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Cluster analysis of molecular components in 100 patients suffering from atopic dermatitis according to the ISAC Multiplex testing

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ABSTRACT

Aim: The cluster analysis for the evaluation of the results in Multiplex ISAC test in atopic dermatitis patients. **Method:** The complete dermatological and allergological examination including the examination of the sensitisation to molecular components with Multiplex ISAC testing was performed. The cluster analysis of molecular components (silhouette value) was processed. **Results and conclusion:** Altogether 100 atopic dermatitis patients were examined - 48 men, 52 women, the average age 40.9 years. We found 10 clusters with different numbers of molecular components. 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 correspond to the association of molecular components into protein families according to their biochemical structure.

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Atopic dermatitis; molecular components; multiplex ISAC testing; protein families; biochemical structure; cluster analysis - silhouette

Introduction

Diagnostic work – up for IgE mediated allergic reactions starts with the history, followed by sensitisation tests (skin, IgE and basophil tests) and optional challenge tests (Top – down approach). Molecular allergens for IgE testing provide additional information, particularly in polysensitized patients and with allergens of low abundance, low stability or associated risks. IgE reactivity to members of the same allergen family reflect the degree of protein homology and IgE cross – reactivity. If it is high, the relevance needs to be sorted out clinically. In case it is low, selected IgE testing of other family members can provide additional information. Proper interpretation should complete diagnostic testing. Positive sensitisations to allergen extracts or molecules are only clinically relevant in case of corresponding symptoms (Matricardi et al., 2016). Testing with molecular components is a part of the development of novel allergy vaccines and identification of non-

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allergenic isoforms (Matricardi et al., 2016). The introduction of allergen molecules has had a major effect on analytic specificity and allergy diagnosis. They are used in both singleplex ImmunoCAP and multiplex ImmunoCAP ISAC assays. The major advantage of ISAC is the comprehensive IgE pattern obtained with a minute amount of serum (van Hage et al., 2017). ImmunoCAP ISAC (Thermo Fisher), based on 112 different molecular components (both extracted and recombinant), is the most studied and most frequently used molecular diagnostic tool based on a microarray (Melioli et al., 2011). The usefulness of ImmunoCAP ISAC has been validated in a wide spectrum of allergic diseases like asthma, allergic rhinoconjunctivitis, atopic dermatitis, eosinophilic esophagitis, food allergy and anaphylaxis. ISAC provides a broad picture of a patient's sensitization profile from a single test, and provides information on specific and cross-reactive sensitizations that facilitate diagnosis, risk assessment, and disease management. Furthermore, it can reveal unexpected sensitizations which may explain anaphylaxis previously categorized as idiopathic and also display for the moment clinically non-relevant sensitizations. ISAC can facilitate a better selection of relevant allergens for immunotherapy compared with extract testing. Microarray technique can visualize the allergic march and molecular spreading in the preclinical stages of allergic diseases, and may indicate that the likelihood of developing symptomatic allergy is associated with specific profiles of sensitization to allergen components. ISAC is shown to be a useful tool in routine allergy diagnostics due to its ability to improve risk assessment, to better select relevant allergens for immunotherapy as well as detecting unknown sensitization. Multiplex component testing is especially suitable for patients with complex symptomatology (Melioli et al., 2011).

Proteins that are described worldwide as allergens can be classified roughly into 30–40 protein families. Based on their molecular, biologic and biochemical properties, allergenic proteins are able to initiate both innate and adaptive immune responses during the sensitisation process that ultimately result in the production of allergen – specific IgE. The most important families and selected allergenic member proteins are shown in Table 1.

The list of the molecular components in ImmunoCAP ISAC (Phadia, Thermo Fisher Scientific, Uppsala, Sweden) sorted by protein group is recorded in Table 2. We distinguish these superfamilies: Prolamin (family Cereal prolamins, Bifunctional inhibitors,

Table 1. The most important allergen superfamilies and families with their corresponding sources (Matricardi et al., 2016).

Superfamily	Family	Sources
Prolamin	cereal prolamin	grains of cereal grasses
	bifunctional inhibitors	grains of cereal grasses
	2S albumin	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

Table 2. The list of the molecular components sorted by protein group. (ImmunoCAP ISAC - Phadia, Thermo Fisher Scientific, Uppsala, Sweden).

Allergen Source	Molecular component	Protein Group
Food components		
Egg white	nGal d 1	Ovomucoid
	nGal d 2	Ovalbumin
	nGal d 3	Conalbumin/ Ovotransferrin
Egg yolk/ chicken meat	nGal d 5	Livetin/Serum albumin
Cow's milk	nBos d 4	Alpha-lactalbumin
	nBos d 5	Beta-lactoglobulin
	nBos d 8	Casein
	nBos d lactoferrin	Transferrin
Cod	rGad c 1	Parvalbumin
Shrimp	nPen m 2	Arginine kinase
	nPen m 4	Sarcoplasmic calcium binding protein
Cashew nut	rAna o 2	Storage protein, 11S globulin
Brazil nut	rBer e 1	Storage protein, 2S albumin
Hazelnut	nCor a 9	Storage protein, 11S globulin
Walnut	rJug r 1	Storage protein, 2S albumin
	nJug r 2	Storage protein, 7S globulin
Sesame seed	nSes i 1	Storage protein, 2S albumin
Peanut	rAra h 1	Storage protein, 7S globulin
	rAra h 2	Storage protein, 2S albumin
	rAra h 3	Storage protein, 11S globulin
	nAra h 6	Storage protein, 2S albumin
Soybean	nGly m 5	Storage protein, Beta-conglycinin
	nGly m 6	Storage protein, Glycinin
Buckwheat	nFag e 2	Storage protein, 2S albumin
Wheat	rTri a 14	Lipid transfer protein (nsLTP)
	rTri a 19.0101	Omega-5 gliadin
	nTri a aA_Ti	Alpha-amylase/ Trypsin inhibitor
Kiwi	nAct d 1	Cysteine protease
	nAct d 5	Kiwelling
Aeroallergen components		
Grass pollen		
Bermuda grass	nCyn d 1	Grass group 1
Timothy grass	rPhl p 1	Grass group 1
	rPhl p 2	Grass group 2
	nPhl p 4	Berberine bridge enzyme
	rPhl p 5	Grass group 5
	rPhl p 6	Grass group 6
	rPhl p 11	Ole e 1-related protein
Tree pollen		
Birch	rBet v 1	PR-10 protein
Japanese cedar	nCry j 1	Pectate lyase
Cypress	nCup a 1	Pectate lyase
Olive pollen	rOle e 1	Common olive group 1
	rOle e 9	Beta-1,3-glucanase
Plane tree	rPla a 1	Putative invertase inhibitor
	nPla a 2	Polygalacturonase
Weed pollen		
Ragweed	nAmb a 1	Pectate lyase
Mugwort	nArt v 1	Defensin
Goosefoot	rChe a 1	Ole e 1-related protein
Wall pellitory	rPar j 2	Lipid transfer protein (nsLTP)
Plantain	rPla l 1	Ole e 1-related protein
Saltwort	nSal k 1	Pectin methylesterase
Animal		
Dog	rCan f 1	Lipocalin
	rCan f 2	Lipocalin
	rCan f 5	Arginine esterase
Horse	rEqu c 1	Lipocalin
Cat	rFel d 1	Uteroglobin

(Continued)

Table 2. Continued.

Allergen Source	Molecular component	Protein Group
Mouse	rFel d 4	Lipocalin
Mold	nMus m 1	Lipocalin
Alternaria	rAlt a 1	Acidic glycoprotein
	rAlt a 6	Enolase
Aspergillus	rAsp f 1	Mitogillin family
	rAsp f 3	Peroxisomal protein
	rAsp f 6	Mn superoxide dismutase
Cladosporium	rCla h 8	Mannitol dehydrogenase
Mite		
House dust mite	rBlo t 5	Mite group 5
	nDer f 1	Cysteine protease
	rDer f 2	NPC2 protease
	nDer p 1	Cysteine protease
	rDer p 2	NPC2 family
Storage mite	rLep d 2	NPC2 family
Cockroach		
Cockroach	rBla g 1	Cockroach group 1
	rBla g 2	Aspartic protease
	rBla g 5	Glutathione S-transferase
Other components		
Venom		
Honey bee venom	rApi m 1	Phospholipase A2
	nApi m 4	Melittin
Paper wasp venom	rPol d 5	Venom, Antigen 5
Common wasp venom	rVes v 5	Venom, Antigen 5
Parasite		
Anisakis	rAni s 1	Serine protease inhibitor
Latex		
Latex	rHev b 1	Rubber elongation factor
	rHev b 3	Small rubber particle protein
	rHev b 5	Acidic protein
	rHev b 6.01	Prohevein
Cross-reactive components		
Serum albumin		
Cow's milk/meat	nBos d 6	Serum albumin
Dog	nCan f 3	Serum albumin
Horse	nEqu c 3	Serum albumin
Cat	nFel d 2	Serum albumin
Tropomyosin		
Anisakis	rAni s 3	Tropomyosin
Cockroach	nBla g 7	Tropomyosin
House dust mite	rDer p 10	Tropomyosin
Shrimp	nPen m 1	Tropomyosin
Lipid transfer protein (nsLTP)		
Peanut	rAra h 9	Lipid transfer protein (nsLTP)
Hazelnut	rCor a 8	Lipid transfer protein (nsLTP)
Walnut	nJug r 3	Lipid transfer protein (nsLTP)
Peach	rPru p 3	Lipid transfer protein (nsLTP)
Mugwort	nArt v 3	Lipid transfer protein (nsLTP)
Olive pollen	nOle e 7	Lipid transfer protein (nsLTP)
Plane tree	rPla a 3	Lipid transfer protein (nsLTP)
PR-10 protein		
Birch	rBet v 1	PR-10 protein
Alder	rAln g 1	PR-10 protein
Hazel pollen	rCor a 1.0101	PR-10 protein
Hazelnut	rCor a 1.0401	PR-10 protein
Apple	rMal d 1	PR-10 protein
Peach	rPru p 1	PR-10 protein
Soybean	rGly m 4	PR-10 protein
Peanut	rAra h 8	PR-10 protein

(Continued)

Table 2. Continued.

Allergen Source	Molecular component	Protein Group
Kiwi	rAct d 8	PR-10 protein
Celery	rApi g 1	PR-10 protein
Thaumatine-like protein		
Kiwi	nAct d 2	Thaumatine-like protein
Profilin		
Birch	rBet v 2	Profilin
Latex	rHev b 8	Profilin
Annual mercury	rMer a 1	Profilin
Timothy grass	rPhl p 12	Profilin
CCD		
Sugar epitope from Bromelain	nMUXF3	Cross-reactive Carbohydrate Determinants
Polcalcine (Calcium binding 2-EF-hand protein)		
Birch	rBet v 4	Polcalcine
Timothy grass	rPhl p 7	Polcalcine

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 (Matricardi et al., 2016; Nicolaou et al., 2010; Vieths et al., 2002).

The aim of our study is to evaluate the results of examination with Multiplex ImmunoCAP ISAC in atopic dermatitis according to the Cluster analysis. This statistic method is commonly used to group similar samples across a diverse range of applications. Typically, the goal of clustering is to form groups of samples that are more similar to each other than to samples in other groups (Frades & Matthiesen, 2010). 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). In our study, we evaluate if these results correspond to the association of molecular components into protein families according to their biochemical structure. Our results can demonstrate the importance of molecular components in atopic dermatitis patients.

Patients and methods

In the period 2018–2019, 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 long term therapy with cyclosporin or systemic corticoids, pregnancy, breastfeeding. Patients with atopic dermatitis having other systemic diseases were excluded from the study as well. 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 molecular components

The serum level of the sIgE was measured by the component-resolved diagnosis microarray-based sIgE detection assay ImmunoCAP ISAC (Phadia, Thermo Fisher Scientific, Uppsala, Sweden). ImmunoCAP ISAC is a solid-phase multiple immunoassay which enables to determine 112 different components from 51 allergen sources (Jakob et al., 2015). The allergens are applied in triplicates to ensure the test reproducibility. The specific IgE values are presented in arbitrary units called ISAC Standardized Units (measuring range of 0.3 - 100 ISU-E). The level of specific IgE higher than 0.3 ISU-E was assessed as positive. The level of molecular components was evaluated in ISU -E: < 0.3 - negative, 0.3- >0.9 low positivity, 0.9 > 15 moderate positivity, ≥ 15 ISU -E very high positivity (Choi et al., 2014; Jakob et al., 2015).

Statistic

We analysed the data to determine 10 clusters with molecular components according to the positivity to molecular components and to their level of specific IgE. The level of molecular components in ISU -E was evaluated: < 0.3 - negative, 0.3-0.9 ISU -E low positivity, 0.9-15 ISU -E moderate positivity, above 15 ISU -E very high positivity. 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 (Kaufman & Rousseeuw, 1990; Rousseeuw, 1987).

Interpreting silhouettes

A silhouette value is constructed for each object. The value can range from minus one to one. 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. Why isn't it in the neighbouring cluster? Hence, the silhouette value summarizes how appropriate each object's cluster is.

Determining the number of clusters

One useful summary statistic is the average value of s across all objects. This summarizes 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 maximizes the average silhouette. We denote the maximum average silhouette across all values of k as SC . Kaufman and Rousseeuw (Kaufman & Rousseeuw, 1990) present the following table to aid in the interpretation of SC . We evaluated our data according to this table, it is shown in Table 3.

Table 3. Cluster analysis - Proposed Interpretation (Kaufman & Rousseeuw, 1990).

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

Results

Patients

Altogether 112 different components from 51 allergen sources were examined in 100 atopic dermatitis patients included in the study (48 men and 52 women with the average age 40.9 years and with the average SCORAD 39, s.d.13.1 points). The mild form of AD was recorded in 14 patients (14%), moderate form in 58 patients (58%), severe form in 28 patients (28%). In the included patients, 55 patients (55%) suffer from asthma bronchiale and 78 patients (78%) suffer from rhinitis. The positive results in sensitisation to molecular components was recorded in 93 patients (93%). The characteristics of patients is shown in Table 4.

We found 10 clusters with different numbers of molecular components.

The results of ISAC Multiplex testing evaluated by Clustering by Medoid Partitioning Report are shown in Table 5. We show the average of Silhouette Value: **Cluster 1:** Phl p 1 (Silhouette Value 0.000), **Cluster 2:** Phl p 4 (Silhouette Value 0.000), **Cluster 3:** Phl p 2, 5, 6, Cyn d 1 (Silhouette Value 0.1278), **Cluster 4:** Bet v 1 (Silhouette Value 0.000), **Cluster 5:** Alt a 1 (Silhouette Value 0.000), **Cluster 6:** Der f 2, Der p 2, Der p 1, Der f 1 (Silhouette Value 0.4167), **Cluster 7:** Pen m 2, Mus m 1, Lep d 2, Can f 5, Phl p 11, Pen m 1, Bla g 7, Der p 10, Ole e 1, Blot 5, Ani s 3 (Silhouette Value - 0.0893), **Cluster 8:** Can f 1, Fel d 1, Fel d 4, Equ c 1 (Silhouette Value - 0.0357), **Cluster 9:** Pru p 1, Cor a 1.010, Mal d 1, Aln g 1, Cor a 1.040 (Silhouette Value 0.3692), **Cluster 10:** 82 molecular components in ISAC multiplex testing (Silhouette Value 0.3743). Groups of 10 Clusters of molecular components with their biochemical name, protein family and allergen source and number of patients with positive results to molecular components are recorded in Table 6.

Discussion

The sense of our study was to evaluate the results of examination of molecular components with ISAC Multiplex testing in atopic dermatitis patients with the use of the Clustering

Table 4. The characteristic of patients with atopic dermatitis

- 100 patients examined – 48 men, 52 women, the average age 40.9 years
- the average SCORAD 39 s.d. 13.1 points
- the positive results in sensitisation to molecular components - 93 patients (93%)
- mild form of AD patients 14 (14%)
- moderate form of AD patients 58 (58%)
- severe form of Ad patients 28 (28%)
- number of patients with asthma bronchiale – 55 (55%)
- number of patients with allergic rhinitis - 78 (78%)

Table 5. Results of ISAC Muxplex testing evaluated by Clustering by Medoid Partitioning Report. There are 10 clusters. Cluster average is shown extra bold.

Row Detail Section						
Row	Cluster	Nearest Neighbor	Average Distance Within	Average Distance Neighbor	Silhouette Value	Silhouette Bar
rPhlp1	1	3	0.00	74.59	0.0000	
Cluster Average	1	(1)	0.00	74.59	0.0000	
nPhlp4	2	3	0.00	74.62	0.0000	
Cluster Average	2	(1)	0.00	74.62	0.0000	
Phlp5	3	9	53.09	69.49	0.2360	
Phlp6	3	10	48.22	59.64	0.1916	
Cynd1	3	9	58.66	64.85	0.0954	
rPhlp2	3	9	56.77	56.10	−0.0118	
Cluster Average	3	(4)	54.19	62.52	0.1278	
Betv 1	4	9	0.00	67.67	0.0000	
Cluster Average	4	(1)	0.00	67.67	0.0000	
Alta1	5	10	0.00	69.05	0.0000	
Cluster Average	5	(1)	0.00	69.05	0.0000	
Derf2	6	9	35.94	69.38	0.4820	
Derp2	6	9	37.22	70.83	0.4745	
Derp1	6	7	43.28	68.43	0.3676	
Derf1	6	7	38.00	57.81	0.3427	
Cluster Average	6	(4)	38.61	66.61	0.4167	
Penm2	7	10	46.75	47.46	0.0151	
Musm1	7	10	45.67	45.26	−0.0088	
Lepd2	7	10	37.32	35.99	−0.0356	
Canf5	7	10	48.96	47.18	−0.0363	
Phlp11	7	10	44.86	43.18	−0.0373	
Penm1	7	10	28.14	24.81	−0.1184	
Blag7	7	10	27.17	23.85	−0.1221	
Derp10	7	10	27.17	23.85	−0.1221	
Olee1	7	10	39.58	34.51	−0.1279	
Blot5	7	10	34.58	29.55	−0.1455	
Anis 3	7	10	28.46	21.54	−0.2431	
Cluster Average	7	(11)	37.15	34.29	−0.0893	
Canf1	8	10	64.71	68.78	0.0591	
Feld1	8	3	62.72	65.55	0.0432	
Feld4	8	10	49.20	44.46	−0.0964	
Equc1	8	10	51.63	43.94	−0.1488	
Cluster Average	8	(4)	57.06	55.68	−0.0357	
Prup1	9	10	28.47	53.32	0.4660	
Cora1.010	9	10	30.61	54.65	0.4400	
Mald1	9	10	32.84	58.47	0.4384	
Alng1	9	10	31.69	55.45	0.4285	
Cora1.040	9	10	34.56	56.22	0.3853	
Arah8	9	10	30.56	47.70	0.3593	
Glym4	9	10	36.30	38.90	0.0668	
Cluster Average	9	(7)	32.15	52.10	0.3692	
Fage2	10	7	14.70	31.00	0.5260	
Plaa1	10	7	14.70	31.00	0.5260	
Bosd4	10	7	14.70	31.00	0.5260	
Alng4	10	7	14.70	31.00	0.5260	
Apim4	10	7	14.70	31.00	0.5260	
Actd5	10	7	14.70	31.00	0.5260	
Pla1	10	7	14.70	31.00	0.5260	
Bere1	10	7	14.70	31.00	0.5260	
rhevb602	10	7	14.70	31.00	0.5260	
Lolp1	10	7	14.70	31.00	0.5260	
TriaGliad	10	7	14.70	31.00	0.5260	
Cora9	10	7	15.17	30.99	0.5106	
Bosd5	10	7	15.19	31.00	0.5102	

(Continued)

Table 5. Continued.

Row Detail Section						
Row	Cluster	Nearest Neighbor	Average Distance Within	Average Distance Neighbor	Silhouette Value	Silhouette Bar
Blag1	10	7	15.28	31.02	0.5075	
Olee7	10	7	15.64	31.38	0.5017	
Glym5	10	7	15.39	30.58	0.4965	
Tri14	10	7	15.83	31.15	0.4920	
Blag5	10	7	16.08	31.27	0.4857	
Bosd	10	7	16.18	31.41	0.4848	
Hevb3	10	7	16.60	31.57	0.4741	
Bosd6	10	7	16.78	31.75	0.4714	
Glym6	10	7	16.85	31.55	0.4661	
Phlp12	10	7	17.13	31.91	0.4632	
Apim1	10	7	16.55	30.80	0.4626	
TriaaA	10	7	16.67	30.84	0.4596	
Arah6	10	7	17.24	31.68	0.4558	
Salk 1	10	7	16.69	30.58	0.4541	
Gald5	10	7	17.73	31.93	0.4448	
Hevb1	10	7	17.68	31.73	0.4430	
Bosd8	10	7	18.00	32.23	0.4415	
Equc3	10	7	18.21	32.29	0.4360	
Blag2	10	7	18.10	32.00	0.4342	
Hevb5	10	7	18.42	32.45	0.4322	
Anis 1	10	7	17.33	30.52	0.4321	
Clah8	10	7	17.75	31.01	0.4276	
Sesi1	10	7	18.89	32.48	0.4184	
Penm4	10	7	17.77	30.50	0.4175	
Cora8	10	7	19.85	33.77	0.4122	
gald2	10	7	19.65	33.22	0.4084	
Arah2	10	7	19.78	32.81	0.3969	
Arah3	10	7	19.91	32.96	0.3961	
Plaa3	10	7	20.84	34.51	0.3961	
Arah1	10	7	19.97	32.65	0.3883	
Gald1	10	7	19.39	31.46	0.3837	
Artv 3	10	7	20.88	33.71	0.3807	
Hevb6.01	10	7	20.85	33.32	0.3743	
Jugr3	10	7	21.90	34.91	0.3726	
Arah9	10	7	21.44	34.17	0.3725	
Aspf1	10	7	19.39	30.89	0.3724	
Pold5	10	7	20.82	33.17	0.3722	
Jugr1	10	7	21.29	33.88	0.3715	
Prup3	10	7	22.23	34.51	0.3560	
Parj2	10	7	21.09	32.74	0.3560	
Feld2	10	7	21.32	32.65	0.3472	
Gald3	10	7	21.31	32.17	0.3377	
Canf3	10	7	21.82	32.89	0.3366	
Gadc1	10	7	22.21	33.16	0.3302	
Olee9	10	7	23.96	35.12	0.3178	
Betv 4	10	7	23.24	33.77	0.3118	
Vesv 5	10	7	23.68	34.20	0.3077	
Aspf3	10	7	21.71	31.31	0.3065	
Betv2	10	7	24.30	34.79	0.3017	
Actd1	10	7	24.03	34.35	0.3005	
Jugr2	10	7	22.89	32.50	0.2958	
Anao2	10	7	27.31	37.49	0.2715	
Mera1	10	7	26.40	35.94	0.2654	
Cryj1	10	7	24.66	33.01	0.2530	
Cupa1	10	7	25.67	34.32	0.2521	
Actd8	10	7	29.74	37.96	0.2165	
Phlp7	10	7	26.86	34.18	0.2141	

(Continued)

Table 5. Continued.

Row Detail Section						
Row	Cluster	Nearest Neighbor	Average Distance Within	Average Distance Neighbor	Silhouette Value	Silhouette Bar
Chea1	10	7	28.64	36.14	0.2074	
Alta6	10	7	31.07	39.18	0.2069	
Apig1	10	7	31.64	39.74	0.2037	
Hevb8	10	7	35.18	43.19	0.1856	
MUXF3	10	7	29.71	36.11	0.1771	
Amba1	10	7	41.61	49.24	0.1550	
Plaa2	10	7	32.62	38.12	0.1442	
Plal1	10	7	41.95	48.63	0.1373	
Actd2	10	7	43.67	50.11	0.1285	
Aspf6	10	7	45.31	50.68	0.1060	
Canf2	10	7	41.26	45.96	0.1024	
Artv 1	10	7	61.00	64.60	0.0559	
Cluster Average	10	(82)	22.01	34.29	0.3743	
Overall Average		(116)	26.19	39.47	0.2960	= SC

analysis. It needs to be emphasized that in this paper we focus only on sensitization rates and not their clinical relevance without using specific provocation tests.

The clustering problem has been addressed in many contexts and disciplines. Cluster analysis encompasses different methods and algorithms for grouping objects of similar kinds into respective categories. Clustering techniques are widely used in the analysis of large datasets to group together samples with similar properties. There are many algorithms for performing clustering, and the results can vary substantially (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 lie 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. Silhouette combines two clustering criteria, compactness and separation, which imply that spherical cluster shapes are preferred over others—a property that can be seen as a disadvantage in the presence of complex, nonspherical clusters, which is common in real situations (Rousseeuw, 1987).

We did not find similar studies dealing with this question in adolescents and adults suffering from atopic dermatitis according to the databases in Medline, Pubmed, and Web of Science.

Our results confirm the fundamental and exceptional position of the components such as Phl p 1, Bet v 1, Alt a 1 – these components were recorded as a principal components without any other components in the separated clusters. The interesting cluster is Cluster No. 4 with the single component of Phl p 4. Important role play also the molecular components of NPC2 family (Niemann-Pick disease type C2), cysteine protease, tropomyosin, uteroglobin, lipocalin and PR-10 proteins.

The single molecular component Phl p 1 was recorded in Cluster 1. Sensitization to Phl p 1 usually precedes sensitization to other grass pollen allergen and is the most prevalent

Table 6. Results of Multiplex ISAC testing - molecular components in 10 clusters with their biochemical name, protein family and allergen source, number of patients with positive results to molecular components. Examination in 100 patients (= 100%).

Cluster	Molecular components	Biochemical name, protein family	Allergen	Biological function	Number of patients with positive results in %
Cluster 1	Phl p 1	Beta-expansin	Timothy	Expansins	61%
Cluster 2	Phl p 4	Berberine bridge enzymes, CCD-bearing protein	Timothy	Berberine bridge enzymes	52%
Cluster 3	Phl p 5	Grass group 5	Timothy	Ribonucleases	43%
	Phl p 6	Grass group 6	Timothy	unknown	42%
	Phl p 2	Grass group 2	Timothy	unknown	39%
	Cyn d 1	Beta-expansin CCD-bearing protein	Bermuda grass	Expansins	49%
Cluster 4	Bet v 1	PR-10 protein	Birch	Ribonucleases	57%
Cluster 5	Alt a 1	Alt a 1-related protein	Alternaria	unknown	29%
Cluster 6	Der f 2	NPC2 family	House dust mite	NPC2 family	45%
	Der p 2	NPC2 family	House dust mite	NPC2 family	46%
	Der p 1	Cysteine protease	House dust mite	Cysteine proteases	36%
	Der f 1	Cysteine protease	House dust mite	Cysteine proteases	34%
Cluster 7	Pen m 2	Arginine kinase	Shrimp	Arginine kinases	22%
	Mus m 1	Lipocalin	Mouse	Lipocalins	20%
	Lep d 2	NPC2 family	Storage mite	NPC2 family	13%
	Can f 5	Arginine esterase	Dog	Arginine esterases	26%
	Phl p 11	Ole e 1-related protein	Timothy	Trypsin inhibitors	20%
	Pen m 1	Tropomyosin	Shrimp	Muscle contraction proteins	8%
	Bla g 7	Tropomyosin	Cockroach	Muscle contraction proteins	5%
	Der p 10	Tropomyosin	House dust mite	Muscle contraction proteins	5%
	Ole e 1	Commom olive group 1	Olive	Trypsin inhibitors	14%
	Blo t 5	Mites group 5/21	House dust mite	unknown	9%
Cluster 8	Ani s 3	Tropomyosin	Anisakis	Muscle contraction proteins	4%
	Can f 1	Lipocalin	Dog	Lipocalins	39%
	Fel d 1	Uteroglobin	Cat	Uteroglobins	42%
	Fel d 4	Lipocalin	Cat	Lipocalins	29%
Cluster 9	Equ c 1	Lipocalin	Horse	Lipocalins	27%
	Pru p 1	PR-10 protein	Peach	Ribonucleases	46%
	Cor a 1.0101	PR-10 protein	Hazel pollen	Ribonucleases	45%
	Mal d 1	PR-10 protein	Apple	Ribonucleases	47%
	Aln g 1	PR-10 protein	Alder	Ribonucleases	43%
	Cor a 1.0401	PR-10 protein	Hazelnut	Ribonucleases	42%
	Ara h 8	PR-10 protein	Peanut	Ribonucleases	38%
	Gly m 4	PR-10 protein	Soy	Ribonucleases	32%
Cluster 10	Fag e 2	2S albumin	Buck wheat	2S albumins	0%
	Pla a 1	Putative invertase inhibitor	London plane tree	Invertase inhibitors	0%
	Bos d 4	Alpha-lactalbumin	Cow's milk	Albumins	0%
	Api m 4	Melittin	Honey bee	Hemolysins	0%
	Act d 5	Kiwellin	Kiwi	unknown	0%
	Ber e 1	2S albumin	Brazil nut	2S albumins	0%
	Tri a 19.0101	omega-5 gliadin	Wheat	Gliadins	0%
	Cor a 9	11S globulin	Hazelnut	Legumine-like Proteins	1%
	Bos d 5	Beta-lactoglobulin	Cow's milk	Lipocalins	1%
	Bla g 1	Microvilli-like protein	Cockroach	unknown	2%
	Ole e 7	nsLTP	Olive	Lipid transfer proteins	1%

(Continued)

Table 6. Continued.

Cluster	Molecular components	Biochemical name, protein family	Allergen	Biological function	Number of patients with positive results in %
	Gly m 5	7S globulin, Beta-conglucinin	Soy	Vicilin-like proteins	2%
	Tri a 14	nsLTP	Wheat	Lipid transfer proteins	1%
	Bla g 5	Glutathione-S-transferase	Cockroach	Glutathione-S-transferase	3%
	Bos d lactoferrin	Transferrin	Cow's milk		2%
	Hev b 3	Small rubber particle protein	Latex	Small Rubber Particle Proteins	2%
	Bos d 6	Serum albumin	Cow's milk/meat	Albumins	2%
	Gly m 6	11S globulin, Glycinin	Soy	Legumin-like Proteins	2%
	Phl p 12	Profilin	Timothy	Actin-binding Proteins	3%
	Api m 1	Phospholipase A2	Honey bee	Phospholipases	2%
	Tri a aA_TI	Alpha-amylase/trypsin inhibitor	Wheat		3%
	Ara h 6	2S albumin, Coglutin	Peanut	2S albumins	2%
	Sal k 1	Pectin methylesterase	Saltwort	Pectinesterases	2%
	Gal d 5	Serum albumin	Egg yolk/chicken meat	Albumins	3%
	Hev b 1	Rubber elongation factor	Latex	Elongation factors	3%
	Bos d 8	Casein	Cow's milk	Caseins	2%
	Equ c 3	Serum albumin	Horse	Albumins	3%
	Bla g 2	Inactive aspartic protease	Cockroach	Aspartic proteases (inactive)	3%
	Hev b 5	Hev b 5-like	Latex	unknown	2%
	Ani s 1	Serine protease inhibitor	Anisakis	Serine protease inhibitors	1%
	Cla h 8	Mannitol dehydrogenase	Cladosporium	Mannitol dehydrogenases	4%
	Ses i 1	2S albumin	Sesame	2S albumins	5%
	Pen m 4	Sarcoplasmic calcium-binding protein	Shrimp	Calcium-binding proteins	2%
	Cor a 8	nsLTP	Hazelnut	Lipid transfer proteins	4%
	Gal d 2	Ovalbumin	Egg white	Albumins	5%
	Ara h 2	2S albumin, Coglutin	Peanut	2S Albumins, Trypsin inhibitors	3%
	Ara h 3	11S globulin, Glycinin	Peanut	Legumine-like proteins	4%
	Pla a 3	nsLTP	London plane tree	Lipid transfer proteins	5%
	Ara h 1	7S globulin, Vicilin	Peanut	7S Vicilin-like globulins	4%
	Gal d 1	Ovomucoid	Egg white	Trypsin inhibitors	7%
	Art v 3	nsLTP	Mugwort	Lipid transfer proteins	5%
	Hev b 6.01	Prohevein	Latex	Chitin-binding proteins	6%
	Jug r 3	nsLTP	Walnut	Lipid transfer proteins	7%
	Ara h 9	nsLTP	Peanut	Lipid transfer proteins	7%
	Asp f 1	Mitogillin family	Aspergillus	Ribonucleases	6%
	Pol d 5	Antigen 5	Mediterranean paper wasp	unknown	6%

(Continued)

Table 6. Continued.

Cluster	Molecular components	Biochemical name, protein family	Allergen	Biological function	Number of patients with positive results in %
	Jug r 1	2S albumin	Walnut	2S albumins	3%
	Pru p 3	nsLTP	Peach	Lipid transfer proteins	8%
	Par j 2	Phospholipid transfer protein	Wall pellitory	Lipid transfer proteins	10%
	Fel d 2	Serum albumin	Cat	Albumins	7%
	Gal d 3	Conalbumin, Ovotransferrin	Egg white	Transferrins	6%
	Can f 3	Serum albumin	Dog	Albumins	8%
	Gad c 1	Beta-parvalbumin	Cod	Calcium-binding Proteins	6%
	Ole e 9	Glucanase	Olive	Glucanases	17%
	Bet v 4	Polcalcin	Birch	Calcium-binding proteins	4%
	Ves v 5	Antigen 5	Yellow jacket	unknown	10%
	Asp f 3	Peroxisomal protein	Aspergillus	Peroxisomal membrane proteins	6%
	Bet v 2	Profilin	Birch	Actin-binding proteins	9%
	Act d 1	Cysteine protease	Kiwi	Cysteine proteases	11%
	Jug r 2	7S globulin, Vicilin, CCD-bearing protein	Walnut	7S Vicilin-like globulins	8%
	Ana o 2	Legumin-like protein	Cashew nut	Legumine-like proteins	4%
	Mer a 1	Profilin	Annual Mercury	Actin-binding proteins	13%
	Cry j 1	Pectate lyase, CCD-bearing protein	Japanese cedar	Pectate lyases	15%
	Cup a 1	Pectate lyase, CCD-bearing protein	Cypress	Pectate lyases	14%
	Act d 8	PR-10 protein	Kiwi	PR-10 proteins	24%
	Phl p 7	Polcalcin	Timothy	Calcium-binding proteins	7%
	Che a 1	Ole e 1-related protein	Goosefoot	Trypsin inhibitors	8%
	Alt a 6	Enolase	Alternaria	Glycolytic enzymes	16%
	Api g 1	PR-10 protein	Celery	Ribonucleases	22%
	Hev b 8	Profilin	Latex	Actin-binding proteins	13%
	MUXF3	Sugar epitope from Bromelain	CCD	Glycan side-chains	22%
	Amb a 1	Pectate lyase	Ragweed	Pectate lyases	5%
	Pla a 2	Polygalacturonase, CCD-bearing protein	London plane tree	Polygalacturonase	24%
	Pla l 1	Ole e 1-related protein	English plantain	Trypsin inhibitors	15%
	Act d 2	Thaumatocin-like protein	Kiwi	Thaumatins	21%
	Asp f 6	Mn superoxide dismutase	Aspergillus	Mn Superoxide Dismutases	21%
	Can f 2	Lipocalin	Dog	Lipocalins	17%
	Art v 1	Defensin-like protein	Mugwort	Defensins	29%

component sensitization in grass pollen -allergic patients (Hatzler et al., 2012). Phl p 1 is a beta-expansin, bound to the cell wall and important for pollen tube penetration; 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). Phl p 1 is in most patients the “initiator” molecule. Moreover, even in the few grass pollen - allergic patients who start

their sensitization process with other molecules, IgE against Phl p 1 are produced quite soon. Therefore, IgE to Phl p 1 is an essential marker in grass pollen-allergic patients to establish “true sensitization”. The presence of IgE to Phl p 1 confirms that the patient with a positive skin test or IgE assay to grass pollen extract is truly sensitized to grass pollen. The absence of IgE to Phl p 1 does not exclude “true” sensitization to grass pollen, which might be due (in a few cases) to isolated IgE sensitization to other major allergenic proteins (e.g. Phl p 5) but makes it rather unlikely. The molecular component Phl p 4 is recorded in Cluster 2; it is a tryptase-resistant glycoprotein, berberine bridge enzyme, involved in the synthesis of alkaloids. It can be classified as a major allergen. It shows IgE cross-reactivity with other group 4 grass pollen allergens, including with Cyn 4 to some extent. Moreover, cross-reactivity to the major ragweed allergen Amb a 1 and to Oilseed Rape pollen has been demonstrated. Natural Phl p 4 contains cross-reactive carbohydrate determinants (CCD), which may lead to IgE cross-reactivity with a wide range of plants and plant products. This explains why in several epidemiological studies IgE positivity to Phl p 4 scores over 90% of the grass pollen-allergic patients. However, when the recombinant version of the molecules is used for assays, over 50% of the positivity is not confirmed anymore. Phl p 4 may also serve as a marker for sensitization to Bermuda grass pollen due to its similarity with Cyn d 4, a major allergen of Bermuda grass pollen, but this needs to be investigated in relevant patient populations (Matricardi et al., 2016). As 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 our study.

Molecular components such as Phl p 5, Phl p 6, Cyn d 1 and Phl p 2 were found in Cluster 3. Phl p 5 is another major pollen allergen of temperate grasses with low sensitization prevalence, but often with high IgE levels. Phl p 5 is a cytoplasmatic ribonuclease, important in the enzymatic degradation of RNA. 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 sensitization process, their concentration grows in many patients rapidly and higher and their contribution to patients' symptoms has been demonstrated. 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. Phl p 5-specific IgE may have some prognostic value for indicating disease severity or likely progression from allergic rhinitis to asthma, but this needs to be confirmed with well-designed studies. 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 sensitization 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 SPT reaction is the expression of true sensitization 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). The only component found in Cluster 4 is Bet v 1. Birch, followed by alder and hazel, represents the most potent cause of tree pollen allergy. In Europe, the prevalence of positive skin prick test to birch pollen allergens ranges from 5% in The Netherlands to 54% in Switzerland, while Scandinavian countries have the highest number of patients with exclusive sensitization to Bet v 1 (Asam et al., 2015; Breiteneder et al., 1988). Bet v 1 in birch pollen is responsible for most of the IgE binding to the allergen source. For

tree pollen allergic patients in Northwestern and Central Europe, Bet v 1 is of decisive clinical importance because there is no “competing” major allergen (Matricardi et al., 2016). 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 recognized by virtually all allergic patients, thus representing the major cause of clinical allergy (D’Amato et al., 1998). PR-10 proteins defend plants against fungi and other microorganisms (Asam et al., 2015; Breiteneder et al., 1988). Their homologs are also present in a large number of plant-derived foods, and thus frequently cause cross-sensitization and consequently plant-food allergy (oral allergy syndrome, in most cases). In our study, molecular components from PR – 10 protein family (Pru p 1, Cor a 1.010, Mal d 1, Aln g 1, Cor a 1.040, Ara h 8 and Gly m 4) are recorded in Cluster 9.

Exceptional position was recorded for molecular component Alt a 1, which was found as a single component in Cluster 5. Sensitisation to *Alternaria alternata* spores are considered a well-known biological contaminant and a very common potent aero-allergen 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 considered a marker of primary sensitization to *Alternaria alternata*. Moreover, *Alternaria alternata* sensitization seems to be a triggering factor in the development of poly-sensitization, 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). According to our previous results, the sensitisation to molecular fungal allergen was recorded altogether in 58% atopic dermatitis patients (Čelakovská et al., 2019). The study and understanding of *Alternaria alternata* allergen information may be the key to explaining why sensitization to *Alternaria alternata* is a risk factor for asthma and also why the severity of asthma is associated to this mold. Recent research on the identification and characterization of *Alternaria alternata* allergens has allowed for the consideration of new perspectives in the categorization of allergenic molds, assessment of exposure and diagnosis of fungi-induced allergies (Chruszcz et al., 2012; Gabriel et al., 2016).

The molecular components such as Der f 2, Der p 2, Der p 1 and Der f 1 were recorded in Cluster 6. Der p 1, Der f 1 are cystein proteasa, Der p 2, Der f 2 are from NPC2 protein family (epidermal secretory proteins). For *D. pteronyssinus* and *D. farinae*, the fecal particles are the cardinal form in which the allergens became airborne (de Blay et al., 1997; Tovey, Chapman, and Platts-Mills, 1981; Tovey, Chapman, Wells, et al., 1981). In addition, for Der p 1, there is specific evidence about the effects of reducing exposure from 13 to 0.2 µg/g on symptoms among mite-allergic individuals (Banerjee et al., 2015; de Blay et al., 1997; Tovey, Chapman, & Platts-Mills, 1981; Tovey, Chapman, Wells, et al., 1981). The nature of these particles is relevant to both the induction of the IgE response and the subsequent contribution to inflammation of the nose and lungs. For dust mite particles, it is clear that they not only carry a high concentration of several allergens but also are an important source of other substances both nucleic acids and proteins that act as toll receptor ligands or PAMPs. While this has been well recognized for mites, it may be just as important for cockroach allergens (Banerjee et al., 2015; de Blay et al., 1997; Matricardi et al., 2016; Spieksma & Voorhorst, 1969; Tovey, Chapman, & Platts-Mills, 1981; Tovey, Chapman, Wells, et al., 1981).

The components such as Pen m 2, Mus m 1, Lep d 2, Can f 5, Phl p 11, Pen m 1, Bla g 7, Der p 10, Ole e 1, Blo t 5 and Ani s 3 are found together in the Cluster 7. Phl p 11 belongs to the Ole e 1 related proteins and hence exhibits a broad range of cross-reactivities to pollen from different plants as olive, ash, privet, saffron crocus, thistle, plantain, and corn. It is an acidic polypeptide with homology to the tryptase inhibitor of soybean. 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, sensitization to mite tropomyosin Der p 10 is low and has been considered an effect of cross-reactivity but also a marker for broad sensitization 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 sensitization to Der f 10 was found around 80% in Japan. In addition, sensitization 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 sensitization to tropomyosin from mite cockroach, *Ascaris* (Ahumada et al., 2015), and mosquito could influence the prevalence and severity of asthma in places where coexposition to several sources of tropomyosin occurs. The frequency of IgE sensitization 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). Molecular components such as Can f 1 (Lipocalin), Fel d 1 (Uteroglobin), Fel d 4 (Lipocalin) and Equ c 1 (Lipocalin) were found in Cluster 8. The majority of the mammalian allergens are lipocalins (Hilger, Swiontek, et al., 2012). Lipocalins are proteins that are ubiquitous; they are present also in arthropods, plants and bacteria, and have very diverse functions. They are characterized by a common tertiary structure composed of a central β -barrel formed of eight antiparallel β -strands. The internal binding pocket carries small hydrophobic molecules such as retinol, steroids, pheromones, and odorants. Mammalian allergens isolated so far are mostly odorant and pheromone binding lipocalins. Only few natural ligands have been characterized. Lipocalin allergens are present in dander, saliva, and urine. They stick to particles and become easily airborne and transported to public places such as schools or day care centers (Hilger, Kuehn, et al., 2012; Hilger, Swiontek, et al., 2012; Konradsen et al., 2014; Nilsson et al., 2012; Saarelainen et al., 2008; Zahradnik & Raulf, 2014). Lipocalins are small, secreted molecules of 150–250 amino acids. Representatives of this cross-reactive group are Equ c 1, Fel d 4 and Can f 6. However, even between molecules of low general sequence identity such as Fel d 4 and Can f 2 (25% identity), single epitopes may have short stretches of sequence identity and lead to patient-dependent IgE cross-reactivity. (Hilger, Kuehn, et al., 2012; Nilsson et al., 2012; Saarelainen et al., 2008). In Cluster 9, these components were found: Pru p 1 (Peach), Cor a 1.010 (Hazelnut), Mal d 1 (Apple), Aln g 1 (Alder), Cor a 1.040 (), Ara h 8 (Peanut), Gly m 4 (Soy) – all these components belong to the family PR –10 protein. In 1980, pathogenesis-related proteins (PR proteins) were defined as “proteins

encoded by the host plant but induced only in pathological or related situations” and subsequently grouped into families. Today, the list of PR proteins comprises 14 families. When the sequence of Bet v 1 was discovered in 1989, the PR-10 family had not been defined yet but it was noted that Bet v 1 was homologous to a PR protein from pea Bet v 1 is constitutively expressed in pollen at rather high concentrations. Hence, the term PR-10 for the Bet v 1 homologous allergens is not entirely correct. These constitutively expressed proteins are referred to as PR-10 like proteins.. Positive specific IgE to Bet v 1 homologs plant-food allergens (i.e. Pru p 1 from peach) demonstrates allergic sensitization, being clinically relevant in case of corresponding symptoms (Antoniw et al., 1980; Gepp et al., 2014; Geroldinger-Simic et al., 2011; Kitzmüller et al., 2015; van Loon & van Strien, 1999).

In Cluster 10, 82 molecular components from different pollen and food allergen sources were recorded. Phl p 7 and Phl p 12 are minor allergens, representing panallergens from the plant world. Phl p 7, polcalcin, is a calcium-binding protein present in many different pollens, hence representing a broad cross-reacting allergen: birch, alder, juniper, ragweed, mugwort, olive, goosefoot, etc. Phl p 7 sensitization can be used as a marker for a more general pollen sensitization. Phl p 12 is a member of the profilin family, an actin-binding protein that is present throughout the whole plant world. As profilins are ubiquitous in plant cells, profilin sensitization gives rise to a long range of cross-reacting plants and plant products as birch, soybean, corn, latex, and plant foods. Phl p 12 is the highly cross-reacting profilin of *Phleum pratense*. As a heat-labile, relatively weak allergenic molecule, IgE sensitization to profilin comes later in the molecular spreading process, reaches only moderate levels of IgE antibodies and only in a minority of patients. Hence, IgE to Phl p 12 mark in general patients with a higher atopic background and/or longer disease duration. Patients with a positive skin test/IgE to grass pollen extract but no IgE to Phl p 1 and Phl p 5 must be tested for IgE to Phl p 12 as these antibodies – that can be induced by other pollens containing profilin – are the first cause of “false” positivity to assays based on grass pollen extract. In the presence of IgE to Phl p 12, patients should be asked about oral allergy syndrome triggered by the ingestion of fruit and vegetables containing profilin, with a focus on apple, celery, and birch. Phl p 7, is the highly cross-reacting polcalcin of *Phleum pratense*. This is a heat-stabile, relatively potent allergen that can induce quite high IgE antibody levels. An IgE response to Phl p 7 is observed only infrequently among grass pollen-allergic patients and usually many years after the disease onset. IgE to Phl p 7 marks a relatively distinct category of grass pollen-allergic patients, with more severe symptoms, a higher prevalence of asthma, and a higher frequency of additional allergic comorbidities. Moreover, many other pollens and allergenic sources contain polcalcin so that the origin sensitization to polcalcin in a grass pollen-allergic patient must be carefully searched. These allergenic sources could be indeed responsible of a more severe disease (Bousquet et al., 2007; Davies, 2014; Devanaboyina et al., 2014; Popescu, 2014).

In our previous studies we evaluated the occurrence of sensitisation to food and inhalant allergens in patients suffering from AD. The challenge test was performed according to the results of examinations sIgE and APT atopy patch tests with suspected foods. The diagnostic work-up should comprise not only the laboratory methods, but also the diagnostic hypoallergenic diet and the challenge test in patients with suspected food allergy (Čelakovská, Ettlerová, Ettler, and Bukač, 2015; Čelakovská, Ettlerová, Ettler, Vaněčková,

et al., 2015). Our previous study demonstrates that there is a significant association between the severity of AD and the incidence of perennial rhinitis, asthma bronchiale, and the worsening of atopic dermatitis in relation to food (Čelakovská & Bukač, 2011).

According to the literature, quantitative competition immunoassays with appropriate combinations of antibodies give consistent dose-response patterns which may be used to identify and estimate amounts of cross-reacting compounds. Previously reported methods of analyzing cross-reaction patterns include multiple regression, principal components analysis and minimum estimates of variance (MEV). Four other techniques which are preferable in theory have been surveyed: discriminant analysis (DA), maximum likelihood estimates (MLE), classification and regression trees (CART), and computational neural networks (NN). MLE and simple back-propagation neural networks can estimate the concentration, as well as the identity, of individual compounds. These four methods worked well with unfitted, unscaled data from monoclonal assays of triazines, phenylureas and avermectins. Immunoassays must be properly designed to provide adequate data for pattern recognition. Cross-reactivity pattern analysis will make multi-analyte, multi-antibody immunoassays feasible for many applications in toxicology and hazard assessment (Karu et al., 1994).

According to the literature, little is known about pollen-food allergy syndrome (PFS) in China. Ma Shikun et al. investigated the clinical characteristics, as well as sensitization patterns, of PFS in China. Clinical parameters and serum Immunoglobulin E (IgE) responses to prevalent pollens, plant foods and corresponding allergen components were evaluated. The top three most common pollen-associated allergenic foods were peach, apple and pear. Peach was the most common allergenic food in PFS patients. Patients with PFS in China showed an LTP-dominant sensitization profile and usually presented systemic reactions upon consumption of the allergenic foods (Ma et al., 2018).

Food allergies, including kiwi fruit allergy, have been the subject of extensive research in the last few years. The aim of Gavrovic-Jankulovic 's study was to examine a possible relationship between the developmental stage of kiwi fruit and its allergenic potency. The protein and allergen patterns of kiwi fruit extracts in September, October, November and December fruit in the period from 2000–2002 were analysed. Two major allergens of kiwi fruit, Act c 1 and Act c 2, mainly accounted for the highest allergenic potential of November kiwi extract *in vivo* and *in vitro*. Not only the content of major allergens, but also the ratio of different proteins and even isoforms of the same allergen (Act c 2) change with fruit ripening. These findings should be taken into account during preparation of extracts for allergy diagnosis (Gavrovic-Jankulovic et al., 2005).

The prevalence of fish allergies has become a serious health problem and has increased alarmingly over the past few years. Liu, Chu-Yi 's study attempted to identify and purify the major allergen implicated in the allergic response to largemouth bass (*Micropterus salmoides*), a freshwater fish widely consumed in China. According to their results, nucleoside diphosphate kinase B was identified as a novel fish allergen in largemouth bass. This finding is important for allergy diagnoses and the treatment of freshwater fish-allergic disorders (Liu et al., 2014).

The quality of an applied protein extract is important in both serological and *in vivo* diagnosis of allergy, and for allergen detection methods. In Dooper 's study the effects of the extraction procedure and hazelnut source on antibody binding to hazelnut (*Corylus avellana*) proteins were investigated. This study indicates that for the production of hazelnut protein extracts, the use of fresh hazelnuts is important. A quick, vigorous

extraction in a tris buffer might contribute positively, at least for extraction of Cor a 9 (Dooper et al., 2008).

Conclusion

The results of ISAC multiplex examination in 100 atopic dermatitis patients were processed with cluster analysis, we found 10 clusters with different numbers of molecular components. Our results correspond to the association of molecular components into protein families according to their biochemical structure. Fundamental position have the components Phl p1, Bet v 1, Alt a 1 followed by molecular components of NPC2 family, cystein proteasa, tropomyosin, uteroglobin, lipocalin and PR-10 protein. These molecular components play the most important role in atopic dermatitis patients.

Disclosure statement

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

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