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Functional characterization of complete and immunodominant epitopes of a novel pollen allergen from *Parthenium hysterophorus*

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Key words

Parthenium hysterophorus; allergic rhinitis; bronchial asthma; Skin Prick Test; basophil activation test.

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Impact statement

The allergenic epitopes deduced from 40 kDa pectin methylesterase allergenic protein of P. hysterophorus was found to induce prompt phenotypic responses by SPT and cellular immune responses comparable with that of the 40kDa allergenic protein and crude pollen extract.

Summary

Background. *Parthenium hysterophorus pollen induces chronic clinical conditions such as allergic rhinitis and bronchial asthma. Among the plethora of proteins in the pollens, only few were reported to induce allergy. Currently sensitization to P. hysterophorus pollen allergen is diagnosed by skin prick test (SPT) using the entire pollen extract instead of using the specific allergen.* ***Methods.*** *In P. hysterophorus sensitized patients, SPT was done using the crude pollen extract, 40kDa allergenic pollen protein and two commercially synthesized allergen epitopes (17 and 24) of P. hysterophorus. Dot-blot of allergen epitopes was done using P. hysterophorus sensitized sera. Crude pollen extract (1, 1.25, 2.5, 5 and 10µg/mL), 40kDa allergenic protein (3µg/mL), and allergen epitopes (3µg/mL) were used to perform Basophil Activation Test (BAT).* ***Results.*** *Crude pollen extract at 2.5, 5, 10 µg/mL and 40kDa allergenic protein at 3 µg/mL concentrations induced wheal and flare reaction by around 15 minutes, whereas commercially synthesized allergen epitopes at 3 µg/mL induced wheal and flare reactions in <10 minutes. Allergen epitopes (3µg/mL) revealed strong reactivity with sensitized patient's IgE in dot-blot analysis. Basophil activation Test using crude pollen extract (2.5, 5, 10 µg/mL), 40 kDa allergenic protein (3 µg/mL), and allergenic epitopes (3 µg/mL) indicated significant basophil activation (as measured by CD63 expression) in sensitized patients.* ***Conclusions.*** *The 40 kDa allergenic protein and its allergenic epitopes (17 & 24) induced phenotypic and cellular immune responses in P. hysterophorus sensitized individuals. The tested allergenic epitopes (17 and 24) induced faster wheal and flare reactions in comparison with the crude extract and the 40kDa allergenic protein. The novel 40kDa allergenic protein and its allergen epitopes identified here may be useful for the development of component-resolved diagnosis (CRD) while also serving as a potential therapeutic lead for desensitization treatment for P. hysterophorus pollen induced allergy.*

Introduction

Allergy is one of the leading illness, affecting more than 20% of the Indian population (1). Allergic rhinitis and asthma are the common and serious manifestations of allergy, causing considerable distress and burden by being chronic in nature, with remissions and relapses in the affected population but are rarely fatal (2). In the absence of specific treatment, palliative measures using epinephrine, antihistamines, and corticosteroids for symptom relief are usually offered to the patients during clinical exacerbations of allergy (3).

As a diagnostic procedure for allergies, skin prick test (SPT) is commonly used to confirm allergic sensitization to established allergens. Although, SPT is minimally invasive, economical, and provides immediate results (4, 5) some patients might develop anaphylactic reactions (6). The crude allergenic extracts used for SPT can lead to cross reactivity between related allergens. Besides, crude allergic extracts are heterogenous and contain undefined nonallergenic materials and contaminants (7). Batch to batch and manufacturer associated variations in the major and minor components of the allergens in the extracts used for SPT affect the sensitivity and specificity of the test. Variable responses are observed in patients based on their sensitization to different determinants, making precise standardization of methods essential for diagnosing clinical allergies (8, 9). Therefore, the use of well-standardized allergens is recommended for diagnosis. Improved standardization of allergens using allergen epitopes helps to discriminate between cross reactivity, enhancing the specificity of the diagnostic assay and to assess disease severity (10). In 2001, a project funded by the European Union, CREATE, introduced the idea of standardizing and optimizing allergenic extracts based on the content of the major allergens (11). The development of recombinant allergens has also contributed to the standardization of allergenic extracts for use in diagnosis (4). In India, the data on the specific allergens from the source is very sparse and dose dependent allergenic extracts are not commonly used in clinical practice for allergy diagnosis. In India, allergic respiratory disorders are common and pollen aeroallergens from various plant sources were implicated as etiologies (12). *P. hysterophorus*, a ubiquitous and invasive weed of global significance, is abundant in more than 30 countries. Though *P. hysterophorus* is not included in the panel of respiratory allergens routinely tested in Europe, it has been identified as the leading cause of allergic rhinitis and asthma in India, including Puducherry over the last three decades, reaching epidemic proportion (13). Earlier studies conducted on *P. hysterophorus* did not provide information on allergen concentration used for SPT and cell-specific immune response by basophil activation test (BAT). Therefore, in this study, *P. hysterophorus* pollen crude extract, 40kDa allergenic protein and its in-silico predicted allergen epitopes were subjected to in-vitro BAT and SPT to obtain quantitative and qualitative conclusions on allergen specific effector cell responses.

Materials and methods

Study subjects

Patients with allergic rhinitis fulfilling Allergic Rhinitis and its Impact on Asthma (ARIA) guidelines (14) and allergic asthma fulfilling Global initiative for Asthma (GINA) guidelines (15) and who tested positive to *P. hysterophorus* allergens by SPT, were enrolled from the Clinical Immunology, Otorhinolaryngology and Pulmonary Medicine outpatient clinics from 2014 to 2018 between May and September. A panel of 26 allergens (16 plant pollens, 3 fungal, 4 insects, 3 animal dander) were tested by skin prick test (SPT) as a part of routine diagnosis (**supplementary table I**) to identify the allergen specific sensitization in the patients. The patients who developed wheal and flare reaction (> 3 mm diameter) within 15 minutes after SPT were considered to be sensitized to the particular allergen. Histamine dihydrochloride (Sigma-Aldrich, USA) at 5 mg/mL and sterile PBS (Sigma-Aldrich, USA) were used as positive and negative controls respectively.

Patients with chronic obstructive pulmonary disease (COPD), autoimmune diseases such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), dermatomyositis, metabolic diseases such as diabetes mellitus, dyslipidemia, thyroid dysfunction, hypertension, diseases of the skin, such as psoriasis, vitiligo and those on long term immunosuppression drugs were excluded from the study. Healthy controls were individuals without any family history of chronic infections, allergic or autoimmune diseases.

After the initial screening, patients who tested allergic to *P. hysterophorus* by SPT were included after obtaining a written informed consent. Ten milliliters of peripheral venous blood were collected (5 mL in plain sterile vials, 5 mL in heparinized tubes). Heparinized blood sample was used for BAT. Serum was separated from the clotted blood and stored at -80 °C until further use. The study was approved by JIPMER Ethics Committee (Human Studies), Protocol No. JIP/IEC/2014/10/482 dated January 30, 2015. As a negative control for all the functional assays, heparinized blood sample and serum obtained from healthy individuals who tested negative by SPT to all the 26 allergens was used.

The total IgE level in the serum of *P. hysterophorus* sensitized patient was measured using the commercial IgE kit (N Latex IgE mono kit, Siemens, Germany) by Nephelometry (BN ProSpec® System, Siemens, Germany). Subjects with ≥ 100 IU/mL of total IgE were considered as "sensitized" while those tested < 100 IU/mL were considered as "unsensitized". The serum and heparinized blood samples thus obtained from SPT-sensitized patients with total IgE ≥ 100 IU/mL were subjected to functional analysis.

Characterization of specific allergenic protein from pollens of P. hysterophorus

The inflorescences from the *P. hysterophorus were collected from various locations in Puducherry between 2014-2018. From the inflorescence, pollen collection and extraction of pollen proteins were* performed following the published protocol (16). The pollen protein extract was lyophilized (ModulyoD Freeze Dryer, Thermo Scientific, USA) and stored at -80 °C until further use. When needed, the lyophilized pollen protein extract was reconstituted in sterile Milli-Q water, and its protein concentration was measured using a UV-visible spectrophotometer (Picodrop, PICOPET 01, UK). The proteins in the pollen extract were resolved on 12.5% SDS-PAGE and transferred onto a nitrocellulose membrane (Sigma Aldrich, USA) by semi-dry blot method (Trans-Blot SD Semi Dry Transfer Cell, Bio-Rad, USA). The unbound sites in the membrane were blocked using 5% bovine serum albumin (BSA). Following three washes using Phosphate Buffered Saline with Tween 20 (PBST), the membrane was incubated overnight at 4 °C, with the diluted serum (1:500) containing IgE from the sensitized patient. After washing the membrane was incubated at 37 °C for 3 hours with diluted anti-human IgE antibody HRP conjugate (1:500) (Abcam, USA) (17, 18). The membrane was incubated with Clarity Western peroxide reagent and Clarity Western Luminol/Enhancer reagent (Clarity Western ECL blotting substrate, Bio-Rad, USA). Images were acquired using the Chemi-Doc™ XRS⁺ system (Bio-Rad, USA).

After identifying the reactive allergenic pollen protein by immunoblotting, the protein was isolated from the SDS-PAGE gel by excising and protein stripping by cold acetone method (19). Protein precipitate was treated with cold acetone (1:4 v/v) and sample was incubated at -20 °C for 1 hour and centrifuged for 10 min at 10,000 g. Precipitated protein free of SDS was then dissolved in 500 µl of 1x PBS by vortexing and was subjected to ultra-performance liquid chromatography (UPLC) using the Acquity Ultra Performance LC system (Waters, USA) in the reversed phase mode and protein was separated on the Acquity UPLC BEH300 C4 column (Waters, USA). The concentration of the purified protein was quantified (Picodrop, PICOPET 01, UK) and was stored at 4 °C for further analysis. The amino acid sequence of the identified protein was analyzed using commercial service (Sandor proteomics, Hyderabad, India).

Allergen epitope identification

Immune epitope database (http://tools.immuneepitope.org/bcell/) and analysis resource tools were used to predict epitopes from the 40 kDa allergenic protein (20). Various immune epitope database tools were used to analyze peptide parameters such as solubility (Parker Hydrophilicity Prediction), flexibility (Karplus and Schulz flexibility scale), accessibility (Emini surface accessibility scale), Beta-turns (Chou and Fasman Beta-Turn prediction), antigenicity (Kolaskar and Tongaonkar antigenicity scale), and linear epitopes (Bepipred 1.0 and 2.0) (20-22). NetSurfP 2.0 server was used to predict the surface accessibility, and secondary structure of peptides (23). The peptides that exhibited high flexibility, hydrophilicity, antigenicity, and surface accessibility were selected as candidate molecules for further analysis. The total net charge of peptides and their binding potential (Boman index) was also calculated using the antimicrobial peptide database (https://aps.unmc.edu/prediction) (24). Based on the data derived from Immune Epitope Database tools, NetSurfP 2.0 server, and antimicrobial peptide database, two peptides (17 and 24) were selected.

In vitro peptide synthesis

The selected peptides were synthesized using a commercially available service ('S' BioChem company, Kerala, India) and the peptides were synthesized by Solid Phase Peptide Synthesis (SPPS) method using Specific Automated Peptide Synthesizer Autopep-001A (CS Bio, California). Briefly, 4-(2',4'-Dimethoxyphenyl-Fmoc-aminomethyl) phenoxy resin 100-200 mesh was used to provide a C-terminus free carboxyl group to the peptide. Deprotection of peptide was performed using 20% piperidine in dimethylformamide. The resin was removed by filtration and washed with hexane, dimethylformamide, chloroform, and methanol, and dried. The synthesized peptide was isolated from the solution using excess peroxide free pure cold diethyl ether (25, 26). After isolation, crude peptide was dissolved in 5% acetonitrile solution and purified using reverse phase HPLC on a RPC18 column (M/s Shimadzu Corporation, Japan). The molecular mass of the synthesized peptide was determined using ESI-MS (Waters, USA).

Dot blot analysis

The commercially synthesized peptides (allergen epitopes) were diluted from the stock to a final concentration of 3 µg/µL in sterile PBS and 10 µL of peptides were separately blotted onto the 0.2 µm nitrocellulose membranes (Sigma Aldrich, USA). The membranes were blocked using 5% BSA and then incubated at 4 °C for 2 hours with 10 mL of diluted serum (1:500) containing IgE from *P. hysterophorus* sensitized patient and then washed twice using 1x Tris-buffered saline with 0.1% Tween 20 detergent (TBST). The membranes were then incubated with anti-human IgE antibody (Abcam, USA) HRP conjugate (1:500) at 37 °C for 3 hours (27). Post washing with PBST, clarity western peroxide reagent and clarity western Luminol/Enhancer reagent was added to the membrane (Clarity Western ECL blotting substrate, Bio-Rad, USA) and images were acquired using ChemiDoc™ XRS⁺ system (Bio-Rad, USA). As a negative control, serum from an apparently healthy person, non-reactive by SPT was used.

Evaluation of reactivity of pollen allergenic extract, 40kDa allergenic protein and in vitro synthesized peptides using SPT

To optimize the diagnostic dose for SPT, the crude pollen extract (1, 1.25, 2.5, 5, 10 µg/mL), 40 kDa allergenic protein and aller-

gen epitopes at 3 µg/ml were used for SPT. The time taken to develop wheal and flare reactions for the respective test preparation was recorded and measured respectively and compared with the positive control (5 mg/mL Histamine dihydrochloride, Sigma Aldrich, USA).

Basophil degranulation test

Basophil degranulation assay was carried out using the Fast Immune™ CD63/CD123/Anti–HLA-DR reagent kit (BD Biosciences, California, USA). Variable concentrations of crude pollen extract, 40 kDa allergenic protein, and commercially synthesized peptides were used for the assay. In this exploratory study, we used 1, 1.25, 2.5, 5 and 10 µg/mL of crude pollen extract to perform SPT. A crude pollen extract of 2.5, 5 and 10 µg/mL was found to induce the wheal and flare reactions in sensitized individuals. Based on this observation, the minimal concentration of 3 µg/mL of 40 kDa protein and 3 µg/mL of allergen epitopes (17 and 24) was considered to be sufficient for SPT and basophil activation test. Heparinized blood samples were collected from patients tested positive by SPT to *P. hysterophorus* and healthy donors. Briefly, 100 µL of blood was mixed with 20 µL of basophil stimulation buffer 20 µL of crude pollen extract (1, 1.25, 2.5, 5 and 10 µg/mL), 40 kDa allergenic protein (3 µg/mL) and allergen epitopes (3 µg/mL) was added separately to the above tube and incubated at 37 °C in a water bath for 15 min. N-Formylmethionyl-leucyl-phenylalanine (fMLP) and basophil stimulation buffer (BSB) were used as positive and negative controls respectively. Degranulation was stopped by chilling the tubes with the addition of 1 mL of ice-cold PBS with 10 mmol/L EDTA on ice and were centrifuged for 5 min. The CD63 FITC/CD123 PE/Anti–HLA-DR PerCP antibody cocktail (20 µL) was added to each tube and incubated in the dark on ice for 20 min. Samples were then lysed using 1X BD FACS™ lysing solution at room temperature for 15 min and centrifuged. Supernatants were analyzed by BD FACS™ flow cytometer with a 488-nm laser to detect the $CD63^+$ basophils. Data was acquired with a threshold to eliminate most of $CD123^-$ cells and at least 500 $CD123^+$ cells were acquired per sample. Basophils were identified as low side scatter (SSC), $CD123^+$ and $HLA\text{-}DR^-$ cells. The quantitative determination of activated basophils was measured on CD63 FITC.

Statistical analysis

Descriptive variables are represented as mean and standard deviation (SD) or the median with interquartile range (IQR). Kruskal-Wallis test was used to compare the difference between the percentages of activated basophils in patients. A P-value < 0.05 was considered statistically significant.

Results

A total of 484 patients were screened in this study. Among them, only 18 patients (mean age 37.9 ± 13.5 years) tested reactive to *P. hysterophorous* allergens by SPT. Of these 18 patients, five (mean age 39.6 ± 10.5 years) had a history of direct exposure by virtue of their profession. In addition to *P. hysterophorus*, these five patients were also tested positive to *Ambrosia artemisiifolia* (short ragweed), *Casuarina equisetifolia,* and *Dermatophagoides pteronyssinus* or *Dermatophagoides farinae* aeroallergens and had elevated total serum IgE levels (> 100 IU/mL). Five individuals (mean age 32.8 ± 1.6 years) tested negative by SPT to all the 26 allergens, were recruited as healthy controls. The serum from the healthy control was used as negative control for all the downstream assays.

Prediction and in vitro synthesis of potential allergen epitopes from 40kDa allergenic protein of P. hysterophorus

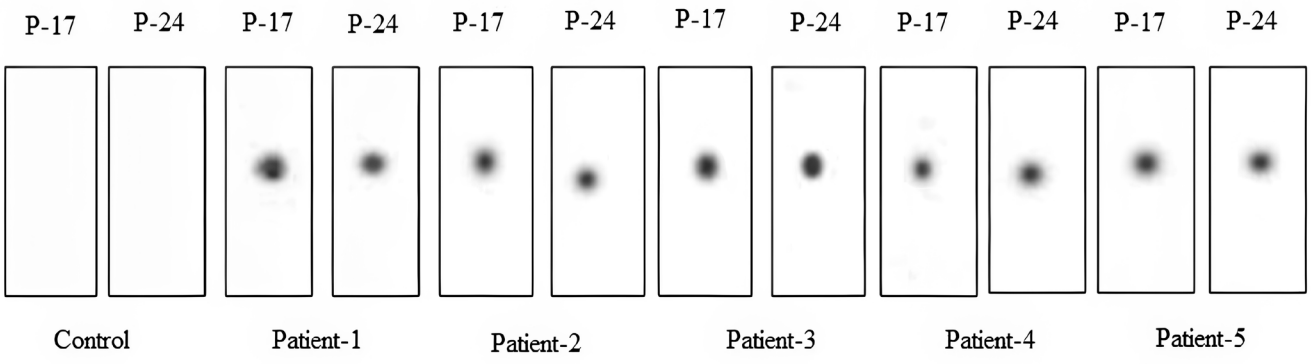
SDS-PAGE and immunoblotting analysis of *P. hysterophorus* pollen extract using sensitized sera revealed binding of IgE to a 40 kDa pollen protein. The 40 kDa protein was further characterized by amino acid sequencing as pectin methylesterase (data not shown). Using bioinformatic tools, a total of 48 peptide sequences from 40 kDa pectin methylesterase were identified. The peptides that exhibited high flexibility, hydrophilicity, antigenicity, and surface accessibility were selected. Peptides 17 and 24 fulfilled the required physicochemical features such as length, molecular weight, and protein binding potential to be considered as potential allergen epitopes (**supplementary figures 3-5**).

The predicted allergen epitopes were commercially synthesized. The matrix assisted laser desorption ionization-time of flight (MALDI-TOF) analysis of peptide-17 showed the ESI mass spectrum of 2.78e8 detected four charge states of the peptide: m/z 965.25 [M+2H]2+, m/z 482.98 [M+4H]4+, m/z 796.00 [M+5H]5+, m/z 663.50 [M+6H]6+ and the molecular mass of the peptide was found to be 1929.18 daltons (**supplementary figure 6A**). The HPLC analysis of the peptide showed a single sharp peak with a retention time of 7.626 min indicating a peptide with > 99% purity (**supplementary figure 6B**). The MALDI-TOF analysis of peptide-24 revealed the ESI mass spectrum of 5.72e7 detected four charge states of the peptide: m/z 1330.25 [M+2H]2+, m/z 665.70 [M+4H]4+, m/z 796.00 [M+5H]5+, m/z 663.50 [M+6H]6+ verifying a molecular mass of peptide to be 2660.12 daltons (**supplementary figure 7A**). The HPLC analysis of peptide-24 showed a single sharp peak with a retention time of 10.770 min representing peptide with > 99% purity (**supplementary figure 7B**).

Evaluation of the reactivity of the synthesized peptides with specific IgE

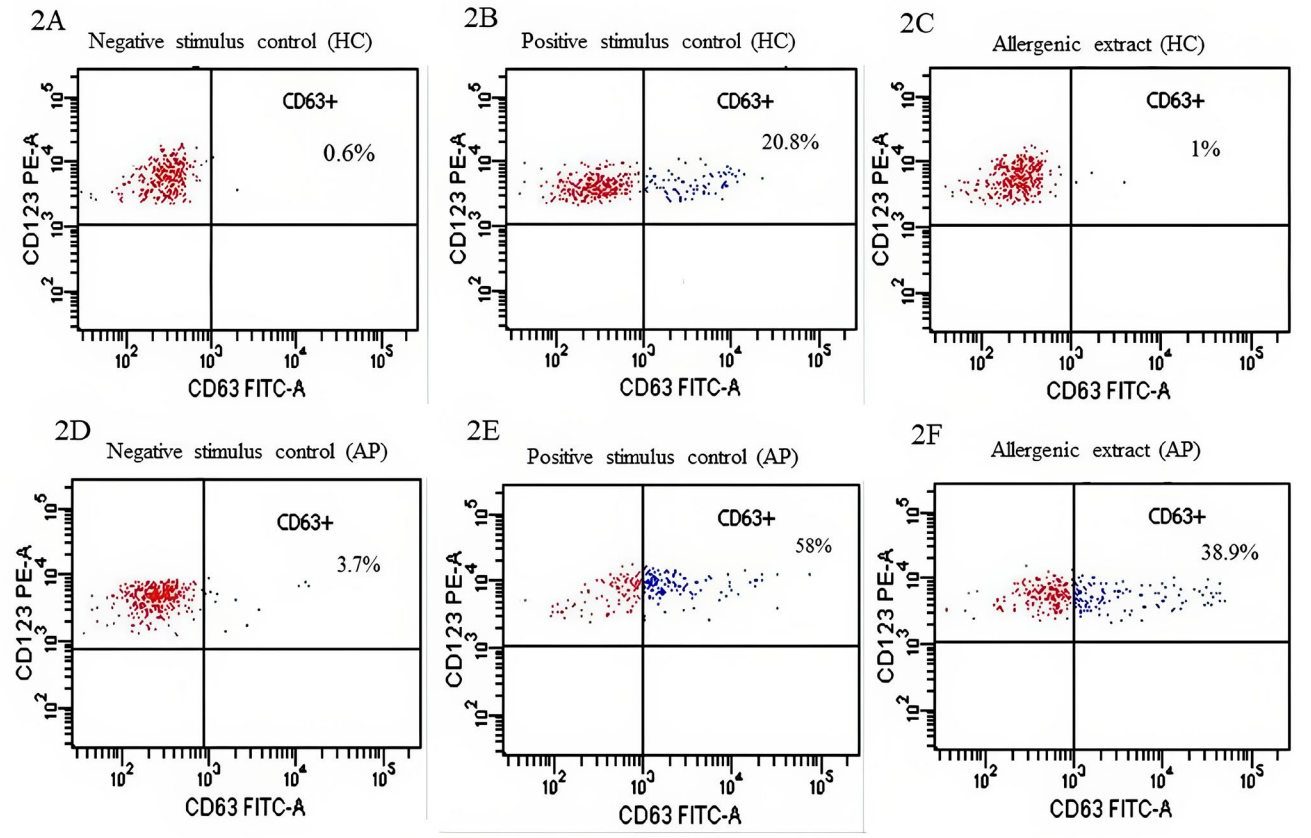
The *in vitro* synthesized peptides (17 and 24) were diluted from the stock to a final concentration of 3µg/µL and 10µL of peptides (17 and 24) blotted on nitrocellulose membranes. The *P. hysterophorus* sensitized patient's sera exhibited strong IgE (1:500) reactivity with the peptides (17 and 24) by dot-blot analysis, whereas no reaction was observed with the negative control (healthy individ-

Figure 1 *- Dot-blot analysis of peptide-17 and 24 (3 µg/mL) using P. hysterophorus sensitized sera (1:500).*



Control: Dot-blot analysis of peptide-17 (P-17) and peptide-24 (P-24) using a healthy control serum. Patient's-1-5: Dot-blot analysis of peptide-17 and 24 using *P. hysterophorus* sensitized patient sera of allergic rhinitis and bronchial asthma patients.

Figure 2 *- Effect of negative and positive stimulus controls and test (crude allergenic extract) on basophils of healthy control and allergic patients.*



(A-C) Percentage of activated basophils from healthy control stimulated with negative control (BSB: basophil stimulation buffer), positive control (fMLP: N-Formylmethionine-leucyl-phenylalanine), and test (allergenic extract: 2.5 µg/mL); **(D-F)** Percentage of activated basophils from allergic patient stimulated with negative control (BSB), positive control (fMLP) and test (allergenic extract: 2.5 µg/mL).

ual serum) which clearly indicates that peptides reacted with specific IgE of sensitized patients and the details are given in **figure 1**.

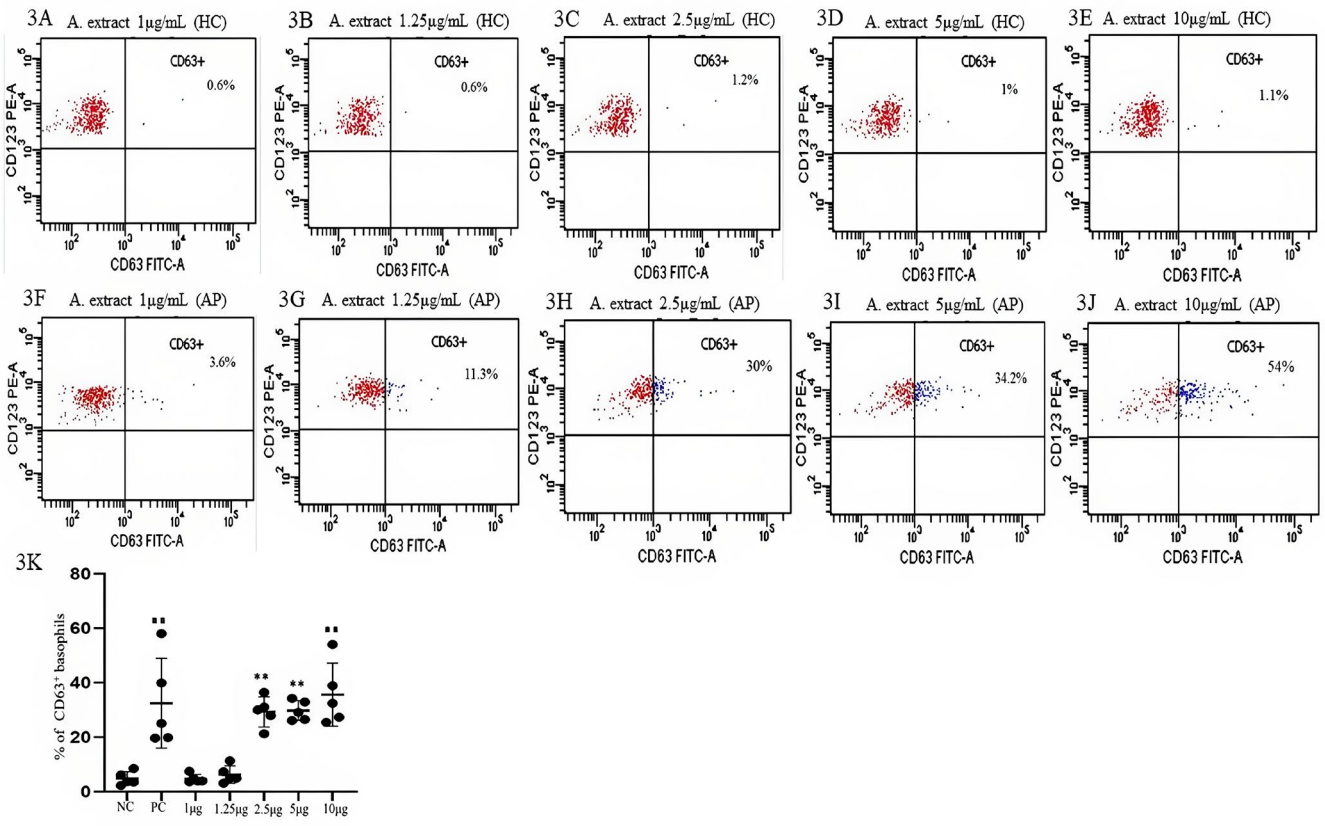
SPT reactivity of pollen crude extract, 40kDa allergenic protein and allergen epitopes

SPT performed in sensitized patients (n = 5), using different concentrations of (1, 1.25, 2.5, 5 and 10 µg/mL) antigenic extract, 40 kDa allergenic protein (3 µg/mL) and allergen epitopes 17 and 24 (3 µg/mL) showed that tested patients exhibited significant reactivity with the crude antigenic extract at concentration ranging from 2.5, 5 and 10 µg/mL. Sensitized patients also exhibited strong reactivity to 40 kDa allergenic protein and commercially synthesized allergen epitopes (3 µg/mL). In sensitized patients, strong wheal and flare reactions were observed within 10 minutes with the allergen epitopes, while wheal and flare reactions were observed after 15 minutes using the crude pollen extract and 40 kDa protein. The results of SPT carried out using crude antigenic extract, 40kDa allergenic protein and allergenic epitopes and their wheal and flare reactions are given in **supplementary table II**.

Basophil activation test

The gating strategy for basophils is described in **supplementary figures 1** and **2**. Effect of negative and positive stimulus controls and 2.5 µg/mL crude pollen extract on basophils of healthy control and allergic patient was tested. In sensitized patients a higher percentage of basophil activation ($CD63^+/CD123^+/HLA\text{-}DR^-$) was seen using positive control compared to healthy individuals (58% *vs* 20.8%) and with crude pollen extract at 2.5µg/mL concentration, 38.9% basophil activation was noted in sensitized patients compared to 1% in healthy controls (**figure 2A-F**). On testing different concentrations of crude pollen extract in controls no activation of basophils were noted (**figure 3 A-E**) while a dose dependent rise in the basophil activation was observed with 2.5 (30%, p = 0.05), 5 (34.2%, p = 0.05) and 10µg/µL (54%, p

Figure 3 *- Effect of 1, 1.25, 2.5, 5 and 10 µg/mL of P. hysterophorus pollen allergenic extract on basophils of healthy control and allergic patient.*



(A-E) Percentage of activated basophils of healthy control stimulated with 1, 1.25, 2.5, 5 and 10 µg/µL of allergenic extract; **(F-J)** Percentage of activated basophils of allergic patient stimulated with 1, 1.25, 2.5, 5 and 10 µg/µL of allergenic extract; **(K)** The percentage of activated basophils between negative, positive controls and different allergenic extract concentrations (1, 1.25, 2.5, 5 and 10 µg/mL) were and tested using Kruskal-Wallis test. $p < 0.05$ is considered significant.

= 0.05) concentrations in patients (**figure 3 F-J**). Likewise, the observed basophil activation frequency with 40kDa allergenic protein, allergenic epitopes (17&24) at 3µg/µL each was 47.4% (p = 0.05), 28% (p = 0.05) and 42.3% (p = 0.05) respectively in patients compared to controls (**figure 4**).

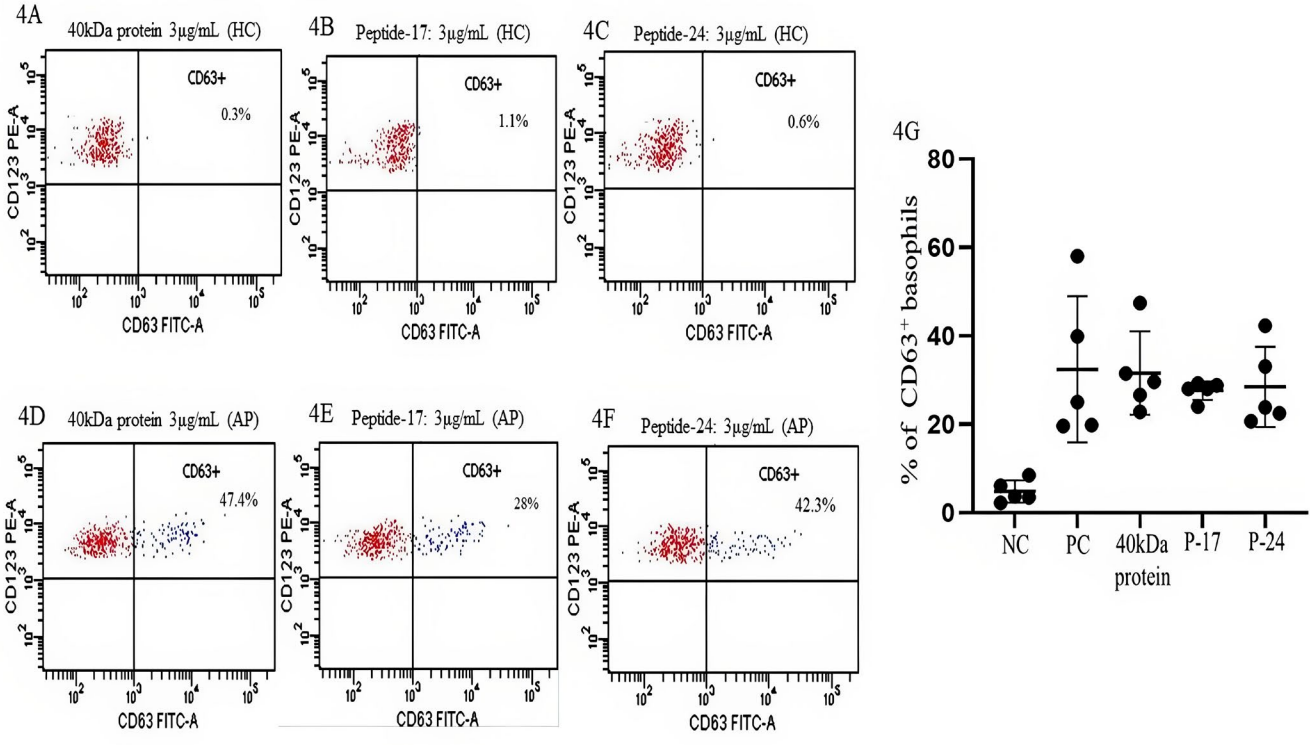
Discussion and conclusions

In this study, we assessed the immune response induced by the 40 kDa allergenic protein of *P. hysterophorus* pollen and two immunodominant allergenic epitopes identified from the 40 kDa allergen in allergic rhinitis and asthma patients.

Immunoblotting analysis of *P. hysterophorus* pollen protein extract using sensitized sera revealed 40 kDa protein to be allergenic and in silico studies revealed that it is a member of the pectin methylesterase family (data not shown). Pectin methylesterase family members from pollen and other sources have been reported to induce allergy (28, 29). Salamanca *et al.* reported a 37.4 kDa Ole e 11 as a pectin methylesterase from olive tree, had 57% and 54% similarity with pectin methylesterase of *Arabidopsis thaliana* and Sal k 1 of *Salsola kali* (Russian thistle) pollens respectively (28). Barderas *et al.* reported a 43 kDa pectin methylesterase from Russian thistle to be highly allergenic with significant sensitization rates in the Spanish population (30). Pectin methylesterase of Japanese hop pollen, has 23.2-50.2% of sequence similarities with Ole e 11, and Sal k 1 (31). These data highlight the importance of cross-reactive amino acids in pectin methylesterase family members and their impact in susceptible individuals which necessitates the characterization of clinically important allergen epitopes for accurate allergy diagnosis.

Defensins are antimicrobial glycoproteins, that plays a critical role in the plant immune system and have been reported to induce allergy (32, 33). A diverse number of defensins were reported in the members of the Asteraceae family members, especially from the *Artemisia sp., Ambrosia* sp., and *P. hysterophorus* (33). Par h 1 a defensin-polyproline-linked protein from *P. hysterophorus* has

Figure 4 *- Effect of 3 µg/mL of 40 kDa protein, peptide-17 and 24 on basophils of healthy control and allergic patient.*



(A-C) Percentage of activated basophils from healthy control stimulated with 3 µg/µL of 40 kDa protein and peptides (17 and 24); **(D-F)** Percentage of activated basophils from allergic patient stimulated with 3 µg/µL of 40 kDa protein and allergen epitopes (17 and 24); **(G)** The percentage of activated basophils between negative, positive controls and 40kDa allergenic protein and allergen epitopes (17 and 24) were tested using Kruskal-Wallis test. $p < 0.05$ is considered significant.

high sequence similarity with Amb a 4 and Art v 1, defensins of *Ambrosia artemisiifolia* and *Artemisia vulgaris* respectively (33). In *P. hysterophorus* Gupta *et al*. identified 28, 31, and 45 kDa proteins of which only 31 kDa Par h I was allergenic based on their reactivity to sera from patients with allergic rhinitis and bronchial asthma (17). Our findings are different to the above studies, and it could be due to differences in the geographical regions and the climate/environment induced changes in pollen protein composition. Pollen protein component variations collected from variable geographic regions in India and differences in their ability to cause disease severity has already been reported (34-36). Although all the *P. hysterophorus* sensitized patients were polysensitized to other aeroallergens, resource limitations precluded us from evaluating the cross-reactivity of *P. hysterophorus* specific pectin methylesterase with the other allergens. Nevertheless, specific 40 kDa allergenic protein characterized in this study, may be useful for in-vitro and *in vivo* diagnosis of *P. hysterophorus* induced allergy in future.

Immune epitope prediction database tools were used to predict the specific IgE binding epitopes from the 40 kDa protein. A total of 48 peptides were predicted out of which only two peptides (17 and 24), possessed the required criteria such as length, molecular weight, and protein binding potential. The selected peptides were also shown to form the alpha-helical structure. Hence, these two peptides were used for downstream SPT and cellular assays.

Using immune epitope database tools, Carrera *et al.* had identified the B-cell epitopes from the major fish allergens beta parvalbumins (37). Using BepiPred 1.0, Chen *et al*. reported seven B-cell epitopes from the major cockroach allergens Per a 6 of *Periplaneta americana* and Bla g 6 of *Blattella germanica* (38). Similarly, three B-cell IgE binding epitopes were identified from the osmotin protein of tobacco (*Nicotiana tabacum*). The B-cell epitopes of osmotin displayed higher reactivity with allergen-specific IgE by dot-blot analysis (39). Molecular analysis of sesame allergen, 14 kDa β-globulin revealed two IgE binding epitopes, which exhibited strong reactivity in dot-blot analysis using sensitized patient sera (40). T and B cell epitopes of pectin methylesterase from Russian thistle were predicted using immunoinformatic tools. Molecular docking studies of Sal k 1 with MHC-II identified Sal k 1 as a promising molecule for allergen specific immunotherapy as it revealed strong and stable interactions (41). From the major Sal k 1 allergen, two isoforms Sal k 4.03 and Sal k 4.02 were identified using immunoinformatic tools. IgE binding assay of these isoforms revealed that the Sal k 4.03 bound better to specific IgE than Sal k 4.02, indicating a hypoallergenic nature useful to devise desensitization therapy (42).

In our study, immunoblotting assays confirmed that the sensitized patient's IgE specifically reacted with the 40 kDa allergen. Dot-blot assay using allergen epitopes (17 and 24) displayed stronger binding with the IgE of *P. hysterophorus* sensitized individuals. Our study results are in parallel with the above reports of IgE binding epitope identification and characterization.

In our study, varied concentrations of *P. hysterophorus* pollen extract (1, 1.25, 2.5, 5, and 10 µg/mL) induced strong wheal and flare reactions in sensitized patients by SPT. In contrast, a 40 kDa allergenic protein and allergenic epitopes elicited skin reactions at a standard concentration of 3 µg/mL. However, the time to develop wheal and flare reactions was slightly different. While SPT was performed using allergen epitopes (17 and 24), we observed development of wheal and flare reaction within 10 minutes. However, a delay in the development of responses by 5 minutes was observed when the crude pollen extract and purified allergenic 40 kDa protein was used. Peeters *et al*. studied the effect of peanut-specific purified allergens (Ara h 1, Ara h 2, Ara h 3, and Ara h 6) in eliciting skin reactions by SPT. It was shown that the sensitized patients with severe symptoms developed significant reactions with the low concentrations (0.1 µg/mL) of Ara h 2 and Ara h 6 and with higher concentrations of Ara h 1 and Ara h 3 (100 µg/mL) (43). In our study, we found that 40 kDa allergenic protein and allergen epitopes induced the visible skin reaction at 3 µg/mL, a slightly higher concentration. The salient finding of our study is the faster immune response elicited by the allergen epitopes compared to the 40 kDa protein. This could be due to instantaneous recognition of the allergenic epitopes by high affinity allergen-specific IgE in the sensitized patients and activation of allergen-specific mast cells (44). Ebo *et al*. reported that purified Mal d 1 (a major apple allergen) could activate basophils even at 1 µg/ml (45). Likewise, a marked increase in the percentage of CD63 expressing basophils was reported using 1µg/mL of wasp recombinant allergens (Ves v 1, Ves v 2, Ves v 3, and Ves v 5) (46). In our study, the allergenic crude extract at lower concentrations failed to induce basophil activation, while at higher concentrations, a dose-dependent increase in the activation of basophils was noted. Compared to the crude extract, a significantly higher percentage of basophils were activated by 40 kDa allergenic protein and allergenic epitopes (17 and 24) in sensitized individuals. Although the above studies have reported basophil activation with lower concentrations, we did not carry out the basophil activation assays using variable concentrations which is a limitation of our study. Resource limitations and lack of data from the published literature precluded us from using variable concentrations of 40 kDa allergenic protein and allergen epitopes (17 and 24) of *P. hysterophorus* to analyze their use in *in vivo* and *in vitro* assays. Therefore, we used a standard concentration of 3 µg/mL of 40 kDa allergenic protein and allergenic epitopes. However, 3 µg/mL of 40 kDa allergenic protein and allergenic epitopes significantly induced phenotypic and cellular immune responses. Future studies would help to optimize the minimum concentration of these molecules required for activating basophils as well as diagnosis of *P. hysterophorus* pollen allergy.

Although there are reports of other protein components in the *P. hysterophorus* pollen being allergenic from various geographi-

cal locations in India (17, 47), we for the first-time report that a 40 kDa Pectin methylesterase protein induced allergic responses among patients from Puducherry. Both the complete allergen and the predicted epitopes were tested to elicit cellular and phenotypic responses in sensitized individuals indicating its enhanced specificity. As these predicted allergen epitopes are specific and unique for the allergen, use of them for diagnosis would negate the cross reactivity with the similar allergens to a greater extent. However, we could not perform further *in silico* and *in vitro* studies to assess its cross reactivity with the other allergens and test its use to diagnose *P. hysterophorus* pollen sensitization. Also assessing cellular and phenotypic responses by using various concentrations would have helped us to arrive at the effective concentrations to be used for the effector functional studies. A major limitation of this study is its smaller sample size and hence these data should be validated in a larger cohort to confirm its clinical utility. Likewise, provocation studies and chemical modification of the peptides in future, might help to identify and develop allergen epitopes with poor avidity to IgE, which could be tested for its use in desensitization therapy to treat the sensitized patients thereby reducing their allergic symptoms, anaphylaxis related complications, and associated costs to the individual and society in future. In summary, the 40 kDa allergenic protein and its allergenic epitopes (17 and 24) were demonstrated to induce phenotypic and cellular immune responses in *P. hysterophorus* sensitized individuals. The allergenic epitopes identified here may also be tested in a larger cohort for validating its use in the rapid diagnosis of *P. hysterophorus* pollen induced allergy.

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Contributions

VSN, SRB, MPA, CMM: conceptualization. VSN: resources. SRB: investigation. SRB, VSN, MMT: data curation. BNRM, KV, SNB: data curation, formal analysis. SRB, TM: formal analysis. CMM, MMT, TM, MPA, VSN: writing – review & editing. SRB, CMM: writing – original draft. VSN: funding acquisition.

Conflict of interests

The authors declare that they have no conflict of interests.

References

1. Krishna MT, Mahesh PA, Vedanthan PK, Mehta V, Moitra S, Christopher DJ. The burden of allergic diseases in the Indian subcontinent: barriers and challenges. Lancet Glob Health. 2020;8(4):e478-9. doi: 10.1016/S2214-109X(20)30061-9.
2. Kim H, Bouchard J, Renzi PM. The link between allergic rhinitis and asthma: a role for antileukotrienes? Can Respir J. 2008;15(2):91-8. doi: 10.1155/2008/416095.
3. Rowe BH, Edmonds ML, Spooner CH, Diner B, Camargo CA Jr. Corticosteroid therapy for acute asthma. Respir Med. 2004;98(4):275-84. doi: 10.1016/j.rmed.2003.11.016.
4. Jeong KY, Hong CS, Lee JS, Park JW. Optimization of allergen standardization. Yonsei Med J. 2011;52(3):393-400. doi: 10.3349/ymj.2011.52.3.393.
5. Heinzerling L, Frew AJ, Bindslev-Jensen C, Bonini S, Bousquet J, Bresciani M, et al. Standard skin prick testing and sensitization to inhalant allergens across Europe--a survey from the GALEN network. Allergy. 2005;60(10):1287-300. doi: 10.1111/j.1398-9995.2005.00895.x.
6. Lockey RF, Benedict LM, Turkeltaub PC, Bukantz SC. Fatalities from immunotherapy (IT) and skin testing (ST). J Allergy Clin Immunol. 1987;79(4):660-77. doi: 10.1016/s0091-6749(87)80164-1.
7. Valenta R, Karaulov A, Niederberger V, Zhernov Y, Elisyutina O, Campana R, et al. Allergen Extracts for In Vivo Diagnosis and Treatment of Allergy: Is There a Future? J Allergy Clin Immunol Pract. 2018;6(6):1845-55.e2. doi: 10.1016/j.jaip.2018.08.032.
8. Dreborg S, Einarsson R. The major allergen content of allergenic preparations reflect their biological activity. Allergy. 1992;47(4 Pt 2):418-23. doi: 10.1111/j.1398-9995.1992.tb02082.x.
9. van Ree R. Indoor allergens: relevance of major allergen measurements and standardization. J Allergy Clin Immunol. 2007;119(2):270-7; quiz 278-9. doi: 10.1016/j.jaci.2006.10.033.
10. Scheurer S. Improvement of the diagnosis of allergy by using purified allergens. Clin Exp Allergy. 2006;36(12):1483-6. doi: 10.1111/j.1365-2222.2006.02597.x.
11. van Ree R, CREATE Partnership. The CREATE project: EU support for the improvement of allergen standardization in Europe. Allergy. 2004;59(6):571-4. doi: 10.1111/j.1398-9995.2004.00499.x.
12. Singh AB, Shahi S. Aeroallergens in clinical practice of allergy in India- ARIA Asia Pacific Workshop report. Asian Pac J Allergy Immunol. 2008;26(4):245-56.
13. Lakshmi C, Srinivas C. Parthenium the terminator: An update. Indian Dermatol Online J. 2012;3(2):89-100. doi: 10.4103/2229-5178.96698.
14. Bousquet J, Khaltaev N, Cruz AA, Denburg J, Fokkens WJ, Togias A, et al. Allergic Rhinitis and its Impact on Asthma (ARIA) 2008 update (in collaboration with the World Health Organization, GA(2)LEN and AllerGen). Allergy. 2008;63 Suppl 86:8-160. doi: 10.1111/j.1398-9995.2007.01620.x.
15. Reddel HK, Bacharier LB, Bateman ED, Brightling CE, Brusselle GG, Buhl R, et al. Global Initiative for Asthma Strategy 2021: Executive Summary and Rationale for Key Changes. Am J Respir Crit Care Med. 2022;205(1):17-35. doi: 10.1164/rccm.202109-2205PP.
16. Singh AB, Dahiya P. Antigenic and allergenic properties of Amaranthus Spinosus pollen--a commonly growing weed in India. Ann Agric Environ Med. 2002;9(2):147-51.
17. Gupta N, Martin BM, Metcalfe DD, Rao PVS. Identification of a novel hydroxyproline-rich glycoprotein as the major allergen in Parthenium pollen. J Allergy Clin Immunol. 1996;98(5):903-12. doi: 10.1016/s0091-6749(96)80006-6.
18. Ghosh D, Mueller GA, Schramm G, Edwards LL, Petersen A, London RE, et al. Primary Identification, Biochemical Characterization, and Immunologic Properties of the Allergenic Pollen Cyclo-

philin Cat r 1. J Biol Chem. 2014;289(31):21374-85. doi: 10.1074/jbc.M114.559971.
19. Niu L, Zhang H, Wu Z, Wang Y, Liu H, Wu X, et al. Modified TCA/acetone precipitation of plant proteins for proteomic analysis. PLoS One. 2018;13(12):e0202238. doi: 10.1371/journal.pone.0202238.
20. Fleri W, Vaughan K, Salimi N, Vita R, Peters B, Sette A. The Immune Epitope Database: How Data Are Entered and Retrieved. J Immunol Res. 2017;2017:5974574. doi: 10.1155/2017/5974574.
21. Dhanda SK, Mahajan S, Paul S, Yan Z, Kim H, Jespersen MC, et al. IEDB-AR: immune epitope database-analysis resource in 2019. Nucleic Acids Res. 2019;47(W1):W502-6. doi: 10.1093/nar/gkz452.
22. Kim Y, Ponomarenko J, Zhu Z, Tamang D, Wang P, Greenbaum J, et al. Immune epitope database analysis resource. Nucleic Acids Res. 2012;40(Web Server issue):W525-30. doi: 10.1093/nar/gks438.
23. Klausen MS, Jespersen MC, Nielsen H, Jensen KK, Jurtz VI, Sønderby CK, et al. NetSurfP-2.0: Improved prediction of protein structural features by integrated deep learning. Proteins. 2019;87(6):520-7. doi: 10.1002/prot.25674.
24. Ravichandran G, Kumaresan V, Bhatt P, Arasu MV, Al-Dhabi NA, Arockiaraj J. A Cumulative Strategy to Predict and Characterize Antimicrobial Peptides (AMPs) from Protein Database. Int J Pept Res Ther. 2017;23(2):281-90.
25. Selvam R, Sudha E, Rajkumar PR, Subashchandran KP. Synthesis of biologically important neutral amylo-β peptide by using improved Fmoc solid-phase peptide synthetic strategy. J Chem Biol. 2015;8(2):61-6. doi: 10.1007/s12154-015-0128-2.
26. Selvam R, Subashchandran KP. Synthesis of Biologically Active Hydrophobic Peptide by Using Novel Polymer Support: Improved Fmoc Solid Phase Methodology. Int J Pept Res Ther. 2015;21(1):91-7.
27. Pang SL, Ho KL, Waterman J, Rambo RP, Teh AH, Mathavan I, et al. Crystal structure and epitope analysis of house dust mite allergen Der f 21. Sci Rep. 2019;9(1):4933. doi: 10.1038/s41598-019-40879-x.
28. Salamanca G, Rodríguez R, Quiralte J, Moreno C, Pascual CY, Barber D, et al. Pectin methylesterases of pollen tissue, a major allergen in olive tree. FEBS J. 2010;277(13):2729-39. doi: 10.1111/j.1742-4658.2010.07689.x.
29. Palazzo P, Tuppo L, Giangrieco I, Bernardi ML, Rafaiani C, Crescenzo R, et al. Prevalence and peculiarities of IgE reactivity to kiwifruit pectin methylesterase and its inhibitor, Act d 7 and Act d 6, in subjects allergic to kiwifruit. Food Research International. 2013;53(1):24-30.
30. Barderas R, García-Sellés J, Salamanca G, Colás C, Barber D, Rodríguez R, et al. A pectin methylesterase as an allergenic marker for the sensitization to Russian thistle (Salsola kali) pollen. Clin Exp Allergy. 2007;37(7):1111-9. doi: 10.1111/j.1365-2222.2007.02744.x.
31. Jang SW, Jeong KY, Yuk JE, Lee J, Park KH, Park JW. Allergen Homologues, Pathogenesis-Related 1, Polygalacturonase, and Pectin Methyl Esterase from a Japanese Hop. Protein Pept Lett. 2021;28(4):362-71. doi: 10.2174/0929866527666200813201924.
32. Lay FT, Anderson MA. Defensins--components of the innate immune system in plants. Curr Protein Pept Sci. 2005;6(1):85-101. doi: 10.2174/1389203053027575.
33. Pablos I, Eichhorn S, Machado Y, Briza P, Neunkirchner A, Jahn-Schmid B, et al. Distinct epitope structures of defensin-like proteins linked to proline-rich regions give rise to differences in their allergenic activity. Allergy. 2018;73(2):431-41. doi: 10.1111/all.13298.
34. Emberlin J, Detandt M, Gehrig R, Jaeger S, Nolard N, Rantio-Lehtimäki A. Responses in the start of Betula (birch) pollen seasons to recent changes in spring temperatures across Europe. Int J Biometeorol. 2002;46(4):159-70. doi: 10.1007/s00484-002-0139-x.
35. Galán C, García-Mozo H, Vázquez L, Ruiz L, de la Guardia CD, Trigo MM. Heat requirement for the onset of the Olea europaea L. pollen season in several sites in Andalusia and the effect of the expected future climate change. Int J Biometeorol. 2005;49(3):184-8. doi: 10.1007/s00484-004-0223-5.
36. Rasmussen A. The effects of climate change on the birch pollen season in Denmark. Aerobiologia. 2002;18(3):253-65.
37. Carrera M, González-Fernández Á, Magadán S, Mateos J, Pedrós L, Medina I, et al. Molecular characterization of B-cell epitopes for the major fish allergen, parvalbumin, by shotgun proteomics, protein-based bioinformatics and IgE-reactive approaches. J Proteomics. 2019;200:123-33. doi: 10.1016/j.jprot.2019.04.005.
38. Chen H, Yang HW, Wei JF, Tao AL. In silico prediction of the T-cell and IgE-binding epitopes of Per a 6 and Bla g 6 allergens in cockroaches. Mol Med Rep. 2014;10(4):2130-6. doi: 10.3892/mmr.2014.2399.
39. Sharma P, Gaur SN, Arora N. In silico identification of IgE-binding epitopes of osmotin protein. PLoS One. 2013;8(1):e54755. doi: 10.1371/journal.pone.0054755.
40. Wolff N, Yannai S, Karin N, Levy Y, Reifen R, Dalal I, et al. Identification and characterization of linear B-cell epitopes of beta-globulin, a major allergen of sesame seeds. J Allergy Clin Immunol. 2004;114(5):1151-8. doi: 10.1016/j.jaci.2004.07.038.
41. Shams MH, Sohrabi SM, Jafari R, Sheikhian A, Motedayyen H, Baharvand PA, et al. Designing a T-cell epitope-based vaccine using in silico approaches against the Sal k 1 allergen of Salsola kali plant. Sci Rep. 2024;14(1):5040. doi: 10.1038/s41598-024-55788-x.
42. Mas S, Barderas R, Colás C, Quiralte J, Rodríguez R, Villalba M. The natural profilin from Russian thistle (Salsola kali) contains a low IgE-binding ability isoform--molecular and immunological characterization. FEBS J. 2012;279(23):4338-49. doi: 10.1111/febs.12024.
43. Peeters K a. BM, Koppelman SJ, van Hoffen E, van der Tas CWH, den Hartog Jager CF, Penninks AH, et al. Does skin prick test reactivity to purified allergens correlate with clinical severity of peanut allergy? Clin Exp Allergy. 2007;37(1):108-15. doi: 10.1111/j.1365-2222.2006.02628.x.
44. Narayanan M, Freidl R, Focke-Tejkl M, Baranyi U, Wekerle T, Valenta R, et al. A B Cell Epitope Peptide Derived from the Major Grass Pollen Allergen Phl p 1 Boosts Allergen-Specific Secondary Antibody Responses without Allergen-Specific T Cell Help. J Immunol. 2017;198(4):1685-95. doi: 10.4049/jimmunol.1501741.
45. Ebo DG, Hagendorens MM, Bridts CH, Schuerwegh AJ, De Clerck LS, Stevens WJ. Flow cytometric analysis of in vitro activated basophils, specific IgE and skin tests in the diagnosis of pollen-associated food allergy. Cytometry B Clin Cytom. 2005;64(1):28-33. doi: 10.1002/cyto.b.20042.
46. Balzer L, Pennino D, Blank S, Seismann H, Darsow U, Schnedler M, et al. Basophil Activation Test Using Recombinant Allergens: Highly Specific Diagnostic Method Complementing Routine Tests in Wasp Venom Allergy. PLoS One. 2014;9(10):e108619. doi: 10.1371/journal.pone.0108619.
47. Pablos I, Eichhorn S, Briza P, Asam C, Gartner U, Wolf M, et al. Proteomic profiling of the weed feverfew, a neglected pollen allergen source. Sci Rep. 2017;7(1):6049. doi: 10.1038/s41598-017-06213-z.