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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Bet v 1-specific IgE levels and PR-10 reactivity discriminate silent sensitization from phenotypes of birch allergy

To the Editor,

For certain forms of allergy (eg, food allergy, respiratory allergy, skin allergy), molecular allergy diagnosis can provide clinically useful information for the prediction of symptoms. These predictive

algorithms are based on determination of specific IgE levels to defined allergen molecules as well as on IgE reactivity profiles. Such information can be established in a single step by multiplex technologies like allergen microarrays. For example, levels of IgE to certain food and respiratory allergens were found to be associated with severity of clinical phenotypes (eg, anaphylaxis, asthma).^{1,2} Similar observations were made regarding the occurrence of more severe respiratory symptoms in polysensitized subjects.³ Regarding allergy to birch pollen which affects more than 100 million patients

Abbreviations: AD, atopic dermatitis; AR, allergic rhinitis; AS, allergic asthma; BAMSE cohort, Barn Allergi Miljö Stockholm Epidemiologi cohort; IgE, immunoglobulin E; ISAC, immuno solid-phase allergen chip; ISU, ISAC Standardized Units; MeDALL, mechanisms of the development of allergy; OAS, oral allergy syndrome; PR-10 allergen, pathogenesis-related allergen; r, recombinant.

worldwide, mainly in Northern Europe and Central Europe, certain parts of North America, Australia, and Japan,⁴ the association between IgE sensitization to allergen molecules and clinical phenotypes is less clear. However, the major birch pollen allergen, that is, Bet v 1, and the cross-reactive allergens in pollen- and plant-derived food responsible for the birch pollen-associated oral allergy syndrome have been well characterized.⁵ In the Swedish BAMSE birth cohort, the evolution of IgE reactivities to micro-arrayed Bet v 1 and Bet v 1-related PR-10 proteins was investigated recently. In this study, it was demonstrated that Bet v 1-specific IgE levels measured early in life can be used to calculate the likelihood if children are prone to develop respiratory symptoms to birch later in life.⁶ However, data are controversial if specific IgE levels to Bet v 1 and Bet v 1-related PR-10 allergens are associated with certain symptoms of birch pollen-related allergy. One study indicates that Bet v 1-specific IgE levels may predict oral allergy syndrome and another one suggests Mal d 1-specific IgE as biomarker,^{7,8} whereas another study did not find such an association, neither for Bet v 1 nor for Mal d 1.⁹ There is also no evidence that certain IgE reactivity profiles to Bet v 1 and Bet v 1-related PR-10 allergens are associated with respiratory or skin manifestations of birch pollen-associated allergy and certain clinical phenotypes.

The aim of the present study was to investigate whether IgE reactivity profiles and/or threshold levels of IgE to Bet v 1 and Bet v 1-related PR-10 allergens can be determined that allow to differentiate between silent IgE sensitization, certain phenotypes of birch pollen-associated allergy, and/or multimorbidity. Children from Moscow region (Russia), a highly birch pollen-exposed area, were screened by using a validated ISAAC questionnaire for birch pollen allergy (Figure S1 and Methods, Appendix S1). This approach is frequently used for preliminary clinical characterization of trial participants (see references S1 and S2 of the Appendix S1) and allowed us to establish one group of children with birch pollen-related allergy symptoms (group 1: *n* = 103) and a second group devoid of allergic symptoms (group 2: *n* = 97; Table 1). There was no significant difference between the two groups regarding age (range: 10–16 years) and gender distribution (Table 1). Following questionnaire-based screening, children were subjected to detailed clinical assessment of their allergy symptoms (Methods, Appendix S1) the result of which are displayed

in Table 1 (allergic rhinoconjunctivitis: AR, *n* = 65; asthma: AS, *n* = 28; oral allergy syndrome: OAS, *n* = 34; atopic dermatitis: AD, *n* = 34). Using the MeDALL allergen microarray (Methods, Appendix S1), we then tested the sera from the symptomatic (group 1) and from the asymptomatic children (group 2) for IgE reactivity to Bet v 1 and a panel of eight additional PR-10 allergens from alder pollen, hazelnut, apple, peanut, peach, celery, kiwi and soy (respiratory PR-10 allergen: Aln g 1; PR-10 food allergens: Cor a 1.0401 Mal d 1, Ara h 8, Pru p 1, Api g 1, Act d 8, Gly m 4) that are cross-reactive to Bet v 1 (Figure 1A,B). Interestingly, Bet v 1-sensitized children were not only found in the symptomatic group (*n* = 65 out of 103), but almost one third of the asymptomatic children also showed Bet v 1-specific IgE reactivity (*n* = 30 out of 97) (Table S2; Figure 1A,B). In line with previous studies, IgE sensitization was dominated by Bet v 1, followed by less frequent sensitizations to respiratory and food PR-10 allergens (Table S2; Figure 1A,B). Median IgE levels to Bet v 1 and Bet v 1-related PR-10 allergens were significantly (*P* < 0.0001) lower in the asymptomatic children (1.12 ISU) as compared to subjects with birch pollen-related allergic symptoms (19.07 ISU; Table S2 and Figure 1). Using a cutoff level of 6.8 ISU (Figure S2), children without symptoms could be discriminated from children with any type of birch pollen-related symptoms with an odds ratio (OR) of 14.1 (Table 1). As for Bet v 1 (Figure 1D), specific IgE levels to the other Bet v 1-related PR-10 allergens were also significantly lower in the asymptomatic children compared to children with different birch pollen-related symptoms (Figure S3). Interestingly, children with birch pollen-related oral allergy syndrome (OAS) had significantly higher Bet v 1-specific IgE levels than children with birch pollen-related rhinoconjunctivitis, asthma, or atopic dermatitis (Figure 1D; *P* < 0.0001). Children with OAS also had higher IgE levels to Bet v 1-related respiratory PR-10 allergens (ie, Aln g 1; Figure S3A) as well as to Bet v 1-related food allergens (ie, Cor a 1.0401, Mal d 1, Ara h 1, Pru p 1, Api g 1, Act d 8, Gly m 4; Figure S3B). In fact, children with Bet v 1-specific IgE > 6.8 ISU had an OR of 18.3 to suffer from OAS (Table 1). This value was much higher than for any other isolated birch pollen-associated symptom or any combination of symptoms (ie, rhinitis, asthma, atopic dermatitis; Figure S2B,C,E; Table 1). Diagnostic sensitivity and specificity of the defined threshold levels of Bet v 1-specific IgE are indicated in Table 1.

TABLE 1 Odds ratios (OR, 95% CI) for different types of birch-related symptoms in Bet v 1-sensitized subjects (*n* = 95) with IgE to Bet v 1 above the respective cutoff values

Type of symptoms	Symptoms Yes/No	<i>n</i>	Bet v 1-spec. IgE			OR	95% CI	Sensitivity (%) (95% CI)	Specificity (%) (95% CI)
			cutoff (ISU)	<i>n</i> , below cutoff	<i>n</i> , above cutoff				
All types (AR, AS, AD, OAS)	Yes	65	6.8	17	48	14.1	4.6–42.6	73.8 (62.1–83)	83.3 (66.4–92.7)
	No	30		25	5				
AR	Yes	65	1.3	14	51	5.4	2.1–13.9	78.5 (67–86.7)	60 (40.8–76.8)
	No	30		18	12				
OAS	Yes	34	6.8	3	31	18.3	5.0–66.9	91.2 (77–97)	63.9 (51.4–74.8)
	No	61		39	22				

Bet v 1-sensitized children of group 1 reacted most frequently with the major respiratory PR-10 allergen from alder (Aln g 1:96.9%), followed by the most frequently recognized PR-10 food allergens, that is, Cor a 1.0401 from hazelnut (87.6%) and the major apple allergen Mal d 1 (87.6%), whereas <80% of the children showed IgE reactivity to the other PR-10 food allergens (Table S3). Interestingly, the hierarchy of cross-reactivity did not fully match the sequence identities because the major allergen from peach, Pru p 1, showed higher sequence identity to Bet v 1 (ie, 59%) than the major apple

allergen Mal d 1 (ie, 56%) but was only recognized by 72% of Bet v 1-allergic patients (Tables S3, S4). The latter may indicate that the higher IgE recognition frequency of Mal d 1 could be not only due to structural similarities of the allergens but to more frequent exposure due to higher consumption of apple compared to peach.

We finally analyzed our data for a possible relation between the numbers of recognized PR-10 allergens and allergic multimorbidity. Indeed, we found that patients suffering from 3 to 4 types of allergic symptoms more frequently exhibited broader IgE reactivity,

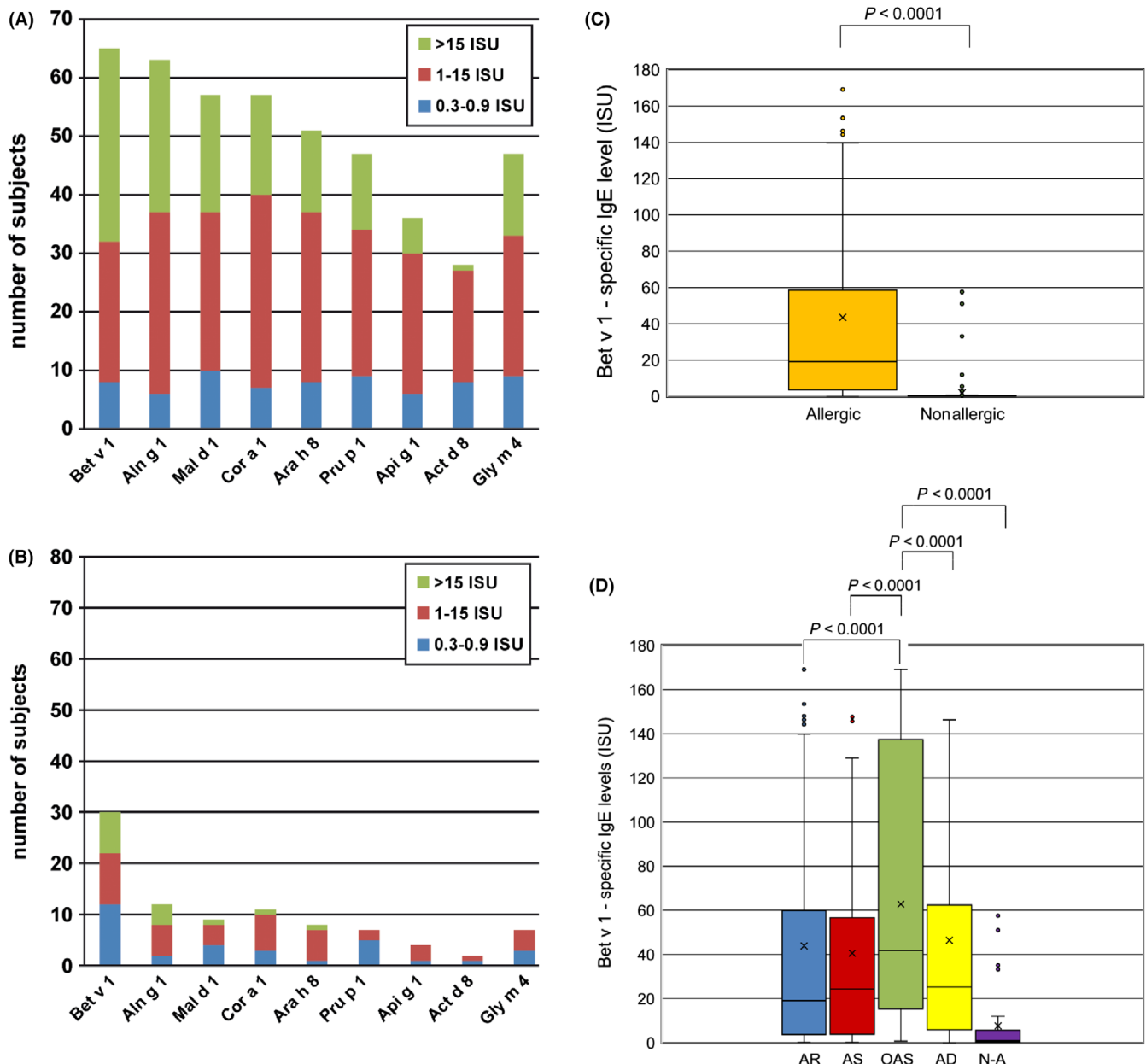


FIGURE 1 Numbers (y-axes) of (A) allergic (n = 103) and (B) nonallergic subjects (n = 97) with IgE-reactivity to Bet v 1 from birch and to Bet v 1-related PR-10 allergens (x-axes): Aln g 1, Mal d 1, Cor a 1.0401, Ara h 8, Pru p 1, Api g 1, Act d 8, Gly m 4. Ranges of allergen-specific IgE levels are color-coded. C and D, Bet v 1-specific IgE levels (y-axes; ISU) in Bet v 1-positive subjects shown for any type of allergic symptoms (C) or for individual manifestations of birch pollen allergy (D), that is, allergic rhinitis (AR: n = 65), asthma (AS: n = 28), oral allergy syndrome (OAS: n = 34), and atopic dermatitis (AD: n = 34), in comparison with sensitized but asymptomatic subjects (N-A: n = 30). Box plots display upper and lower quartiles with medians (horizontal lines), minima, and maxima. Mean values are indicated by an x within the boxes. Outliers (>1.5x the interquartile range from the median) are represented by circles. Significant differences are indicated: $P < 0.0001$

that is, to seven or eight of the PR-10 allergens in addition to Bet v 1 (Figure S4). This was also corroborated by showing a moderate, yet significant association between the sum of IgE levels to the different PR-10 allergens on the microarray with the number of comorbidities (Figure S5; Spearman's $\rho = 0.37$, $P < 0.01$). This indicates that multimorbidity in birch pollen allergy may be related to the number of recognized PR-10 allergens from different allergen sources.

Our study thus demonstrates that it is possible to determine serological cutoff levels for Bet v 1-specific IgE, which discriminate children without symptoms from symptomatic children. Interestingly, Bet v 1-specific IgE levels were significantly higher in patients with OAS than with birch pollen-related respiratory and skin symptoms. Even patients with asthma and atopic dermatitis had significantly lower Bet v 1-specific IgE levels than patients with OAS. Due to almost identical methodologies, we assume that results obtained with the MeDALL chip can be directly extrapolated to ImmunoCAP ISAC (Phadia AB, Thermo Fisher), a commercially available allergen microarray. Furthermore, we found that allergic multimorbidity was associated with sensitization to a broader panel of PR-10 allergens in addition to Bet v 1, an observation that is in line with results obtained for different types of allergen families such as LTP.¹⁰ Finally, our results show an unexpectedly high frequency of clinically silent IgE sensitization to the major birch pollen allergen Bet v 1 and Bet v 1-related PR-10 allergens in children exposed to birch pollen. Therefore, determination of specific IgE levels to Bet v 1 and PR-10 allergens by microarray might be clinically useful as a potential biomarker for the discrimination between silent and symptomatic sensitization and as a surrogate marker for the severity of the clinical phenotype in birch pollen-associated allergy. As this technology consumes only low amounts of serum samples, it is well suited for allergy diagnosis in pediatric patients.

CONFLICTS OF INTEREST

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Keywords

Bet v 1, birch pollen allergy, IgE reactivity, micro-arrayed allergen, threshold

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Food impactions in Eosinophilic esophagitis and acute exposures to fine particulate pollution

To the Editor,

Eosinophilic esophagitis (EoE) is emerging as the most common cause of esophageal food impactions in emergency visits.¹ Seasonal aeroallergens (ie, pollen) have been postulated to influence the presentation of EoE.² Pollution influences expression of TSLP (thymic stromal lymphopoietin), a pivotal EoE-related cytokine.³ We hypothesized that airborne particulate matter may result in excitation of inflammation and, thus, increase the risk for esophageal dysfunction in EoE.

We conducted a case-crossover analysis of Emergency Department (ED) visits among EoE patients in Utah to test the hypothesis that exposure to particulate matter with aerodynamic diameter ≤ 2.5 microns ($PM_{2.5}$) associates with exacerbations of EoE.

Our study was conducted in Utah in which 79% of the residents live in counties prone to wintertime pollution episodes (without pollen influence).⁴ The population was all persons coded for EoE via International Classification of Disease, 9th revision (ICD-9) code 530.13 who could be followed from January 2000 to May 2014 (the time period with robust pollution data). Medical data were obtained from Intermountain Healthcare and University of Utah Health; accounting for approximately 85% of all medical encounters in Utah. Medical record data were linked to residential ZIP code over time using the Utah Population Database.⁵ The ICD-9 code for EoE had been shown to predict esophageal eosinophilia based on chart review of medical records from this database.⁶

Emergency Department visits were the endpoint studied for disease exacerbations because they are connected temporally to

complications of disease (ie, food impactions). We identified the subset of EoE patients with an ED visit with any of the following ICD9 diagnosis or CPT procedure codes: chest pain or heartburn (ICD-9 786.50, 786.59, 787.1), dysphagia (ICD-9 787.20, 787.29), impaction (935.1), and upper gastrointestinal endoscopy (CPT 43215, 43235, 43239, 43247) between January 2000 and May 2014. We defined two outcomes of interest: (a) any of the codes listed above and (b) impaction, see Table 1. Impactions are severe and distinctive to EoE.

Daily air quality data were downloaded from the US Environmental Protection Agency (EPA).⁷ We obtained all available data for $PM_{2.5}$ in Utah in the defined period.

We used household ZIP code as proxy for the location of each person. We estimated $PM_{2.5}$ exposure for each person using the spatial interpolation method, kriging, which calculates a weighted average of $PM_{2.5}$ obtained from air pollution monitors in a region and distance from the ZIP code, giving greater weight to closer monitors.⁸ The kriging method performed better than using the closest monitor (data not shown). We selected $35 \mu\text{g}/\text{m}^3$ as the cut point for defining extreme air pollution because this is the EPA's National Ambient Air Quality Standard (NAAQS) for daily concentrations.⁹

We used a case-crossover design, which is becoming a standard method for researching the health impacts of air pollution. Case-crossover analysis controls for confounding because each case serves as its own control. This controls for variables that do not typically vary within a subject over the course of several weeks such as smoking, disease severity, diet, medications, and endoscopic therapy. We examined $PM_{2.5}$ concentrations during the 1-week period leading up to and including the date of the EoE event (hazard period) compared with the $PM_{2.5}$ concentrations during 1-week periods preceding and following the hazard period (2 control periods per event). Figure S1. Control periods both before and after the hazard period are included to avoid bias that would otherwise be introduced from seasonal and long-term trends in air pollution concentrations.

TABLE 1 Emergency events identified from 242 EoE cases. ED is Emergency Department

Description	Any ED visit	Impaction
# ED visits	242	145
% male	64	70
Median age (IQR) years	38 (28–52)	38 (26–50)

Abbreviation: IQR, interquartile range.

Figure E1

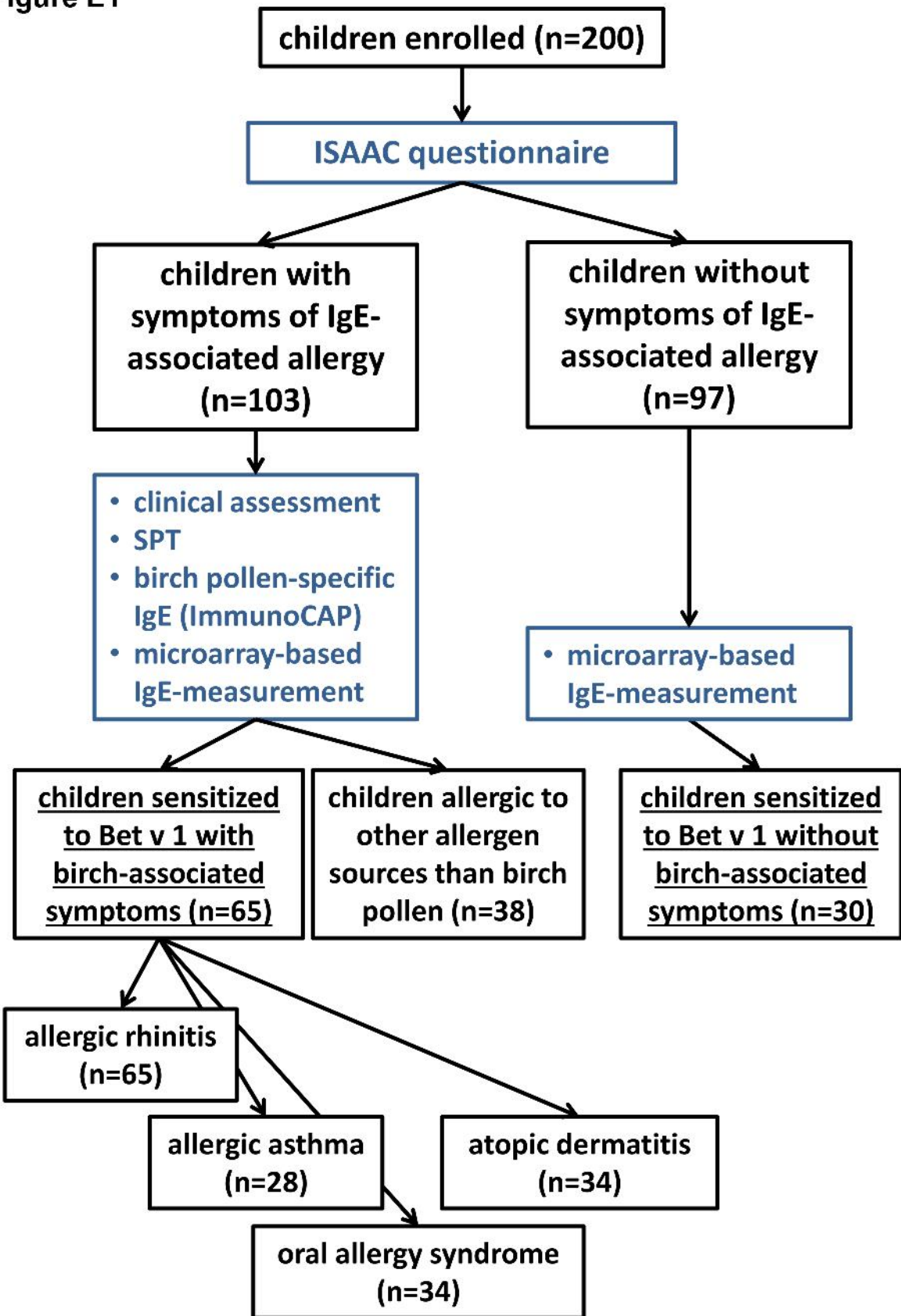


FIG E1. Flow chart illustrating patient enrollment, clinical and serological characterization and prevalences of different clinical manifestations in the group with birch-pollen related symptoms.

Figure E2

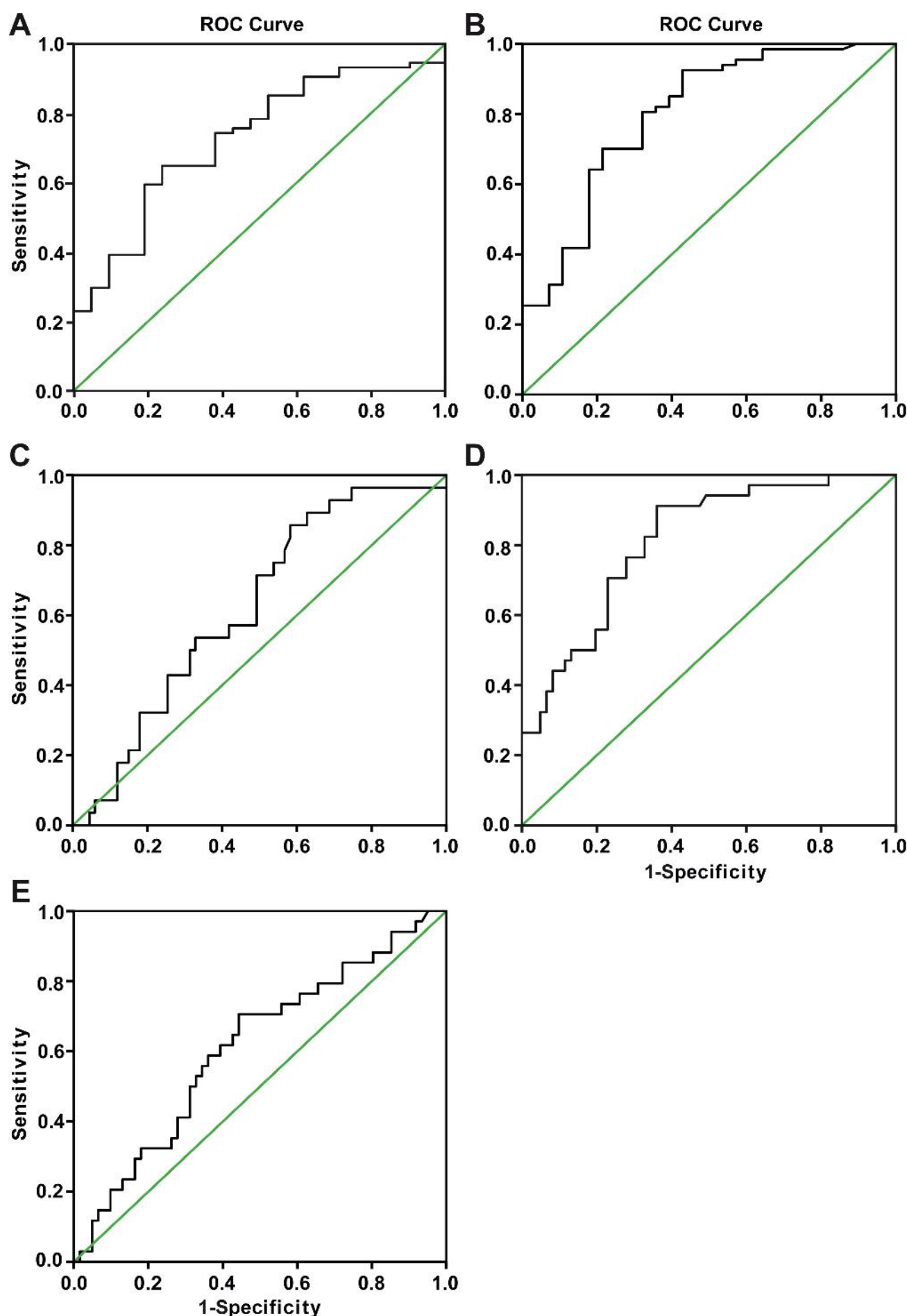


FIG E2. ROC curves for different cut-off levels of Bet v 1 differentiating between Bet v 1-sensitized subjects without symptoms (n=30) and patients with, **A**, any type of birch pollen-induced allergy (n=65) **B**, birch-pollen-induced rhinitis (AR: n=65) **C**, asthma (AS: n=28) **D**, oral allergy syndrome (OAS: n=34) and **E**, atopic dermatitis (AD: n=34).

Figure E3

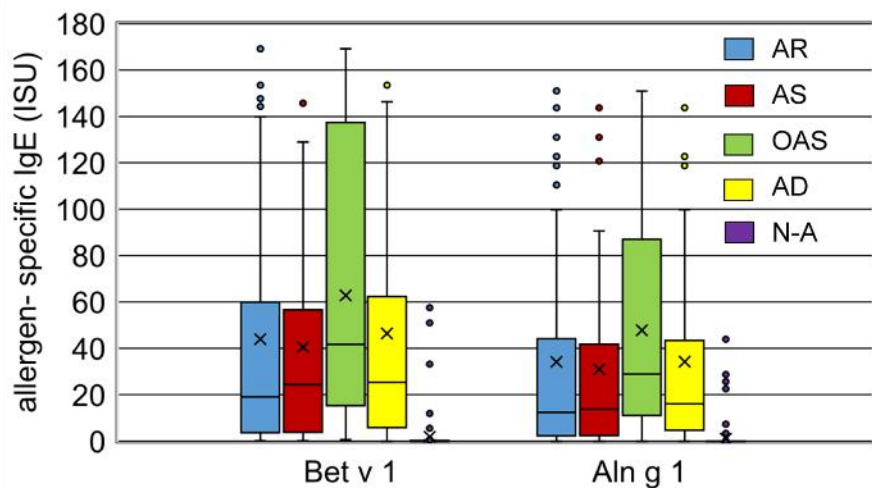
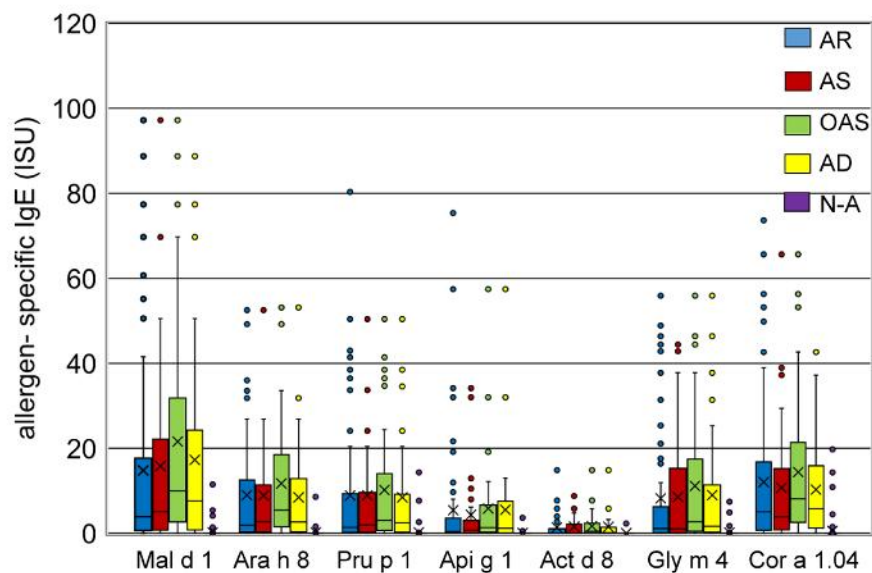
A**B**

FIG E3. Specific IgE levels (box plots, medians: horizontal bars; x: means) (y-axes) to **A**, respiratory PR-10 allergens Bet v 1 and Aln g 1 and **B**, to PR-10 food allergens (Cor a 1.0401, Mal d 1, Ara h 8, Pru p 1, Api g 1, Act d 8, Gly m 4) in patients suffering from different types of birch associated symptoms: allergic rhinitis AR (blue), asthma AS (red), oral allergy syndrome OAS (green), atopic dermatitis AD (yellow) and in Bet v 1-sensitized non-allergic subjects (violet).

Figure E4

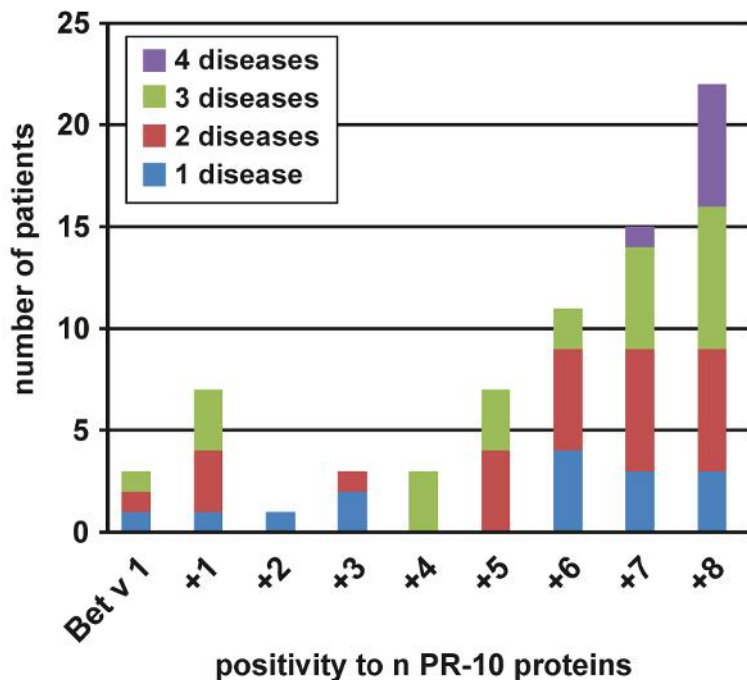
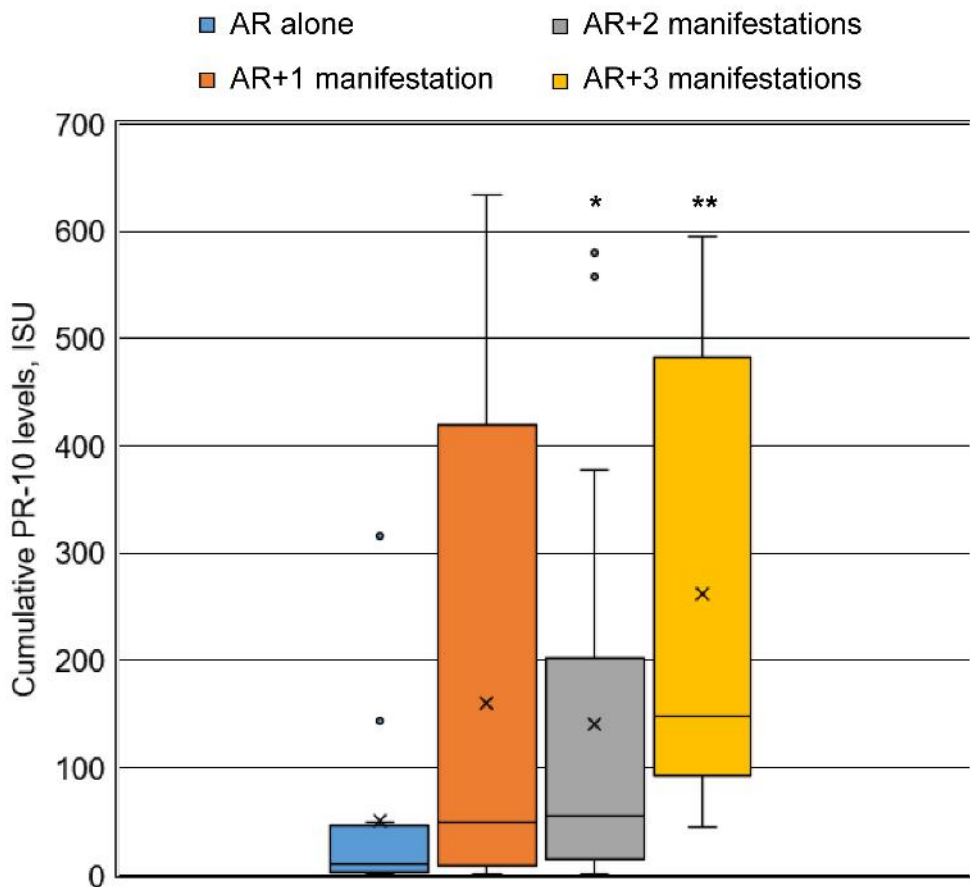


FIG E4. Patterns of sensitization to PR-10 proteins and co-morbidities. Numbers of patients with co-morbidities (blue: 1 disease; red: two diseases; green: 3 diseases; violet: 4 diseases) (y-axis) sensitized to Bet v 1 alone or to Bet v 1 and additional PR-10 proteins (x-axis).

Figure E5



* P < 0.05 (U-test) compared with group AR alone;

** p < 0.05 (U-test) compared with groups AR alone; AR+1; AR+2.

FIG E5. Association of the number of co-morbidities (x-axis) with cumulative levels of IgE specific to PR-10 proteins (y-axis) as box and whisker-plots. In addition, mean levels of IgE are displayed for each group (marked by an X within the respective box-plots). Significant differences between the groups are indicated by asterisks.

Table E1. Demographic and clinical characterization of allergic and non-allergic subjects.

	Group 1 (allergic patients), n=103	Group 2 (non-allergic subjects), n=97
Age, years M±SD	12.24±2.23	12.78±2.23
Gender (m/f)	61/42	50/47
Birch associated symptoms, n=65		
Asthma (AS) (total)	28	0
Allergic rhinitis (AR) (total)	65	0
Oral allergy syndrome (OAS) (total)	34	0
Atopic dermatitis (AD) (total)	34	0
Different types of manifestations, n		
1 type of manifestations		
AR alone	12	0
2 types of manifestations		
AR+AS	7	0
AR+AD	5	0
AR+OAS	7	0
3 types of manifestations		
AR+AS+AD	7	0
AR+AS +OAS	5	0
AR+AD+OAS	13	0
4 types of manifestations		
AR+AS+AD+ OAS	9	0
Skin prick test positivity (n), total IgE		
HDM (n)	50	11
Trees (n)	66	9

Grass	38	3
Weed	30	4
Pets (n)	50	11
total IgE (median, range)	139.0 U/ml, 3.5 – 13000 U/ml	35.5 U/ml, 6.0-1000 U/ml

Table E2. Allergen-specific IgE-levels in Bet v 1-positive subjects with (group 1) and without birch pollen-associated symptoms (group 2)

Allergen molecules	Group 1 (Subjects with birch pollen-associated symptoms) n=103		Group 2 (Subjects without birch pollen-associated symptoms) n=97	
	sIgE (ISU) Me [Q₁;Q₃]	n/% sIgE ≥0.3 ISU	sIgE (ISU) Me [Q₁;Q₃]	n/% sIgE ≥0.3 ISU
Bet v 1	19.07 [3.67; 57.2]	65/63%	1.12 [0.44; 6.37]	30/31%
Aln g 1	13.8 [13.16; 44.2]	63/61%	3.57 [1.34; 24.06]	12/12%
Mal d 1	3.67 [0.61; 17.51]	57/55%	0.66 [0.25; 5.39]	9/9%
Api g 1	2.8 [1.22; 11.3]	36/35%	2.55 [1.0; 4.2]	4/4%
Act d 8	2.42 [0.66; 5.27]	28/27%	2.66 [2.31; 3.02]	2/2%
Cor a 1	6.19 [2.46; 21.13]	57/55%	1.77 [0.51; 14.31]	11/11%
Ara h 8	5.43 [1.55; 18.3]	51/49%	1.97 [1.3; 8.57]	8/8%
Pru p 1	4.3 [1.26; 20.41]	47/45%	0.9 [0.64; 7.68]	7/7%
Gly m 4	3.42 [1.45; 17.6]	47/45%	0.72 [0.5; 4.87]	7/7%

Table E3. Percentages of cross-sensitization to different PR-10 proteins in children with birch-related symptoms (group 1)

Allergen	n	Bet v 1									
Bet v 1	65	100.0	Aln g 1								
Aln g 1	63	96.9	100.0	Cor a 1							
Cor a 1	57	87.6	90.4	100.0	Pru p 1						
Pru p 1	47	72.3	74.6	82.4	100.0	Mal d 1					
Mal d 1	57	87.6	90.4	98.2	100.0	100.0	Act d 8				
Act d 8	28	43.07	44.4	49.1	59.5	49.1	100.0	Ara h 8			
Ara h 8	51	78.4	80.9	89.4	100	89.4	100.0	100.0	Gly m 4		
Gly m 4	47	72.3	74.6	82.4	95.7	78.9	96.4	88.2	100.0	Api g 1	
Api g 1	36	55.3	57.1	63.1	72.3	61.4	92.8	68.6	74.4	100.0	

Table E4. Comparison of sequence identities of the PR-10 proteins represented on the MeDALL-chip

Acc. No	Allergen	Bet v 1									
P15494	Bet v 1	100.00	Aln g 1								
P38948	Aln g 1	81.25	100.00	Cor a 1							
Q9SWR4	Cor a 1	67.50	76.25	100.00	Pru p 1						
Q2I6V8	Pru p 1	59.38	57.50	58.75	100.00	Mal d 1					
P43211	Mal d 1	55.97	54.72	54.72	83.02	100.00	Act d 8				
D1YSM5	Act d 8	50.32	50.96	50.32	54.78	50.00	100.00	Ara h 8			
Q6VT83	Ara h 8	47.13	44.59	43.31	50.32	50.00	39.35	100.00	Gly m 4		
P26987	Gly m 4	46.84	48.10	46.84	53.16	52.87	44.87	70.70	100.00	Api g 1	
P49372	Api g 1	42.21	42.21	38.96	41.56	38.56	47.71	37.50	39.22	100.00	

Online Repository and supplemental information:

Bet v 1-specific IgE levels and PR-10-reactivity discriminate silent sensitization from phenotypes of birch allergy

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Supplementary Figures and Tables

FIG E1. Flow chart illustrating patient enrollment, clinical and serological characterization and prevalences of different clinical manifestations in the group with birch-pollen related symptoms.

FIG E2. ROC curves for different cut-off levels of Bet v 1 differentiating between Bet v 1-sensitized subjects without symptoms (n=30) and patients with, **A**, any type of birch pollen-induced allergy (n=65), **B**, birch-pollen-induced rhinitis (AR: n=65), **C**, asthma (AS: n=28), **D**, oral allergy syndrome (OAS: n=34) and **E**, atopic dermatitis (AD: n=34).

FIG E3. Specific IgE levels (box plots, medians: horizontal bars; x: means) (y-axes) to **A**, respiratory PR-10 allergens Bet v 1 and Aln g 1 and **B**, to PR-10 food allergens (Cor a 1.0401, Mal d 1, Ara h 8, Pru p 1, Api g 1, Act d 8, Gly m 4) in patients suffering from different types of birch associated symptoms: allergic rhinitis AR (blue), asthma AS (red), oral allergy syndrome OAS (green), atopic dermatitis AD (yellow) and in Bet v 1-sensitized non-allergic subjects (violet).

FIG E4. Patterns of sensitization to PR-10 proteins and co-morbidities. Numbers of patients with co-morbidities (blue: 1 disease; red: two diseases; green: 3 diseases; violet: 4 diseases) (y-axis) sensitized to Bet v 1 alone or to Bet v 1 and additional PR-10 proteins (x-axis).

FIG E5. Association of the number of co-morbidities (x-axis) with cumulative levels of IgE specific to PR-10 proteins (y-axis) as box and whisker-plots. In addition, mean levels of IgE are displayed for each group (marked by an X within the respective box-plots). Significant differences between the groups are indicated by asterisks.

Table E1. Demographic and clinical characterization of allergic and non-allergic subjects (n=200).

Table E2. Allergen-specific IgE-levels in Bet v 1-positive subjects with (group 1: n=65) and without allergy symptoms (group 2: n=30).

Table E3. Numbers of Bet v 1 positive allergic patients (group 1: n=65) with IgE-reactivity to the different PR10 proteins (left column) and percentages of IgE-reactivities to the respective other PR10 proteins. Green: 80% to 100%; yellow: 50% to 79%); pink: <50%.

Table E4. Comparison of sequence identities of the different PR-10 proteins investigated. Green: 80% to 100%; yellow: 50% to 79%); pink: <50%.

METHODS

Characterization of children with and without symptoms of birch pollen allergy

Blood samples were obtained from children at the National Institute of Immunology, Moscow, Russia, with permission from the local ethics committee and written informed consent obtained from the parents. Parents filled out the “International Study of Asthma and Allergies in Childhood (ISAAC)” questionnaire^{E1, E2} which had been translated into Russian. Asthma was defined by a positive answer to either “Has your child ever had wheezing or whistling in the chest at any time in the last 12 months?” or “In the last 12 months, has your child had a dry cough at night, apart from a cough associated with a cold or chest infection?” Allergic rhinitis was defined by a positive answer to “Has your child ever had any of the following symptoms for at least one hour on most days (or on most days during the season if your symptoms are seasonal): watery, runny nose, sneezing (especially severe or even bouts

of sneezing), nasal obstruction, nasal itching, conjunctivitis (red, itchy eyes), postnasal drip?”
or “Have you ever had allergic rhinitis?”

Atopic dermatitis was defined by a positive response to “Has your child ever had atopic dermatitis?” or “Has your child ever had an itchy rash that was coming and going for at least six months?”

Oral allergy syndrome was assessed by questions regarding pruritus of the lips, tongue, oral mucosa, burning sensations of the tongue, swelling of the lips or the tongue, swelling of the oral mucosa, laryngeal swelling, inflammation of the tongue or of the oral mucosa, perioral skin symptoms, wheezing, dyspnoea, nausea/vomiting and gastrointestinal disorders that are associated with ingestion of apple, peach, carrot, nuts or other fruits and vegetables. This questionnaire-based assessment of OAS provides comparable diagnostic accuracy compared to oral provocation testing.^{E7}

Blood samples were collected from children (n=103) who, according to ISAAC questionnaire, appeared to suffer from IgE-associated allergy and from children (n=97) who, according to ISAAC questionnaire, did not suffer from allergy-related symptoms. Anonymized serum samples were stored at -20°C until analysis.

Phenotyping of birch pollen-associated allergy symptoms

After filling in the questionnaire by the parents, all children were clinically assessed, including a detailed case history, thorough physical examination and skin prick testing according to current guidelines^{E3}, as well as by measuring birch pollen allergen-specific IgE by ImmunoCAP technology (ThermoFisher, Uppsala, Sweden). The clinical diagnosis of allergic rhinitis was based on recommendations by the European Academy of Allergy and Clinical Immunology^{E4} and ARIA guidelines.^{E5} The diagnosis of asthma was performed according to guidelines of the Global Initiative for Asthma/Global Strategy for Asthma Management and Prevention (available from: www.ginasthma.com).

Atopic dermatitis was diagnosed based on international guidelines.^{E6} The diagnosis of birch pollen-related oral allergy syndrome was based on a questionnaire approach.^{E7}

Microarray-based measurement of specific IgE to Bet v 1 and related PR-10 allergens

IgE reactivity to microarrayed Bet v 1 and related PR-10 allergens was determined using MeDALL allergen-chips which had been developed based on the ImmunoCAP ISAC technology (ThermoFisher, Phadia, Uppsala, Sweden) as described.^{E8} Arrays were scanned using a LuxScan 10K microarray Scanner (CapitalBio Technology, Beijing, China) and analyzed with the microarray evaluation software version 3.1.2 (customized for MeDALL chips). The specific IgE levels measured by the MeDALL chip correspond to specific IgE levels measured with ImmunoCAP® ISAC 112 and are standardized with a reference serum with known levels of specific IgE determined by ImmunoCAP technology. Results are given in ISU (ISAC standardized units). According to previous studies, the threshold for positivity of allergen-specific IgE was defined by an IgE level of 0.3 ISU.

On the MeDALL chip, only 100 femtograms are spotted per allergen. This is more than 10,000,000 times less as compared to ImmunoCAP or other serological test systems that employ allergen in the micromolar range, i.e., in relative excess when related to allergen-specific IgE-levels in serum samples. Therefore, microarrays are more closely related to *in vivo* conditions, where also small amounts of allergen are incorporated, giving rise to competition of IgE and IgG for binding to the same epitopes. However, as was demonstrated previously,^{E8} the MeDALL chip showed equal or even slightly superior sensitivity in direct comparison to ImmunoCAP due to low background signals and a sensitive, fluorescence-based detection system. Intra-assay variation was determined to be approximately 8% in the IgE-range of 1-130 ISU.^{E8} Average inter-assay variation for PR-10 proteins was, based on results of a calibrator serum which had been included in each sample run, 26% (unpublished data) which is in line with results obtained for ImmunoCAP ISAC.^{E9} As microarray surface,

coupling chemistry and reagents are identical or very similar, respectively, to those that are used for ISAC, results from our study most probably can be directly transferred to the latter. However, it cannot be entirely excluded that cut-off levels obtained with the MeDALL chip would be identical to values derived from IgE-levels recorded using ImmunoCAP ISAC.

Statistical analysis

Children were assigned to group 1 of subjects with and to group 2 of subjects without allergy symptoms according to ISAAC questionnaire. In both groups, subjects with Bet v 1-specific IgE levels of 0.3 ISU or greater were identified by chip-based measurement. Children suffering from birch pollen allergy were grouped according to 4 different clinical phenotypes (i.e., AR, OAS, AS, AD) and 4 co-morbidities (i.e., AR+AS, AR+OAS, AR+AS+AD, AR+AS+AD+OAS). Results are given in means $\pm$ SD, medians with quartiles, absolute (n) or relative numbers (%), where appropriate. The level of allergen-specific IgE for each positive allergen molecule was compared between phenotypes using the nonparametric Kruskal-Wallis test. A *P* value of ≤ 0.05 was considered statistically significant.

Receiver operating characteristic curve (ROC) analysis was performed to determine a cutoff for specific IgE which yields highest sensitivity and specificity for the identification of subjects with a high propensity to have symptoms of allergy. Logistic regression models were used to estimate odds ratio (OR), and their 95% CI associated with positive allergen-specific IgE. These data were analyzed by using IBM SPSS Statistics.

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