to 10 for the most conserved alignment position. Based on previous observations,[4,5] we considered there was proper homology when the identity value was greater than 40%.

To identify the position of the most conserved sequences on the 3D structure, we used the PyMOL program. The experimental structures of TPO and EPX have not been solved; therefore, their structures were determined by homology using the Swiss Modeller server (https://swissmodel.expasy.org/). The quality of the models was analyzed by ProSA-web. The models were refined in Deep-View (energy minimization and rotamer replacements). Its quality was

evaluated by several tools, including the Ramachandran graphs, GMQE, QMEAN4 index and energy values (GROMOS96 force field). For epitope identification, we used the ELIPRO server.

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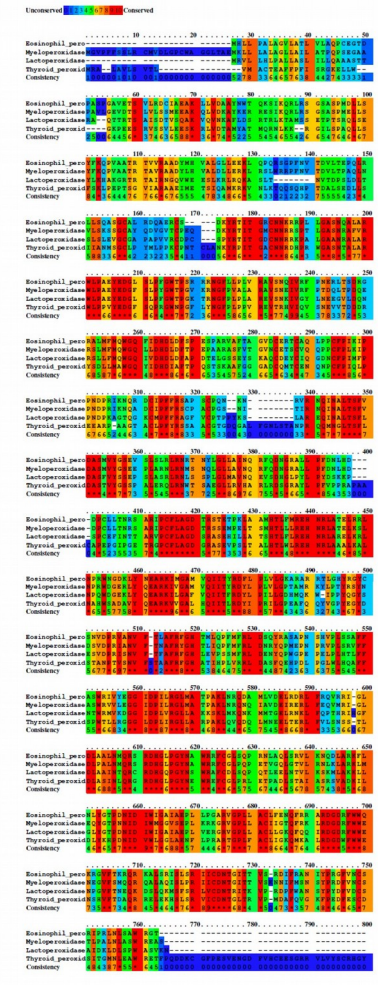
Supplementary Fig. S1. Sequence alignments among oxidases using the PRILINE server are shown. The total aligment score was 56,372 and the score per aligned residue pair was equal to 13.26. The percent sequence identity among the proteins was 50%. The thyroid peroxidase antigenic patches are marked with colored squares (blue, red and green).



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