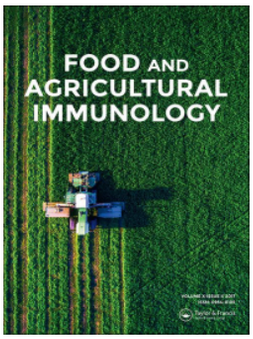

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Cluster analysis of allergen reagents in atopic dermatitis patients according to the specific IgE results in ALEX2 Allergy Explorer test

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ABSTRACT

The aim of this study is the Cluster analysis of specific IgE results in ALEX2 Allergy Explorer test in atopic dermatitis (AD) patients. The complete dermatological and allergological examination including the examination of the sensitisation to allergen reagents with ALEX2 Allergy Explorer testing was performed. The cluster analysis (silhouette value) was processed for results of specific IgE and for clinical parameters. Altogether 100 atopic dermatitis patients were examined – 48 men, 52 women, the average age 40.9 years. Although the distribution of specific IgE results corresponds to the division into protein families, the links between specific IgE to allergens reagents in clusters are weak. Our results strongly point towards cross-reactivity for crustacean-allergic patients to desert locust, house cricket and stable flies. Combining the results of specific IgE for the basic allergen reagents together with the results of specific IgE for less common allergens can help in the estimating of allergic reactions.

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KEYWORDS
Cluster analysis – silhouette; atopic dermatitis; molecular components; multiplex ALEX2 Allergy Explorer testing; protein families; biochemical structure

Introduction

The cluster analysis is commonly used to group similar samples across a diverse range of applications (Frades & Matthiesen, 2010; Kaufman & Rousseeuw, 1990; Rousseeuw, 1987); there is no prior group or cluster membership information for any of the objects in this analysis. The goal of clustering is to form groups of samples that are more similar to each other than to samples in other groups. Silhouette width is a widely used index for assessing the fit of individual objects in the classification, as well as the quality of clusters and the entire classification (Frades & Matthiesen, 2010; Kaufman & Rousseeuw, 1990; Rousseeuw, 1987). In our study, the cluster analysis is used to evaluate the results of specific IgE to allergen reagents (molecular components,

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allergenic extracts) in atopic dermatitis (AD) patients with the use of multiplex examination ALEX 2 Allergy Explorer. We include in cluster evaluation also the severity of AD, onset of AD, the occurrence of asthma bronchiale, allergic rhinitis, duration of lesions and family history about atopy.

Atopic dermatitis together with bronchial asthma and allergic rhinitis, belongs to so-called atopic diseases. A progression from AD to food allergy, allergic rhinitis (AR) and bronchial asthma (AB) may develop in the first several years of life. This process is a phenomenon called "atopic march" (Anto et al., 2017; Heratizadeh, 2016; Ishitsuka & Roop, 2020; Weidinger & Novak, 2016; Werfel et al., 2016). Emerging data suggest that epithelial cell-derived cytokines such as thymic stromal lymphopoietin (TSLP), IL-25, and IL-33 may drive the progression from AD to bronchial asthma and food allergy (Heratizadeh, 2016). Atopic dermatitis was originally considered to be mainly a childhood disease with an imbalance between the Th2 response and the escalated IgE response to external allergens. Today, atopic dermatitis is rated as a long-term disease with varying clinical manifestations and expressivity, with epidermal barrier disorder playing a central role (Heratizadeh, 2016; Ishitsuka & Roop, 2020; Weidinger & Novak, 2016; Werfel et al., 2016). The barrier dysfunction correlates with the downregulation of barrier-related molecules such as filaggrin (FLG), loricrin (LOR), and involucrin (IVL). IL-4 and IL-13 potently inhibit the expression of these molecules by activating signal transducer and activator of transcription (STAT)6 and STAT3 (Anto et al., 2017; Heratizadeh, 2016; Ishitsuka & Roop, 2020; Weidinger & Novak, 2016; Werfel et al., 2016). The epidermal barrier function is formed by coordinated and sequential cross-linking of various barrier proteins, such as filaggrin (FLG) and loricrin (LOR) (Anto et al., 2017; Heratizadeh, 2016; Ishitsuka & Roop, 2020; Weidinger & Novak, 2016; Werfel et al., 2016). The majority of human epidermal barrier proteins map to a 2.5-Mbp cluster termed the "epidermal differentiation complex" (EDC) located on chromosome 1q21. The expression of barrier protein genes in the EDC locus is up- and downregulated by various external and internal stimuli, including Th2 (IL-4 and IL-13) and T22 (IL-22) cytokines (Anto et al., 2017; Heratizadeh, 2016; Ishitsuka & Roop, 2020; Weidinger & Novak, 2016; Werfel et al., 2016). Interleukin-4 and IL-13 significantly inhibit FLG and LOR mRNA and protein expression. FLG and LOR expression is also markedly downregulated by IL-22. In accordance with the loss of function mutation of *FLG* in AD, the downregulated action of Th2 and T22 cytokines explain the disrupted expression of FLG and LOR in the lesional skin of AD patients, leading to epidermal barrier dysfunction (Anto et al., 2017; Heratizadeh, 2016; Ishitsuka & Roop, 2020; Weidinger & Novak, 2016; Werfel et al., 2016). In contrast to the decreased expression of FLG and LOR, the lesional skin of AD patients exhibited increased expression of S100A7, the gene of which is also located in the EDC locus. Although IL-4 and IL-13 inhibit the expression of S100A7 in keratinocytes, IL-22 is a strong inducer of S100A7 gene expression (Anto et al., 2017; Heratizadeh, 2016; Ishitsuka & Roop, 2020; Weidinger & Novak, 2016; Werfel et al., 2016).

A typical manifestation of allergic inflammation is the production of IgE antibodies directed against causative allergens (Anto et al., 2017; Heratizadeh, 2016; Ishitsuka & Roop, 2020; Weidinger & Novak, 2016; Werfel et al., 2016). Various allergens may cause exacerbation of eczematous skin lesions in atopic dermatitis. The main allergenic sources are food, moulds, trees, weeds, grasses, mites, and animals (Anto et al., 2017;

Heratizadeh, 2016; Ishitsuka & Roop, 2020; Weidinger & Novak, 2016; Werfel et al., 2016). The most important allergen families and selected allergenic member proteins are shown in Table 1. We distinguish these superfamilies: Prolamin (family Cereal prolamins, Bifunctional inhibitors, 2S albumin, Non-specific lipid-transfer proteins), EF hand (family Polcalcins, Parvalbumins), Profilin like (family profilin), Tropomyosin like (family Tropomyosin), Cupin (family Vicilins and Legumins), Bet v 1-like (family Bet v 1), Calycin (family Lipocalins) and Double-psi beta-barrel (family DPBB and Pollen allergy). The families of highly cross-reactive molecules taken into consideration are as follows: profilins, PR-10-like molecules, nsLTP, serum albumins, tropomyosins, polcalcins, lipocalins, and parvalbumins. More than 3000 different allergens (plus approximately 1400 isoforms) are currently described, of which almost 1500 were expressed as recombinant proteins (Valenta et al., 1999). Some of these allergens have already been available for in vitro allergy diagnosis, either as highly purified native or recombinant proteins. The use of individual allergenic molecules (instead of extracts) has brought a new area of diagnosis of high-resolution molecular allergy (component-resolved diagnostics– CRD) (Valenta et al., 1999) and changed our understanding of sensitisation and cross-reactivity profiles (Werfel et al., 2015).

Molecular allergy diagnosis using singleplex allergens or multiplex allergen microarrays are considered as the typical methods of precision medicine (Heffler et al., 2018; van Hage et al., 2017). These methods enhance the specificity of IgE-diagnosis in polysensitised respiratory allergies, can be applied in food allergies and atopic dermatitis and may even reveal unexplained anaphylaxis (Werfel et al., 2015). ALEX 2–Allergy Explorer is an in vitro assay for the measurement of allergen-specific IgE antibodies in human plasma (van Hage et al., 2017). The major advantage of ALEX 2 is the comprehensive IgE pattern obtained with a minute amount of serum (Heffler et al., 2018; van Hage et al., 2017). The macroarray nanotechnology-based immunoassay used as a molecular allergy explorer (ALEX®; MacroArray Diagnostics, Wien, Austria) is the latest launched *in vitro* multiplex tool for precision medicine in allergy diagnosis. This new array contains 295 allergen reagents (117 allergenic extracts and 178 molecular components), with a large majority of aeroallergen families and cross-reactive food allergens being represented. This *in vitro*

Table 1. The most important allergen superfamilies and families with their corresponding sources (Breiteneder et al., 1988).

Superfamily	Family	Sources
Prolamin	Cereal prolamin	Grains of cereal grasses
	Bifunctional inhibitors	Grains of cereal grasses
	2Salbumin	Tree nuts, peanuts, legumes, seeds
	Non-specific lipid transfer protein	Fruits, tree nuts, peanuts, vegetables, tree and weed pollen, latex
EF hand	Polcalcins	Tree, grass and weed pollen
	Parvalbumins	Fish
Profilin like	Profilin	Tree, grass and weed pollen, fruits, vegetables, latex
Tropomyosin like	Tropomyosin	Crustaceans, mollusk, fish parasite Anisakis, mites, cockroaches
Cupin	Vicilins	Tree nuts, peanuts, legumes, seeds
	Legumins	Tree nuts, peanuts, legumes, seeds
Bet v 1 like	Bet v 1	Fagales tree pollen, fruits, vegetables, legumes, tree nuts
Calycin	Lipocalins	Mammals, mites, cockroaches
Double-psi–beta-barrel	DPBB1	Grass pollen
	Pollen aller 1	Grass pollen

allergy explorer is the first *in vitro* multiplex allergy test allowing simultaneous measurement of total IgE and specific IgE against a plethora of allergen extracts and molecular allergens. The combination of second- and third-level assays in the same immunoassay allows to evaluate the presence of IgE sensitisation, whether it is genuine or cross-reactive, and saves time and costs, particularly in polysensitised patients and/or with pollen-food syndromes (Heffler et al., 2018). The ALEX® *in vitro* allergy test core technology is based on a two-phased manufacturing process and it represents a multiplex ELISA-based test with proven immunoassay chemistry and detection methods (Heffler et al., 2018).

In our previous studies, we evaluated in patients suffering from AD the occurrence of sensitisation to molecular components with the use of ISAC Multiplex testing. The highest observed sensitisation rate was 61.0% to grass specific molecule Phl p 1, the second most frequent sensitisation was 57. 0% to Betulaceae-specific molecule Bet v 1. Frequently observed sensitisations were those to PR-10 proteins, NPC2 proteins family, Uteroglobin and Lipocalin. In severe form of AD mainly sensitisation to molecular components of NPC2 proteins family, Uteroglobin, Lipocalin, Aspergillus and sensitisation to Phl p was recorded with the significant higher occurrence (Čelakovská et al., 2020a; Čelakovská et al., 2020b).

The aim of our study is to evaluate the results of specific IgE to a large number of allergen reagents (117 allergenic extracts and 178 molecular components) in atopic dermatitis patients with cluster analysis. Our results can demonstrate the importance of single molecular components in atopic dermatitis patients and show, how molecular components are grouped in clusters according to the level of specific IgE and according to the occurrence of allergic rhinitis, asthma bronchiale, the severity of AD, the onset of AD, duration of lesions and family history about atopy. According to the inclusion of specific IgE results in the clusters, we can focus on the search for other possible allergens.

Methods

Patients and methods

In the period 2018–2020, 100 patients suffering from atopic dermatitis were examined. All these patients were examined in the Department of Dermatology, Faculty Hospital Hradec Králové, Charles University, Czech republic. The diagnosis of atopic dermatitis was made with the Hanifin-Rajka criteria (Hanifin & Rajka, 1980). Exclusion criteria were systemic therapy (cyclosporin, systemic corticoids, biological therapy), pregnancy, breastfeeding. Patients with atopic dermatitis having other systemic diseases were excluded from the study as well. The complete dermatological and allergological examination was performed in patients included in the study. This study was approved by Ethics committee of Faculty Hospital Hradec Králové, Charles University of Prague, Czech Republic.

Examination of specific IgE to allergen reagents

The serum level of the sIgE was measured by the components resolved diagnostic microarray-based sIgE detection assay ImmunoCAP ALEX (Heffler et al., 2018; van Hage et al., 2017). It is based on a state-of-the-art proprietary nano-bead technology. This new array contains 295 allergen reagents (117 allergenic extracts and 178 molecular components),

with a large majority of aeroallergen families and cross-reactive food allergens being represented. The ALEX measuring range for specific IgE is 0.3–50 kU_A/L (quantitative) and for total IgE is 1–2500 kU/L (semiquantitative). The sample requirement is 100 µL serum or plasma. The results are expressed as Class 0 (< 0.3 kU_A/L), Class 1 (0.3–1 kU_A/L), Class 2 (1–5 kU_A/L), Class 3 (5–15 kUA/L), and Class 4 (> 15 kU_A/L). ALEX is commercially available, having attained CE certification, which assures that the quality of the assay, regarding LoD, precision and repeatability as well as specificity and linearity, is in line with *in vitro* diagnostic features. There is no significant interference from high total IgE, haemoglobin, bilirubin or triglycerides. A flexible Raptor analysis software (specifically designed for ALEX®) allows to analyse tailor-made allergen panels, as considered fit for clinical needs (multiplex on-demand).

Bronchial asthma

The diagnosis of bronchial asthma (AB), was determined according to the guidelines of the Global Initiative for Asthma (GINA) at allergy out - patients clinic of the Institute of Clinical Immunology and Allergology, Faculty Hospital Hradec Kralove, Czech Republic (Global Initiative for Asthma. Global Strategy for asthma management and prevention – Update 2015. www.ginasthma.com).

Allergic rhinitis

The evaluation of allergic rhinitis (AR), was made according to the allergy testing and personal history of the Institute of Clinical Immunology and Allergology, Faculty Hospital Hradec Kralove, Czech Republic (). AR was defined as a process which included three cardinal symptoms: sneezing, nasal obstruction, and mucus discharge. Symptoms occur with allergen exposure in the allergic patient (Wang et al., 2018).

Dermatological examination

Complete dermatological examination was performed in patients included in the study. The severity of atopic dermatitis was scored in agreement with SCORAD, with an assessment of topography items (affected skin area), intensity criteria and subjective parameters. To measure the extent of atopic dermatitis, the rule of nines was applied on a front/back drawing of the patient's inflammatory lesions. The extent was graded 0–100 points. The intensity part of the SCORAD index consists of six items: erythema, oedema/papules, excoriations, lichenification, crusts, and dryness. Each item was graded on a scale 0–3. The subjective items included daily pruritus and sleeplessness. Both subjective items were graded on a 10-cm visual analogy scale and the maximum subjective score was 20 points. All items were filled out in the SCORAD evaluation form. The SCORAD index formula was: $A/5 + 7B/2 + C$. In this formula A is defined as the extent (0–100 points), B is defined as the intensity (0–18 points), and C is defined as the subjective symptoms (0–20 points). The severity of atopic dermatitis is evaluated with SCORAD as a mild form to 20 points, as moderate over 20–50 points, as a severe form over 50 points (European Task Force on Atopic Dermatitis, 1993). The evaluation of SCORAD score was performed every two months during the study (European Task Force on Atopic Dermatitis, 1993).

The evaluation of duration of atopic dermatitis

The atopic dermatitis lesions were evaluated as persistent (eczematic lesions persisted in various forms throughout the year) or occasionally (for at least 3 weeks the patient was without any eczematic lesions) according to the dermatologist examination during one last year and according to the patient's information. The evaluation of lesions by a dermatologist (= the main investigator of the study) was performed every two months during the study.

The onset of atopic dermatitis

The onset of atopic dermatitis was evaluated according to the patient's history (the onset of atopic dermatitis under five years of age or later).

The family history about atopy

The family history was evaluated according to the patient's information. We evaluated a positive family history: the occurrence of allergy, atopic dermatitis, asthma bronchiale, rhinoconjunctivitis in parents, brothers, sisters and children. If there was no family history of these diseases, the family history was evaluated as negative.

Control group

When introducing the test into the clinical examination, we also examined 15 healthy volunteers–blood donors (equivalent to age, male and female representation). All of these blood donors had in the multiplex examination (ALEX 2–Allergy Explorer) the specific IgE negative – expressed as Class 0 (<0.3 kUA/L). This study was approved by Ethics committee of Faculty Hospital Hradec Králové, Charles University of Prague, Czech Republic.

Statistics

This statistic method for the evaluation of results was used: Clustering by Medoid Partitioning**,** Method: Kaufman-Rousseeuw, Objective Function: Silhouette, Distance Type: Euclidean, Scale Type: Standard Deviation. Molecular components are ordinal variables (Čelakovská et al., 2020a; Čelakovská et al., 2020b; Heffler et al., 2018). We used this program: NCSS 2019 Statistical Software (2019). NCSS, LLC. Kaysville, Utah, USA, ncss.com/software/ncss.

(1) The cluster analysis (silhouette value) was processed for the whole number of specific IgE results to allergen reagents in ALEX2 Allergy Explorer (295 allergen reagents, 117 allergenic extracts and 178 molecular components). The cluster analysis takes into account the frequency of positive results of specific IgE and the level of specific IgE in classes 0, 1, 2, 3, 4.
(2) The statistical analysis TURF (Total Unduplicated Reach and Frequency) was used to find allergenic extracts and molecular components with high (Class 3) and very high level (Class 4) of specific IgE to cover the largest possible set of patients. With the method of TURF (Total Unduplicated Reach and Frequency), we search the unique combinations (here attributes with the desired property – the level of specific IgE in classes 3 or 4), such as to cover the largest possible set of given

elements (here patients = Reach) for which some occurrences have been detected and at the same time the largest number of occurrences of the searched property on given attributes (Frequency). The cluster analysis (silhouette value) was processed for these allergens reagents and for other parameters, such as the occurrence of allergic rhinitis, asthma bronchiale, the severity of AD, the onset of AD, duration of lesions and family history about atopy.

Interpreting silhouettes

A silhouette value is constructed for each object. The value can range from minus one to one (Table 2). It measures how well an object has been classified by comparing its dissimilarity within its cluster to its dissimilarity with its nearest neighbour. When *s* is close to one, the object is well classified. Its dissimilarity with other objects in its cluster is much less than its dissimilarity with objects in the nearest cluster. When *s* is near zero, the object was just between clusters *A* and *B*. It was arbitrarily assigned to *A*. When *s* is close to negative one, the object is poorly classified. Its dissimilarity with other objects in its cluster is much greater than its dissimilarity with objects in the nearest cluster. The silhouette value summarises how appropriate each object's cluster is (Table 2), (Frades & Matthiesen, 2010; Kaufman & Rousseeuw, 1990; Rousseeuw, 1987).

Determining the number of clusters

One useful summary statistic is the average value of *s* across all objects. This summarises how well the current configuration fits the data. An easy way to select the appropriate number of clusters is to choose that number of clusters which maximises the average silhouette. We denote the maximum average silhouette across all values of *k* as *SC*. Kaufman and Rousseeuw (Frades & Matthiesen, 2010; Kaufman & Rousseeuw, 1990; Rousseeuw, 1987) present the following table to aid in the interpretation of *SC*. We evaluated our data according to this table, which is shown in Table 2.

Results

100 atopic dermatitis patients were included in the study (48 men and 52 women with the average age 40.9 years: min. age 14 years, max. age 67 years and with the average SCORAD 39, SD 13.1 points). The mild form of AD was recorded in 14 patients (14%), moderate form in 61 patients (61%), severe form in 25 patients (25%); 55 patients (55%) suffer from bronchial asthma and 74 patients (74%) suffer from allergic rhinitis. Positive family history about atopy was recorded in 48 patients (48%). The onset of

Table 2. Cluster analysis – Silhouette proposed interpretation (Frades & Matthiesen, 2010).

Cluster average – Silhouette value	Interpretation
0.71–1.00	A strong structure has been found
0.51–0.70	A reasonable structure has been found
0.26–0.50	The structure is weak and could be artificial.
−1–0.25	No substantial structure has been found

AD before 5 years of age in 61 patients (61%) and the persistent eczematic lesions in 57 patients (57%). The characteristics of patients are shown in Table 3.

(1) The cluster analysis (silhouette value) for the whole number of specific IgE results to allergen reagents in ALEX2 Allergy Explorer (295 allergen reagents, 117 allergenic extracts and 178 molecular components); we evaluated the rate of occurrence and the level of specific IgE. Parameters such as the occurrence of bronchial asthma, allergic rhinitis and the severity of AD were included.

Majority of specific IgE results to allergen reagents are found in C**luster 2** (151 allergen reagents from different protein families) and in **Cluster 9** (79 allergen reagents from different protein families). The specific IgE results in the other clusters are recorded to a much smaller number of allergen reagents and molecular components (altogether from 65 allergen reagents and molecular components); the grouping of molecular components correspond to the protein families. The included parameter such as the severity of AD appeared in one separated cluster (C**luster 1).** In **Cluster 3,** we find 5 allergen reagents (molecular components) from PR 10 proteins found in foods (celery, peanuts, carrot, soy). In **Cluster 4,** we find 4 allergen reagents (molecular components) from Cysteine protease, Peritrophin-like protein domain and NPC2 family from allergens house dust mites and storage mites. In **Cluster 5,** there are molecular components Der p 2 (NPC2 family, House dust mite) and Der f 2 (NPC2 family, House dust mite). In **Cluster 6,** are recorded 8 allergen extracts (molecular components) from PR -10 proteins from inhalant allergens (European beech, hazel pollen, Birch, Alder) and from food allergens (hazelnuts, Strawberry, apple). In **Cluster 7,** there are 23 allergen extracts (molecular components) from NPC2 family, lipocalins, tropomyosins, troponin C, 2 S albumin and allergens from shrimp, American cockroach, squid and molecular components from unknown function from Aspergilus fumigatus (Asp f 4) and house dust mite (Der p 5). In **Cluster 8,** we find 9 allergen extracts (molecular components) from arginin kinase and from allergens storage mites and from locust, mealworm, house cricket. In **Cluster 10,** we find 11 allergen extracts (molecular components) such as uteroglobins, lipocalins, lipophilin, manganese superoxide dismutase, beta expansins and grass group 2 from

Table 3. The characteristic of patients.

The characteristic of patients, number of patients	
Patients suffering from atopic dermatitis	100 patients
Sex	48 men, 52 women
The average age	40.9 years (min. age 14 years, max. age 67 years)
The average SCORAD	39 points, s.d. 13.1 points
Severity of AD	Mild form 14 (14%)
	Moderate form 61 (61%)
	Severe form 25 (25%)
Family history about atopy	Positive family history 48 patients (48%)
	Negative family history 52 patients (52%)
Onset of AD	Under 5 years of age 61 patients (61%)
	After 5 year of age 39 patients (%)
Eczematic lesions	Persistent 57 patients (57%)
	Occasional 43 patients (43%)
Bronchial asthma	55 (55%)
Allergic rhinitis	74 (74%)

Timothy and allergens from Bahia Grass and Bermuda grass. In **Cluster 11,** there are 5 allergen extracts (molecular components) such as Grass Group 5/6, Timothy, Beta expansin from Timothy, Secc pollen. The results of total IgE are grouped in **Cluster 12**. The detailed review of statistical analysis with 12 clusters for the whole number specific IgE results is recorded in Supplementary Table S1.

(1) The statistical analysis TURF (Total Unduplicated Reach and Frequency) was used to find allergenic extracts and molecular components with high and very high level of specific IgE to cover the largest possible set of patients.

In Table 4, we show this Cluster analysis for allergen reagents (allergenic extracts and molecular components) with the level of specific IgE in classes 3 or 4 (calculated according to TURF) with positivity at least in five patients (altogether 81 allergen reagents) and for parameters such as the occurrence of allergic rhinitis, asthma bronchiale, the severity of AD, onset of AD, duration of lesions and family history about atopy. The parameter severity of AD is recorded as a single object in **Cluster 1.** In C**luster 2,** there are three objects such as allergic rhinitis, onset of AD, persistent lesions. In Cl**uster 3,** we find 6 allergen reagents (molecular components) from PR 10 proteins found in aeroallergens (Birch, Alder, European beech, Hazel pollen, Cora pollen) except Cora1.0401 from Hazelnut. In C**luster 4,** there are 8 allergen reagents (molecular components) from PR 10 proteins found in foods (celery, peanuts, carrot, soy, apple, Strawberry) and Cyn d from Bermuda grass. In C**luster 5,** we observe molecular components from house dust mites Der p 2 and Der f 2 from NPC2 family, together with the occurrence of bronchial asthma and positive family history about atopy. In C**luster 6,** there are grouped 26 molecular components from lipocalins, arginin kinase, tropomyosin, Peritrophin-like protein domain, cystein protease, troponin C, profilin, conglutinin and allergen extract from shrimp, house dust mites, storage mites from locust, mealworm, house cricket and squid. In C**luster 7,** we find 7 molecular components from beta expansins from Timothy, Bermuda grass, molecular components from grass group 5/6 and allergen Cultivated rye. In C**luster 8,** there are grouped 32 molecular components from lipocalins, uteroglobins, Mn superoxide dismutase, Plant defensins, Ole e 1- like protein family, enolase, NPC2 family, cupin, Beta-parvalbumin, Arginine kinase, Icarapin Variant 2 (Honey bee venom) and allergen extract from Ragweed, Rat, Mugwort, English plantain, European ash and Bahia grass. The detailed review of statistical analysis with 8 clusters (review of clusters with allergenic extracts and molecular components with high and very high level of specific IgE to cover the largest possible set of patients) is recorded in Supplementary Table S2.

Discussion

The clustering problem has been addressed in many contexts and disciplines. Cluster analysis includes problem formulation, distance rate selection, clustering process selection, cluster number decisions, profile cluster interpretation, and finally clustering validity assessment. Grouping processes in cluster analysis can be hierarchical, non-hierarchical or two-stage (Frades & Matthiesen, 2010; Kaufman & Rousseeuw, 1990; Rousseeuw, 1987). The distances between cluster centres in cluster analysis indicate

Table 4. Cluster analysis for 81 allergen reagents (allergenic extracts and molecular components) with the level of specific IgE in classes 3 or 4 (calculated according to TURF) with positivity at least in five patients and for parameters such as the occurrence of allergic rhinitis, asthma bronchiale, the severity of AD, onset of AD, duration of lesions and family history about atopy.

	Molecular components	Protein family	Allergen	Parameters
Cluster 1				AD severity
Cluster 2				5 years of age Persistent lesions Allergic rhinitis
Cluster 3	Fag s 1	PR-10 protein,	European beech	
	Cora1.0103	PR-10 protein,	Hazel pollen	
	Cora1.0401	PR-10 protein,	Hazelnut	
	Bet v 1	PR-10 protein,	Birch	
	Aln g 1	PR-10 protein,	Alder	
	Corra pollen	PR-10 protein,	Cora pollen	
Cluster 4	Api g 1	PR 10 protein	Celery	
	Ara h 8	PR-10 protein,	Peanuts	
	Dau c 1	PR-10 protein,	Carrot	
	Gly m 4	PR-10 protein,	Soy	
	Dau c	PR-10 protein,	Carrot	
	Mal d 1	PR-10 protein,	Apple	
	Fraa1 + 3	PR-10 protein+ non-specific lipid transfer protein type 1, Strawberry	Strawberry	
	Cyn d		Bermuda grass	
Cluster 5	Der p 2	NPC2 family	House dust mite	
	Der f 2	NPC2 family,	House dust mite	
	Family history			
	Bronchial asthma			
Cluster 6	Chispp		Crab	
	Loc m		Locust	
	Ten m (Mealworm)		Mealworm	
	Ach d		House cricket	
	Lol spp		Squid	
	Pan b		Northern shrimp	
	Bla g 9	Arginin kinase,	German cockroach	
	Der p 5	Unknown	House dust mite	
	Cra c 6 (Troponin C, North Sea shrimp)	Troponin C	North Sea shrimp	
	Acas		Acarus siro	
	Der p 20	Arginin kinase	Mites	
	Der p 21	Unknown	House dust mite	
	Tyr p		Storage mite	
	Hom g		Lobster	
	Per a 7	Tropomyosin	American cockroach	
	Der p 7	group 7 mites	group 7 mites	
	Der f 1	Cysteine protease	House dust mite	
	Der p 1	Cysteine protease	House dust mite	
	Der p 23	Peritrophin-like protein domain	House dust mite	
	Ara h 6	Conglutin, 2S albumin	Peanuts	
	Blo t 5		Storage mite	
	Cav p 1 (Lipocalin, Guinea pig)	Lipocalin	Guinea pig	
	Can f 2	Lipocalin	Dog	
	Can f 4	Lipocalin	Dog	
	Ory c 1	Lipocalin	Rabbit	
	Art v 4	Profilin	Mugwort	
Cluster 7	Lol p 1	Beta-espansin	Rye grass	
	Phl p 1	Beta - expansin	Timothy	

(*Continued*)

Table 4. Continued.

	Molecular components	Protein family	Allergen	Parameters
	Phlp5.0101	Grass Group 5/6,	Timothy	
	Phlp6	Grass Group 5/6,	Timothy	
	Secc_polle		Cultivated rye, Pollen	
	Phlp2	Expansin	Timothy	
	Cynd1	Beta expansin,	Bermuda grass	
Cluster 8	Asp f 6	Mn superoxide dismutase	*Aspergillus fumigatus*	
	Fel d 7	Lipocalin	Cat	
	Pas n		Bahia grass	
	Can f 6	Lipocalin	Dog	
	Ory c 3 (Uteroglobin, Rabbit)	Uteroglobin	Rabbit	
	Can f maleu		Male dog urine	
	Amb a		Ragweed	
	Equ c 1	Lipocalin	Horse	
	Fel d 4 (Lipocalin, Cat)	Lipocalin	Cat	
	Malas 11	Mn superoxide dismutase	Malassezia sympodialis	
	Alt a 1	unknown	*Alternaria alternata*	
	Amb a 4	Plant defensin	Ragweed	
	Rat n		Rat	
	Art v		Mugwort	
	Fel d 1	Uteroglobin	cat	
	Frae		European ash	
	Can f 1	Lipocalin	Dog	
	Fra e 1	Ole e 1-like protein family	European ash	
	Phod s 1 (Lipocalin, Siberian hamster)	Lipocalin	Siberian hamster	
	Ole e 1	Ole e 1-family	Olive	
	Api m 10	Icarapin Variant 2,	Honey bee venom	
	Pla l 1	Ole e 1-like protein family	English plantain	
	Pla l		English plantain	
	Mala s 5	unknown	Malassezia sympodialis	
	Can f Fd 1	Uteroglobin	Dog	
	Alt a 6	Enolase	*Alternaria alternata*	
	Lep d 2	NPC2 family	Storage mite	
	Ara h 1	Cupin, vicillin-type, 7S globulin	Peanut	
	Mus m 1	Lipocalin and urinary prealbumin,	House mouse	
	Xip g 1	Beta-parvalbumin	Swordfish	
	Gly d 2	NPC2 family	Storage mite	
	Pen m 2	Arginine kinase	Shrimp	

how each cluster pair is separated. Groupings that are widely separated are different and therefore desirable (Frades & Matthiesen, 2010; Kaufman & Rousseeuw, 1990; Rousseeuw, 1987).

At our study, each cluster is represented by a so-called silhouette, which is based on the comparison of its tightness and separation. This silhouette shows which objects he well within their cluster, and which ones are merely somewhere in between clusters. The entire clustering is displayed by combining the silhouettes into a single plot, allowing an appreciation of the relative quality of the clusters and an overview of the data

configuration. The average silhouette width provides an evaluation of clustering validity. Silhouette width is a widely used index for assessing the fit of individual objects in the classification, as well as the quality of clusters and the entire classification (Frades & Matthiesen, 2010; Kaufman & Rousseeuw, 1990; Rousseeuw, 1987).

In our study, the grouping of results of specific IgE form clusters with different numbers of allergen reagents. In evaluating all results of specific IgE in classes 0, 1, 2, 3, 4, the majority of specific IgE results are found in two clusters (there are 151 allergen reagents in cluster 2 and 79 allergen reagents in cluster 9 from different protein families). The other results of specific IgE are divided into clusters, which correspond to the division into protein families. If we include in the cluster analysis only the results of specific IgE with high and very high levels (classes 3, 4), we observe a similar distribution with grouping of allergen reagents according to the protein families. These allergens play a vital and exceptional role in patients with AD. Although the cluster analysis algorithm divides allergens into clusters, the size of the average Silhouette (Average Silhouette) is 0.0977 for 8 clusters, it means no significant structure was found. For 12 clusters, this value is equal to 0.141. We calculated also algorithm characteristics for a larger number of clusters and the Average Silhouette still does not exceed 0.25. The larger the number of clusters, the more the anamnestic data become independent and form separate clusters of one member. We see it as the algorithm trying to minimise the distances inside the clusters and maximise the Average Silhouette. Although the distribution of molecular components corresponds to the division into protein families, the links between allergens reagents (molecular components) are weak. Only one binding between molecular components was strong (cluster 5), where only 2 components (Der p 2, Der f 2) were selected.

We hypothesised that objects such as allergic rhinitis, asthma bronchiale, the severity of AD, onset of AD, duration of lesions and family history about atopy may be associated with some allergens. In evaluating all results of specific IgE in classes 0, 1, 2, 3, 4, the severity of AD appears in one separated cluster; the objects such as AB and AR are found in cluster 9 together with 79 allergen reagents. In evaluating specific IgE in classes 3 and 4, family history about atopy and bronchial asthma are recorded together with molecular components Der f 2 and Der p 2 in one cluster. The object such as the severity of AD is found in one other cluster and objects such as AR, the onset of AD and duration of lesions are found in another cluster without grouping with results of specific IgE. In evaluating specific IgE in classes 3 and 4 (in 81 allergen reagents), we observe the division of specific IgE results according to the occurrence of panallergens in clusters 3-8. Panallergens comprise various protein families of plant as well as animal origin and are responsible for wide IgE cross-reactivity between related and unrelated allergenic sources. Such cross-reactivity's include reactions between various pollen sources, pollen and plant-derived foods as well as invertebrate-derived inhalants and foodstuff (Breiteneder et al., 1989; Asero et al., 2015; Jensen-Jarolim, 2014; Mastrorilli et al., 2016; McKenna et al., 2016). In our study, molecular components from Pathogenesis-related protein group 10 (PR-10 protein) family found in foods (Api g 1, Ara h 8, Dau c 1, Gly m 4, Mal d 1 and Fra a 1 + 3) are recorded in Cluster 4 and molecular components from aeroallergens (Fag s 1, Cora1.0103, Cora1.0401, Bet v 1, Aln g 1 and Corra pollen) are recorded in Cluster 3. The PR-10 proteins are actually one of the 11 subfamilies of the Bet v 1 family. Pathogenesis-related protein group 10 PR-10 molecules (i.e. Bet

v 1 and homologous allergens) are the major allergens in Fagales pollen and are recognised by virtually all allergic patients, thus representing the major cause of clinical allergy. PR-10 proteins are expressed in high concentrations in reproductive tissues such as pollen, seeds, and fruits. The major role of PR-10 proteins is reported in response to biotic and abiotic stresses and defend plants against fungi and other microorganisms (Asam et al., 2015; Breiteneder et al., 1988; Matricardi et al., 2016). Their homologs are also present in a large number of plant-derived foods, and thus frequently cause cross-sensitisation and consequently plant-food allergy (oral allergy syndrome, in most cases), (Agarwal & Agarwal, 2014; Breiteneder et al., 1989; Asero et al., 2015; Finkina et al., 2017; Jensen-Jarolim, 2014; Mastrorilli et al., 2016; McKenna et al., 2016). The oral allergy syndrome was recorded clinically in majority of patients with sensitisation to these molecular components in our study. For tree pollen allergic patients in Northwestern and Central Europe, the molecular component Bet v 1 is of decisive clinical importance because there is no "competing" major allergen (Asam et al., 2015; Breiteneder et al., 1988; Matricardi et al., 2016; Biedermann et al., 2019 July). We compared the results of our study with the botanical characteristic of our region. Based on data from the nearest phenological station the following species are present from important pollen allergens: owing to the sandy substrate, white birch (Betula pendula) and wood pine (Pinus sylvestris) predominate, as well as common hazel (Corylus avellana).

The results of specific IgE with high and very high level to molecular components Der f 2, Der p 2 (house dust mites, NPC2 family) together with parameters such as bronchial asthma and positive family history about atopy are recorded in Cluster 5; the silhouette value is 0.1316 in cluster average, what means weak structure and weak bond. On the other hand, the results of specific IgE to molecular components Der f 2 and Der p 2 in classes 0, 1, 2, 3, 4 are grouped in one cluster with silhouette value average 0.9572, what means the strong structure and strong bond. IgE-mediated sensitivity to house dust mites allergens plays a major role in allergic rhinitis and allergic asthma in adults and children. Sensitisation to house dust mites allergens may be the initial event in the so-called "allergic march" in children. Our study confirms the basic role of these components (Der p 2, Der f 2) in subgroup of patients suffering from bronchial asthma and with the positive family history about atopy. Sensitisation to crude D*ermatophagoides pteronyssinus* extract does not always indicate genuine house dust mites sensitisation (Reese et al., 1999; Yang et al., 2010), because molecular components from crude extract *Dermatophagoides pteronyssinus*, such as tropomyosin, arginine kinase, and glutathione S-transferase, can contribute to cross-reactivity between crude *Dermatophagoides pteronyssinus* extract and extracts from other invertebrates (Reese et al., 1999; Yang et al., 2010). Der p 2, Der f 2 are from NPC2 (Niemann-Pick Type C2) protein family (epidermal secretory proteins). NPC2 proteins are secreted early in response to an IgE-mediated stimulus and belong to the ML (MD-2-related lipid-recognition) protein family (155 members), which includes the Toll-like receptor co-factors, MD-1 and MD-2, and seven major house dust mite allergens of unknown function (including Der p 2 and Der f 2), (Pathak & Helm, 2010 March 8). Group 2 allergens from the *Dermatophagoides* house dust mites cause IgE-mediated responses in over 80% of the dust mite-allergic individuals and are therefore classified as major allergens (Platts-Mills et al., 1997; Thomas & Smith, 1998). Although it is well characterised in terms of allergenicity, there is still some ambiguity in terms of its biological function. The hypothesis is

that Der p 2 may be involved in the mite's innate antibacterial defence system (Derewenda et al., 2002; Ichikawa et al., 1998; Ichikawa et al., 2005; Inohara & Nunez, 2002; Valerio et al., 2005).

In one cluster (cluster 6 – the results of specific IgE with high and very high level), we observe the grouping of these molecular components and allergen extracts: lipocalins, arginin kinase, tropomyosin, peritrophin-like protein domain, cystein protease, troponin C, profilin, conglutinin and allergen extract from shrimp, house dust mites, storage mites from locust, mealworm, house cricket and squid. In analysis of all results of specific IgE (classes 0–4), we find in one cluster (cluster 8) allergen extracts (molecular components) from arginin kinase and from allergens storage mites and from locust, mealworm, house cricket. Cross-reactivity of insect tropomyosin and arginine kinase has been demonstrated in house dust mite and seafood (e.g. prawn, shrimp) allergic patients (de Gier & Verhoeckx, 2018 August). In addition, many other allergenic species, such as various non-edible insects, arachnids, mites, seafoods, mammals, nematoda, trematoda, plants, and fungi, have been identified with sequence alignment analysis to show potential cross-reactivity with allergens of edible insects (de Gier & Verhoeckx, 2018 August). Based on cross-reactivity studies, there is a possibility that house dust mites- and crustacean-allergic patients may react to food containing Yellow mealworm proteins (Verhoeckx et al., 2014 March; Broekman et al., 2017 September; Pali-Schöll et al., 2019 January 26; Broekman et al., 2016; Srinroch et al., 2015). In the study of Broekman, all shrimp allergic patients were sensitised to multiple insects with similar response profiles for all insects tested. Shrimp allergic patients are most likely at risk of food allergy to mealworm and other insects. Primary mealworm allergy does not mean subjects are likely to react to all insects (Broekman et al., 2017 September). Pali-Schöll et al. studied the cross-reactivity of shrimp-, mite- and flies-allergic patients to different edible insects; the results show that crustacean-, house dust mites- and stable flies-allergic patients cross-recognise desert locust and house cricket proteins, and crustacean-allergic patients also flies proteins (Pali-Schöll et al., 2019 January 26). However, studies on cross-reactivity of crustacean- and house dust mite-allergic patients against house cricket (*Acheta domesticus*) as well as desert locust (*Schistocerca gregaria*) have not been performed (Broekman et al., 2016; Srinroch et al., 2015). The grouping of results of specific IgE to these molecular components (lipocalins, arginin kinase, tropomyosin, peritrophin-like protein domain, cystein protease, troponin C, profilin, conglutinin and allergen extract from shrimp, house dust mites, storage mites from locust, mealworm, house cricket and squid) confirmed cross-reactivity and/or co-sensitisation of insect tropomyosin and arginine kinase in house dust mite and seafood allergic patients. Our results are in agreement with the work of Broekman et al., who shows for the first time that crustacean-and house dust mite-allergic patients are at risk for cross-reactions to desert locust and house cricket (Broekman et al., 2017 September).

In cluster 11 (evaluating all results of specific IgE) and in cluster 7 (evaluating results of specific IgE in classes 3 and 4), we find molecular components such as beta expansins from Timothy, molecular components from grass group 5/6 and allergen Cultivated rye. Sensitisation to Phl p 1 usually precedes sensitisation to other grass pollen allergen and is the most prevalent component sensitisation in grass pollen-allergic patients (Hatzler et al., 2012). Phl p 1 is a beta-expansin and it is a major grass pollen allergen with more than 80% homology to group 1 allergens from the Pooideae subfamily (Focke

et al., 2001; Hatzler et al., 2012; Matricardi et al., 2016). Phl p 1 is in most patients the "initiator" molecule. Moreover, even in the few grass pollen-allergic patients who start their sensitisation process with other molecules, specific IgE against Phl p 1 are produced quite soon. Therefore, specific IgE to Phl p 1 is an essential marker in grass pollen-allergic patients to establish "true sensitisation". The absence of IgE to Phl p 1 does not exclude "true" sensitisation to grass pollen, which might be due (in a few cases) to isolated IgE sensitisation to other major allergenic proteins (e.g. Phl p 5). Specific IgE to Phl p 5 was found in this cluster also; it is another major pollen allergen of temperate grasses with low sensitisation prevalence, but often with high IgE levels (Matricardi et al., 2016). It shows broad IgE cross-reactivity with other group 5 allergens from the Pooideae subfamily of temperate grasses. Although IgE to Phl p 5 usually appear later than those to Phl p 1 in the sensitisation process, their concentration grows in many patients rapidly and higher and their contribution to patients' symptoms has been demonstrated (Matricardi et al., 2016). Testing IgE to Phl p 5 can be useful as a second-line test and has been shown to be useful for distinguishing between allergy to grass and olive pollen in southern Europe (Matricardi et al., 2016). Phl p 6 is another major grass pollen allergen, specific for the Pooideae subfamily. Its function has not yet been described (Matricardi et al., 2016). Phl p 2 is rarely the only molecule inducing grass pollen sensitisation and the presence of IgE antibodies to this Phl p 2 – observed in around 60%–80% of the European grass pollen-allergic patients – just confirms that a positive skin prick reaction is the expression of true sensitisation to grass. Phl p 6 is highly cross-reacting with Phl p 5 and does not add more diagnostic information, once IgE to Phl p 5 has been tested (Matricardi et al., 2016). Bermuda grass pollen is not present in our region, possible cross-reactivity with β-expansins from other grasses could be the explanation for the results with high sensitisation to Cyn d 1 in in our study.

In cluster 10 (evaluating all results of specific IgE), we find 11 allergen extracts (molecular components) – uteroglobins, lipocalins, lipophilin, manganese superoxide dismutase, beta expansins and grass group 2 from Timothy and allergens from Bahia Grass and Bermuda grass. In cluster 8 (results of sIgE in classes 3 and 4), we find molecular components from lipocalins, uteroglobins, manganese superoxide dismutase, Plant defensins, Ole e 1-like protein family, enolase, NPC2 family, cupin, Beta-parvalbumin, Arginine kinase, Icarapin Variant 2 (Honey bee venom) and allergen extract from Ragweed, Rat, Mugwort, English plantain, European ash and Bahia grass. Sensitisation to major cat/dog/horse allergens (e.g. Fel d 1/ Can f 1/Can f 2/Can f 5/Equ c 1) are specific markers of cat/dog/horse sensitisation. Patients may experience symptoms from the upper and/or lower airways to cat/dog/horse. IgE to Fel d 1 and Can f 1 in childhood have shown to be predictive markers of cat or dog allergy in adolescence (Asarnoj et al., 2016). Can f 1 and Can f 5 are the dominant marker allergens for dog, although Can f 1 is cross-reactive with Fel d 7. Can f 2 is a minor allergen and should be tested in combination with Can f 1 and Can f 5. Patients with moderate to high IgE reactivity to serum albumins should be advised that they may experience clinical symptoms in different furry animals. Can f 7, an NPC2-like protein that is homologous with the NPC2 components of house dust mites, have been identified in dogs (Basagana et al., 2008; Cabanas et al., 2000; Khurana et al., 2016; Konradsen et al., 2015; Vachová et al., 2020). Sensitisation to certain allergens seems to be associated with the severity and persistence of clinical symptoms, and sensitisation to more than 1 allergen or sensitisation to

albumins seems to be associated with more severe respiratory symptoms (Konradsen et al., 2015). Commercially available components for horse, mouse and rat are cross-reactive. No components are available at all for small pet animals.

The number of allergens available for fish is very limited and should be extended in order to propose a panel of components from main fish families (Hilger et al., 2017 August). Parvalbumin, enolases, aldolases and collagens are important fish allergens. IgE-reactivity patterns might vary according to regional eating habits and local diets. Parvalbumin seems to be a marker allergen for clinical cross-reactions and a diagnostic test characterised by high sensitivity but low specificity. The presence of IgE to enolases and aldolases, in addition to specific clinical symptoms, might reflect true fish allergy (Hilger et al., 2017 August; Permyakov et al., 2017).

Olive pollen allergy is caused by Ole e 1 in the majority of cases (about 70%). The Ole e 1-like protein family comprises several other allergenic glycosylated proteins from tree pollen (Fra e 1, Lig v 1, and Syr v 1), whose glycan moieties are involved in the allergenic properties of these molecules (Rodrıguez et al., 2001). Besides Ole e 1, several other molecules have been identified and a biologic function can be associated with most of these molecules, such as actin-binding protein (the profilin Ole e 2), polcalcin (Ole e 3 and Ole e 8), glucanase (Ole e 9 and its probable degradation product Ole e 4), superoxide dismutase (Ole e 5) and lipid transfer protein (Ole e 7). Moreover, in olive pollen patients a polysensitisation is more common than monosensitisation. Reactivity to other genera belonging to the Oleaceae family, that is, Fraxinus excelsior or Ligustrum vulgare, is relevant in several regions in Central and southern Europe (Asero, 2011; Niederberger et al., 2002). Olive tree Ole e 1 is a 145 amino acid protein sharing both significant sequence identity (82.76% of identity with 120 Identical positions and 19 similar positions) and IgE cross-reactivity with all the other related trees belonging to the Olive family (Fra e 1 from ash, Lig v 1 from privet and Syr v 1 from lilac) (Asero, 2011; Niederberger et al., 2002). Several Ole e 1-like molecules have been described in goosefoot (Chenopodium album, Che a 1), timothy (Phleum pretense, Phl p 11), ryegrass (Lolium perenne, Lol p 11), English plantain (Plantago lanceolata Pla l 1) and prickly saltwort (Salsola kali, Sal k 5), but the real clinical cross-reactivity of these molecules not belonging to the Olive family with Ole e 1 is somewhat questioned (Asero, 2011; Niederberger et al., 2002).

Plant defensins represent a major innate immune protein superfamily with strong inhibitory effects on infectious diseases of humans, antifungal/antibacterial activities, proteinase and insect amylase inhibitory activities. Gachomo et al. identified members of defensin protein families across plant species and use protein-modeling-based structural reconstitution to reveal specific three-dimensional hidden features of plant defensins mediating defense responses and other interesting biological activities in plants; data revealed that plant defensins are structurally similar to their insect counterparts despite the low amino acid sequence similarity between these two organisms (Gachomo et al., 2012 April). Arginine kinase is one of the key enzymes, responsible for the parasites' metabolic plasticity, which maintains the cell energy homeostasis during environment changes. Arginine kinase catalyses the reversible phosphorylation between phosphoarginine and ADP (Pereira, 2014).

Seafood refers to several distinct groups of edible aquatic animals including fish, crustacean, and mollusc. Many allergenic proteins are very different between these groups;

however, some panallergens, including parvalbumin, tropomyosin and arginine kinase, seem to induce immunological and clinical cross-reactivity (Ruethers et al., 2018 August). Human manganese superoxide dismutase is a homotetramer and represents an essential mitochondrial antioxidant enzyme, which catalyses the dismutation of superoxide radicals (O2˙-) at near diffusion-controlled rates (Demicheli et al., 2018 May 23). The cupin superfamily of proteins, named on the basis of a conserved beta-barrel fold ("cupa" is the Latin term for a small barrel), was originally discovered using a conserved motif found within germin and germin-like proteins from higher plants (Dunwell et al., 2004 January). Globulins are a major class of seed storage proteins which were thought to be enzymatically inactive. These proteins belong to the most ancient cupin superfamily. They can be graded into 11S legumin type and 7S vicilin type based on their sedimentation coefficients. Globulins are known to define the nutritional quality of the seeds, however, they are also involved in sucrose binding, desiccation, defense against microbes, hormone binding and oxidative stress, etc. Major drawback with globulins is their tendency to bind to IgE (Kesari et al., 2017). In evaluating all specific IgE results to all allergen reagents (Cluster 7, Supplementary Table S1) we observe the grouping of molecular components from house dust mite, shrimp, cockroach, storage mites. The shrimp (*Penaeus aztecus*) major allergen, Pen a 1, is one of the most clinically relevant allergenic tropomyosins (Ahumada et al., 2015; Daul et al., 1994; Reese et al., 2006). Tropomyosins from invertebrates are allergenic for genetically susceptible individuals, and due to their extensive cross-reactivity, are considered panallergens. Most allergenic tropomyosins are major shellfish allergens. Symptoms may be induced by very low amounts of the offending food and sometimes by inhalation. In Europe, sensitisation to mite tropomyosin Der p 10 is low and has been considered an effect of cross-reactivity but also a marker for broad sensitisation among house dust mite-allergic patients. Component-resolved diagnosis of *D. pteronyssinus* allergens Der p 1, Der p 2, and Der p 10 has been suggested for selecting patients for house dust mite immunotherapy. The prevalence of sensitisation to Der f 10 was found around 80% in Japan. In addition, sensitisation to Der p 10 was found 55% in Zimbabwe and 34% in Colombia, probably because of perennial exposure to shellfish and helminth infections. Therefore, the clinical impact of non-food allergenic tropomyosins may be greater than previously thought. In fact, it has been suggested that sensitisation to tropomyosin from mite cockroach, Ascaris (Breiteneder et al., 1988), and mosquito could influence the prevalence and severity of asthma in places where coexposition to several sources of tropomyosin occurs. The frequency of IgE sensitisation to tropomyosins in shellfish-allergic patients ranges from 50% to 100%. In addition, Pen a 1 binds up to 75% of all shrimp-specific IgE antibodies, which is supported by histamine release experiments (Daul et al., 1994; Reese et al., 1997; Reese et al., 2006).

In our previous study, we confirmed, that the severity of AD is in significant relation to the sensitisation to molecular components of storage mites, lipocalins, arginin kinase, uteroglobin, manganese superoxide dismutase (Mala s 11), PR 10 proteins, Der p 21, Der p 23 – peritrophin-like domain and to Secc pollen (Čelakovská et al., 2021). Our previous study confirms, that the level of specific IgE to Der p 23 in class 4 was recorded with the significantly higher occurrence in patients suffering from a severe form of AD and as well in patients suffering from bronchial asthma. The close association of Der p 23 with fecal pellets may be one reason why it represents a major allergen and exhibits high

allergenic activity (Čelakovská et al., 2021). In our current study with cluster analysis evaluating all classes of specific IgE we find Der p 23 (house dust mite, Peritrophin-like protein domain) in one cluster (Cluster 4) with specific IgE to Der p 1 (house dust mite, cysteine protease), Der f 1 (house dust mite, cysteine protease), and Lep d 2 (storage mite, NPC2 family). In cluster analysis evaluating the specific IgE with high and very high level (class 3, class 4), the specific IgE results to Der p 23 are grouped with specific IgE to Der p 20, Der p 21 in one cluster (Cluster 6) with specific IgE results to molecular components of arginin kinase, tropomyosin, Peritrophin-like protein domain, cystein protease, troponin C, profilin, conglutinin and allergen extract from shrimp, house dust mites, storage mites from locust, mealworm, house cricket and squid. In 2014, it was reported that IgE antibodies to Der p 11 are more common in sera from patients with atopic dermatitis (Banerjee et al., 2015). In our current study, specific IgE to Der p 11 is grouped with specific IgE results to another 150 allergen reagents. We also confirmed, that the level of specific IgE to Der f 2, Der p 2 (house dust mites, NPC2 family) are significantly higher in subgroup of patients suffering from bronchial asthma (Čelakovská et al., 2021); these results correspond to current cluster analysis.

In our previous study evaluating cluster analysis of specific IgE results in ISAC Multiplex testing, we found 10 clusters with different numbers of molecular components (Čelakovská et al., 2020a; Čelakovská et al., 2020b). Fundamental position have the components Phl p 1 (Timothy), Bet v 1 (Birch), Alt a 1 (Alternaria) followed by molecular components of NPC2 family, cystein proteasa, tropomyosin, uteroglobin, lipocalin and PR-10 protein. Our results corresponded also to the association of molecular components into protein families according to their biochemical structure. Exceptional position was recorded for molecular component Alt a 1, which was found as a single component (Čelakovská et al., 2020a; Čelakovská et al., 2020b). On the other hand, in the current study with evaluating all results of specific IgE in classes 0-4, the specific IgE results to Alt a 1 and Alt a 6 are grouped in cluster 9 together with specific IgE results to 77 other allergen reagents and together with objects such as asthma bronchiale and allergic rhinitis. Sensitisation to Alternaria alternata spores are considered a well-known biological contaminant and a very common potent aeroallergen source that is found in environmental samples. The major allergen, Alt a 1, has been reported as the main elicitor of airborne allergies in patients affected by a mold allergy and is considered a marker of primary sensitisation to Alternaria alternata. Moreover, Alternaria alternata sensitisation seems to be a triggering factor in the development of polysensitisation, most likely because of the capability of Alternaria alternata to produce, in addition to Alt a 1, a broad and complex array of cross-reactive allergens that present homologs in several other allergenic sources (Chruszcz et al., 2012).

Eosinophils and their degradation products eosinophil-derived neurotoxin and eosinophil cationic protein are detectable in the inflammatory infiltrate of AD and correlate with disease severity (Byeon et al., 2020). Therefore, eosinophils are considered as potential effector cells, and therapeutic approaches targeting IL-5, the most important cytokine involved in eosinophil biology, were designed accordingly. In our previous studies, we evaluated the role of eosinophils in AD patients suffering from food hypersensitivity reactions. As for the single foods, the most prominent eosinophilia is recorded in patients suffering from reactions to carrot and the difference is statistically significant in

comparison with patients without these reactions. In patients with food hypersensitivity reactions to celery, tangerines, capsidum, oranges, and kiwi, the count of eosinophils is higher in comparison to patients without these reactions, but the difference is not statistically significant (Celakovska & Bukac, 2017). In our other study, we correlated the eosinophil count with the manifestations such as asthma bronchiale, rhinitis, level of total IgE, sensitisation to mites, animal dander, bird feather, dust, mixture of grass, mixture of trees, mixture of fungi, duration of lesions (persistent or occasional during last year), family history about atopy, and onset of AD (under or above 5 years of age). Two hundred and seventy-two patients suffering from AD at the age of 14 year or older were examined – 100 men and 172 women with an average age of 26.7 ± 9.5 years and with an average SCORAD index of 32.9 ± 14.1. The count of eosinophils in peripheral blood was significantly higher in patients with total IgE ≥ 200 IU/ml, with sensitisation to dust, with persistent eczematous lesions and in patients with the onset of AD under 5 years of age. The count of eosinophils above 5% was recorded as well in patients suffering from asthma bronchiale, rhinitis, sensitisation to mites, and in patients with positive family history about atopy, but the difference was not significant. On the other hand, the count of eosinophils was under 5% in patients with sensitisation to animal dander, bird feather, mixture of grass and trees (Celakovská et al., 2019 January-February).

According to the last studies, interleukin-33 (IL-33) is an inflammatory cytokine that is over-expressed in the keratinocytes of patients with AD. IL-33 transgenic mice, which express IL-33 specifically in keratinocytes, spontaneously develop AD-like eczema, suggesting that IL-33 is sufficient for the development of AD. IL-33 stimulates various cells, including group 2 innate lymphoid cells (ILC2s), to produce type 2 cytokines, such as IL-5 and IL-13, and IL-33-stimulated basophils activate ILC2s via IL-4. ILC2s are enriched in human AD skin lesions, and ILC2 isolated from AD lesions, are activated by IL-33, not by thymic stromal lymphopoietin (TSLP). IL-33 induces IL-31, thereby promoting pruritus and scratching behaviour. Conversely, scratching the skin promotes IL-33 release from keratinocytes. IL-33 reduces the expression of filaggrin and claudin-1; it also reduces the skin barrier function. However, barrier destruction causes percutaneous exposure to allergens or IL-33 release. Thus, IL-33 is a common point of entry into the itch-scratch cycle of AD. These new findings can facilitate the development of novel therapeutic drugs targeting IL-33 (Imai, 2019 October).

Günther et al. found, that AD patients have elevated levels of CCL18 in serum and lesional skin and that CCL18 recruits human memory T cells derived from AD patients to human skin in vivo; their results provide conclusive evidence linking CCL18 protein expression with various stages of AD and demonstrating the capacity of CCL18 to recruit memory T cells into human skin in vivo (Günther et al., 2005).

Altrichter et al. investigated autoantigenic cell structures in atopic drematitis patients and analysed by immunohistology, confocal laser microscopy, and flow cytometry. Their analysis revealed that 28% of AD patients, but not healthy individuals, display serum IgE autoreactivity by western-blot analysis. The individual IgE reaction patterns of the sera pointed to the existence of unique as well as common specificities against epidermal or A431-derived proteins. Immunostainings identified cytoplasmic and, occasionally, also cell membrane-associated moieties as targets for autoreactive IgE antibodies. They concluded that IgE autoreactivity is common, particularly among severe AD patients,

and that non-transformed primary cells are needed for characterisation of the entire spectrum of IgE-defined autoantigens (Altrichter et al., 2008 September).

Conclusion

The results of specific IgE were formed in clusters with different numbers of objects (allergen reagents). In evaluating all results of specific IgE, we observe, that majority of specific IgE results (from 230 allergen reagents) are found in two clusters. The results of specific IgE from other allergen reagents are divided into clusters, which correspond to the division into protein families. Although the cluster analysis algorithm divides allergens into clusters, the links between allergens reagents (molecular components) are weak. The size of the average Silhouette is 0.0977 for 8 clusters, which means no significant structure was found. For 12 clusters, this value is equal to 0.141. Only one binding between molecular components was strong, (Der p 2, Der f 2). In evaluating specific IgE in classes 3 and 4, we observe that family history about atopy and bronchial asthma are recorded together with molecular components Der f 2 and Der p 2 in one cluster. The object such as the severity of AD is found in one other cluster and objects such as allergic rhinitis, onset of AD and duration of lesions are found together in another cluster. In evaluating all classes of specific IgE we find Der p 23 (house dust mite, Peritrophin-like protein domain) in one cluster with specific IgE to Der p 1 (house dust mite, cysteine protease), Der f 1 (house dust mite, cysteine protease), and Lep d 2 (storage mite, NPC2 family). In cluster analysis evaluating the specific IgE with high and very high level (class 3, class 4), the specific IgE results to Der p 23 are grouped with specific IgE to Der p 20, Der p 21 in one cluster together with specific IgE results to molecular components of arginin kinase, tropomyosin, lipocalin, Peritrophin-like protein domain, cystein protease, troponin C, profilin, conglutinin and allergen extract from shrimp, house dust mites, storage mites from locust, mealworm, house cricket and squid. Our results strongly point towards cross-reactivity for crustacean-allergic patients to desert locust, house cricket and stable flies. In another cluster we find the results of sIgE to molecular components from Timothy, Bermuda grass, molecular components from grass group 5/6 and allergen Cultivated rye; our study confirms the privileged role of beta expansins of Timothy. Our results show how sensitisation to allergen reagents can be associated with patients suffering from atopic dermatitis. The cluster analysis combining the results of specific IgE for the basic allergen reagents (molecular components) together with the results of specific IgE for less common allergens (molecular components) can help in the estimating of allergic reactions. According to the inclusion of specific IgE results in the clusters, we can focus on the search for other possible allergens.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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