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Seasonal variation in food allergy to apple

Kirsten Skamstrup Hansen[a,*], Stefan Vieths[b], Helle Vestergaard[a], Per Stahl Skov[a], Carsten Bindslev-Jensen[c], Lars K. Poulsen[a]

[a]*Allergy Unit, Department 7551, National University Hospital, Blegdamsvej 9, DK-2100 Copenhagen, Denmark*
[b]*Department of Allergology, Paul-Ehrlich-Institut, Langen, Germany*
[c]*Department of Dermatology, Odense University Hospital, Odense, Denmark*

Abstract

The aim of the study was to investigate the possibility of a seasonal variation in reactivity to apples in 27 birch pollen allergic patients. Before and during the birch pollen season 1998, the patients were subjected to double-blind, placebo-controlled food challenges (DBPCFCs) with grated fresh Golden Delicious apple followed by an open food challenge with whole fresh apple. The clinical reactions elicited during the challenges were evaluated both by the patients and the investigators. Moreover, the skin reactivity and the in vitro reactivity to apple were evaluated by skin prick test (SPT), leukocyte histamine release (HR), measurement of specific IgE, and immunoblotting experiments. The sensitivity of the DBPCFC, when compared with the result of the open challenge, was 0.74 (14/19) before the season and 0.80 (16/20) during the season. None of the patients reacted to the blinded challenge without a subsequent reaction to the open challenge. One placebo reaction was registered both before and in season, but not in the same patient. The patient scores of the first positive challenges, and the maximal scores of each combined blinded and open challenge session, were significantly increased during the pollen season ($P<0.05$). The scores of the open challenge were significantly higher than the scores of the DBPCFC both before the season and during the in-season challenges ($P<0.05$). Specific IgE against Golden Delicious increased during season ($P<0.05$), while neither SPT, HR, nor immunoblotting experiments could confirm an increase in reactivity. In conclusion, the results of the oral challenge tests indicated an increase in clinical reactivity to apples during the birch pollen season in birch pollen allergic individuals.

Keywords: Food allergy; Apples; Double-blind placebo-controlled food challenge; Seasonal variation

1. Introduction

Allergic reactions to fruit, nuts, and vegetables are common food allergic reactions in the Northern part of Europe because of the high prevalence of sensitization to birch pollen and the cross-reactivity between Bet v 1 and allergens from various plant foods [1–8]. Allergy to apple is an important clinical example. Oral allergy syndrome (OAS), i.e., itching and swelling of the lips, mouth, and pharynx immediately after ingestion of the food, is the most frequent clinical manifestation of the allergic cross-reactions [9]. Other allergic symptoms may develop, but concerning apple, serious systemic reactions seem to be rare [10–12].

Isolation and sequencing of the major allergen Mal d 1 from apple have shown a high degree of sequential homology with Bet v 1 [13–18] and further it has been demonstrated that Mal d 1 and Bet v 1 cross-react at T-cell level [19]. Mal d 1 belongs to a group of plant proteins showing significant

*Corresponding author. Fax: +45-357-481.
E-mail address: ksh@dadlnet.dk (K. Skamstrup Hansen).

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sequence identity with pathogenesis related proteins of the PR-10 family and the expression of Mal d 1 varies between different apple strains [20]. Golden Delicious and Granny Smith apples have a high expression of Mal d 1 compared to, e.g., Jamba and Gloster [20,21], a fact that seems well correlated to the history of many patients, telling that green apples more often cause symptoms than the red ones. The Mal d 1 content, and thereby the allergenicity of the apples, increases during ripening and is influenced by storage [22,23]. In contrast to Bet v 1, which occurs in more than 20 isoforms, minor sequence variation has been found in Mal d 1. Three major isoforms have been identified which share amino acid sequence identities of about 90% and minor variation in their allergenic reactivity [21]. The differences were not strain-specific, indicating that low or high immunoglobulin E (IgE)-binding isoforms are not responsible for differences in allergenicity between apple strains.

An aspect, not well investigated is the possibility of a seasonal variation in the clinical reactivity of the patients upon ingestion of cross-reacting foods, due to a priming effect of the exposure to birch pollen, in parallel to phenomena described for inhalant allergy [24–27].

The study design was prospective and the purpose was to investigate whether the in vivo and in vitro reactivity to apples in birch pollen allergic patients changes during the birch pollen season. Furthermore we wanted to evaluate the range of the symptoms to apple, in a group of birch pollen allergic patients by the use of controlled, and if possible blinded, oral challenge tests.

2. Materials and methods

2.1. Design

Twenty-seven birch pollen allergic patients with clinical allergy to apple or hazelnuts were examined before and during the birch pollen season 1998. The first part of the study was carried out from 12 January to 25 February (one patient was included 13 March), and the second part from 5 to 28 May. Before and during the pollen season, the patients were subjected to a double-blind placebo-controlled food challenge (DBPCFC) with grated fresh apple followed by an open food challenge with whole fresh apple. Moreover, the IgE-reactivity to apple before and in season was evaluated by skin prick test (SPT), histamine release (HR), measurement of specific IgE, and immunoblotting experiments. From 1 February to 31 May the patients recorded symptoms and use of medication.

The study was approved by the local ethics committee [(KF)01-075/97] and all subjects gave written informed consent before entering the study.

2.2. Subjects

The patients were recruited based on a clinical history of rhinoconjunctivitis in the birch pollen season and OAS or other allergic manifestations upon ingestion of apple and or hazelnut, together with positive specific IgE and a positive SPT to birch pollen. The patient group comprised of 16 women and 11 men, with a mean (range) age of 31 years (20–54). The mean (range) duration of symptoms to birch pollen was 13 years (4–30) against 10 years (2–21) of symptoms to apple and or hazelnut. In all 21 patients reported symptoms to apples, 20 to green apples and 17 to red apples. One patient remembered symptoms to red apples, only. Of the 17 with reactions to both green and red apples, three patients stated that the green apples resulted in the most pronounced symptoms. All 21 patients experienced symptoms within the first few minutes after ingestion of apple.

Of the 27 patients, 26 had symptoms upon ingestion of hazelnuts. The 11 most reported offending foods other than apples and hazelnuts were walnut, almond, kiwi, pear, plum, peach, cherry, nectarine, carrot, pineapple, and fresh peas. Fifteen patients also reported symptoms to grass pollen. None of the patients had a history of perennial rhinitis or conjunctivitis, severe atopic dermatitis, or severe asthma but nine patients reported asthmatic symptoms provoked by exercise or allergen exposure. At the time of inclusion in the study, none of the patients received daily asthma medication.

All 27 demonstrated a positive conjunctival provocation test (CPT) to doses in the interval of 1000 to 30 000 SQ of the birch pollen extract (ALK-Abelló, Hørsholm, Denmark) and 10 patients also demon-

strated a positive CPT to grass (3000 to 100 000 SQ).

2.3. Medication

Prior to challenges and SPT, medication was discontinued according to the guidelines from the European Academy of Allergy and Clinical Immunology (EAACI) on skin testing [28]: short acting antihistamines (acrivastine) at least 2 days before, long acting antihistamines 8 weeks, and topical steroids 2 to 3 weeks before testing. None of the patients were treated with systemic steroids, hydroxyzine, ketotifen, or tricyclic antidepressants during or a month prior to the study. One patient was treated with inhaled steroid in a low dose for 2 weeks during the study period, but stopped the treatment 6 days before the last food challenge. Another patient reported asthmatic symptoms at the last challenge visit and started inhaled steroid immediately after the challenge test. Five patients used inhaled β_2-agonist as p.n. medication during the study period. Only three of these used the medication before the last challenge test and all stopped at least 6 days before the challenge. Two patients used β_2-agonist exclusively in connection with physical exercise.

The patients were allowed to use short-acting antihistamine tablets (acrivastine), nasal spray, and eye drops (levocabastine) as rescue medication. The medication was supplied by the Allergy Unit in order to keep record and standardize use.

2.4. Allergen sources and extracts

Two apple extracts and two major-allergen preparations produced by Vieths et al. [15] were used for SPT and HR: (1) a whole-apple extract from French Golden Delicious apples (86 μg/ml), (2) a whole-apple extract from German Gloster apples (44 μg/ml), (3) Mal d 1 (10 μg/ml) purified from the whole-apple extract and (4) Bet v 1 (10 μg protein per ml). Further, a commercial recombinant Mal d 1 preparation (Biomay, Linz, Austria) was applied for determination of specific IgE by an enzyme allergo sorbent test (EAST). The Golden Delicious whole-apple extract was applied for the immunoblotting experiments and EAST. Moreover fresh Golden Delicious (France) and Gloster apples (Denmark) were used for SPT and HR. All oral challenges were performed with fresh Golden Delicious apples (France). The fresh apples were purchased at the local market.

Commercial birch and grass pollen extracts (Soluprick and Aquagen, ALK-Abelló) were used for SPT and CPT.

2.5. DBPCFC and open food challenges

The blinded challenge procedure included three challenges. The first challenge was always a test dose without apple (~placebo challenge) served single-blindedly. The following two challenges, one active and one placebo, were randomised and administered blinded, both to the patient and the investigators. The challenges were served with an interval of minimum 30 min. The next challenge was not served before symptoms had disappeared or diminished significantly. The blinded challenges were prepared and randomised by a dietician and the code was not broken until the challenge procedure was completed and the results entered in an observation sheet.

Recipe for the blinded challenges:

Placebo mixture:	Active mixture:
22.5 g finely grated cabbage	17.0 g finely grated cabbage
7.5 ml concentrated apple juice	3.0 ml concentrated apple juice
	20.0 g finely grated apple

The challenge was served inside a little pita bread (approx. 30.0 g). The apple was grated immediately before the active challenge was served to the patient. The commercial apple juice (1:5, v/v) was a pasteurized product.

The challenge session was concluded with an open challenge test with whole fresh apple to confirm the result of the DBPCFC and to establish the range of symptoms for each patient. For safety reasons, the patients were offered a slice (10 g) of apple (first open challenge) before they were allowed to eat an

apple bite for bite (second open challenge). The total amount of apple ingested by the patients during each challenge session (combined blinded and open procedure) was 140 g (30–190).

Before each challenge session the patients were questioned about concomitant diseases and use of medication. Further heart rate, blood pressure, and lung function (FEV1 and PEF) were measured. After the challenges and in case of any symptoms, lung function measurements were repeated. A decrease in FEV1 $\geq$300 ml, a decrease in PEF $\geq$60 l, and or a decrease $\geq$15% in either of the two were considered significant. The patients were continuously observed by the investigators during the challenge tests and all symptoms were recorded. The patients were asked to evaluate the severity of their reactions to the individual challenges on a scale from zero to three, three being the most pronounced symptoms. Besides the subjective evaluation, the symptoms were graded by the investigators using the following scale: 0, No reaction; 1, mild OAS; 2, moderate OAS and possible feeling of local swelling in the mouth and pharynx; 3, severe OAS and objective signs of rhinitis, conjunctivitis, local urticaria, urticaria with other location, mild angiooedema or mild asthma and 4, severe systemic reaction.

2.6. Skin prick test

The SPT was performed according to EAACI guidelines [28]. Histamine dihydrocloride 10 mg/ml served as positive control and diluent (50% glycerol, saline and buffers) as negative control. Allergen preparations and controls were applied in quadruplicate. SPT with fresh apples was performed by the prick–prick technique [5,29]. The skin wheal areas were determined by computer scanning [30] and a skin prick test was considered positive when the wheal area was $\geq$7 mm^2 (diameter $\geq$3 mm).

2.7. Conjunctival provocation test

In order to substantiate the allergic history and the pollen sensitization indicated by positive SPT and specific IgE, titrated CPT with birch and grass pollen extracts (Aquagen, ALK-Abelló) were performed at inclusion. Increasing doses of allergen extract (300, 1000, 3000, 10 000, 30 000, 100 000 SQ) were applied alternately in the right and left eye with 10 min intervals. The test was considered positive, when at least two different symptoms (congestion of the conjunctival mucosa combined with at least one of the following symptoms: itching, secretion, or swelling) developed [31].

2.8. Specific IgE

Specific IgE against apple and pollen were measured at the Allergy Unit using the CAP system (Pharmacia-Upjohn, Uppsala, Sweden) and the Magic Lite system (ALK-Abelló) according to the instructions from the manufacturer. Specific IgE against the Golden Delicious whole-extract and rMal d 1 were measured at the Paul-Ehrlich-Institut by EAST (Allergopharma, Reinbek, Germany) as described in Ref. [20]. The proteins were coupled to cyanogen bromide activated filter paper disks (Hycor, Kassel, Germany) (3 μg per disk for apple extract and 0.25 μg per disk of rMal d 1) [32].

2.9. Leukocyte histamine release

The histamine release experiment was performed by the glass-fibre method [33]. All allergen preparations were diluted further 10 times before use as stock solutions for HR. Further, fresh Golden Delicious and Gloster apples were applied for HR. For the preparation of fresh apples, 10 g fresh apple was crunched in a Stomacher 80 (Seward Lab System, UK) at high speed for 120 s with 10 ml Pipes-AMC (pH 7.4) [10 m*M* piperazine-*N*,*N*-(bis-ethane sulfonic acid), 140 m*M* sodium acetate, 5 m*M* potassium acetate, 0.6 m*M* $CaCl_2$, 1.1 m*M* $MgCl_2$, glucose 1 mg/ml, human serum albumin 0.3 mg/ml, heparin (Leo, Ballerup, Denmark) 15 IU/ml]. After centrifugation (2000 *g*, 10 min) the supernatant was used as stock solution. The fresh preparations were applied within 15 min. Anti IgE (Behringwerke, Marbourg, Germany) was used as positive control in the following final concentrations: 400, 40, and 4 U/ml. The allergen extracts were added to the plates in nine 3.5-fold dilutions. A histamine release test was considered positive when the maximal release $\geq$14 ng/ml. In order to exclude unspecific release,

identical experiments were performed with blood from a healthy, non-atopic individual: All nine dilutions of the allergen extracts consistently produced a release ≤10 ng/ml. The HR results are presented as a mean value of the maximal release (ng/ml) and as the number of 3.5-fold dilution of the allergen preparation at which the release was 50% of the maximal release (1/2 max HR), e.g., the higher number corresponding to 1/2 max HR the higher allergenic potency of the extract.

2.10. Immunoblotting

The IgE-reactivity of the patients before and in season was characterised by means of sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotting experiments [14,20,34]. A serum without specific IgE against apple and the incubation buffer [Tris-buffered saline (TBS), 0.01 *M* Tris–HCl, pH 7.4, 0.15 *M* NaCl, 0.05% Tween 20, 0.1% bovine serum albumin (BSA)] were applied as negative controls. The Golden Delicious whole-apple extract was applied for this part of the study.

For SDS–PAGE, a 13% acrylamide separation gel and 5% stacking gel were prepared from a commercial stock solution (Rotiphorese Gel30, Roth, Karlsruhe, Germany). An amount of 200 μg protein was loaded on the gel (13.5×10 cm gel, 10 cm sloth→20 μg protein per cm). A low-molecular-mass protein marker (LMW-Marker, Pharmacia, Uppsala, Sweden) was added in a separate slot. Two gels were prepared, each were used for 13 patients. The proteins were transferred to a nitrocellulose (NC) membrane (0.45 μm, Schleicher & Schuell, Dassel, Germany) using a semi-dry electroblotting cell (Pharmacia Biotec Multiphor II, Pharmacia, Uppsala, Sweden). After electroblotting and blocking (TBS, 0.01 *M* Tris–HCl, pH 7.4, 0.15 *M* NaCl, 0.3% Tween 20) the NC membrane was cut into strips of 3 mm. Each strip was incubated with 150 μl serum and 850 μl incubation buffer (TBS, 0.01 *M* Tris–HCl, pH 7.4, 0.15 *M* NaCl, 0.05% Tween 20, 0.1% BSA) overnight at room temperature. For the immunodetection, the strips were incubated with monoclonal anti-human-IgE-alkaline phosphatase (Pharmingen, San Diego, CA, USA) 1:750 for 3 h and then stained with an Alkaline Phosphatase Conjugate Substrate Kit (Bio-Rad, Hercules, CA, USA).

2.11. Total IgE and eosinophils

The total serum IgE-level and the eosinophil blood count were determined at the visits before and in the pollen season.

2.12. Symptom and medication score

The patients were asked to keep a diary during the study period. Possible allergic symptoms were evaluated on a scale from zero to three and all use of rescue medication was recorded.

After the diaries were collected, a combined symptom and medication score was calculated for each patient on each day. Regarding the medicine score, one antihistamine tablet was considered equal to one eye drop in each eye or one puff of nasal spray in each nostril, e.g., a patient with a rhinitis score of two and a conjunctivitis score of one, who had used three tablets and two puffs was given a combined score for the actual day of eight. Asthmatic symptoms and possible treatment were recorded on a separate sheet and not included in the score. The symptom and medication scores were compared with the daily birch pollen counts from The Danish Meteorological Institute.

2.13. Statistics

The results of the SPT, specific IgE, HR, and challenges scores before and during the pollen season were compared by parametric (paired *t*-test, Pearson Product Moment) and non-parametric methods (Wilcoxon Signed Rank Test, Spearman Rank Order). The data on total IgE and eosinophil count before and in season were compared by paired *t*-test and Wilcoxon Signed Rank Test. The sensitivity of the blinded challenge was calculated according to Refs. [35,36]. Disease was defined as a positive clinical history of allergy to birch pollen and apple, positive specific IgE and SPT to birch, combined with a positive open challenge test with apples. SigmaStat 1.0 (Jandel, San Rafael, CA, USA) was applied for statistical analyses.

3. Results

Of the 27 patients included in the study 26 completed, while one patient was withdrawn before the in-season challenges due to lack of compliance. During the birch pollen season, 21 patients managed to record symptoms and use of medication. Fig. 1 shows that the symptom and medication score follows the pollen curve. The curve has a bimodal shape with a small peak ultimo February/primo March and another and larger peak late April. In the period from 1 April to 31 May, 99% of the birch pollen were counted and 83% of the total symptom and medication score for the 21 patients were registered. The total symptom and medication score was maximal during the last days of April and the first days of May, which correlates well with the concomitant peak in birch pollen. The smaller peak of symptoms seen in February/March can be ascribed to a high level of the early spring pollen, primarily alder, that year.

During the period from 1 April to 31 May, the mean (range) number of days with registration of symptoms and or medication was 36 (8–60) days. This results in a score per day with symptoms and or medication of 6.6 (2.8–11.8) or a daily symptom and

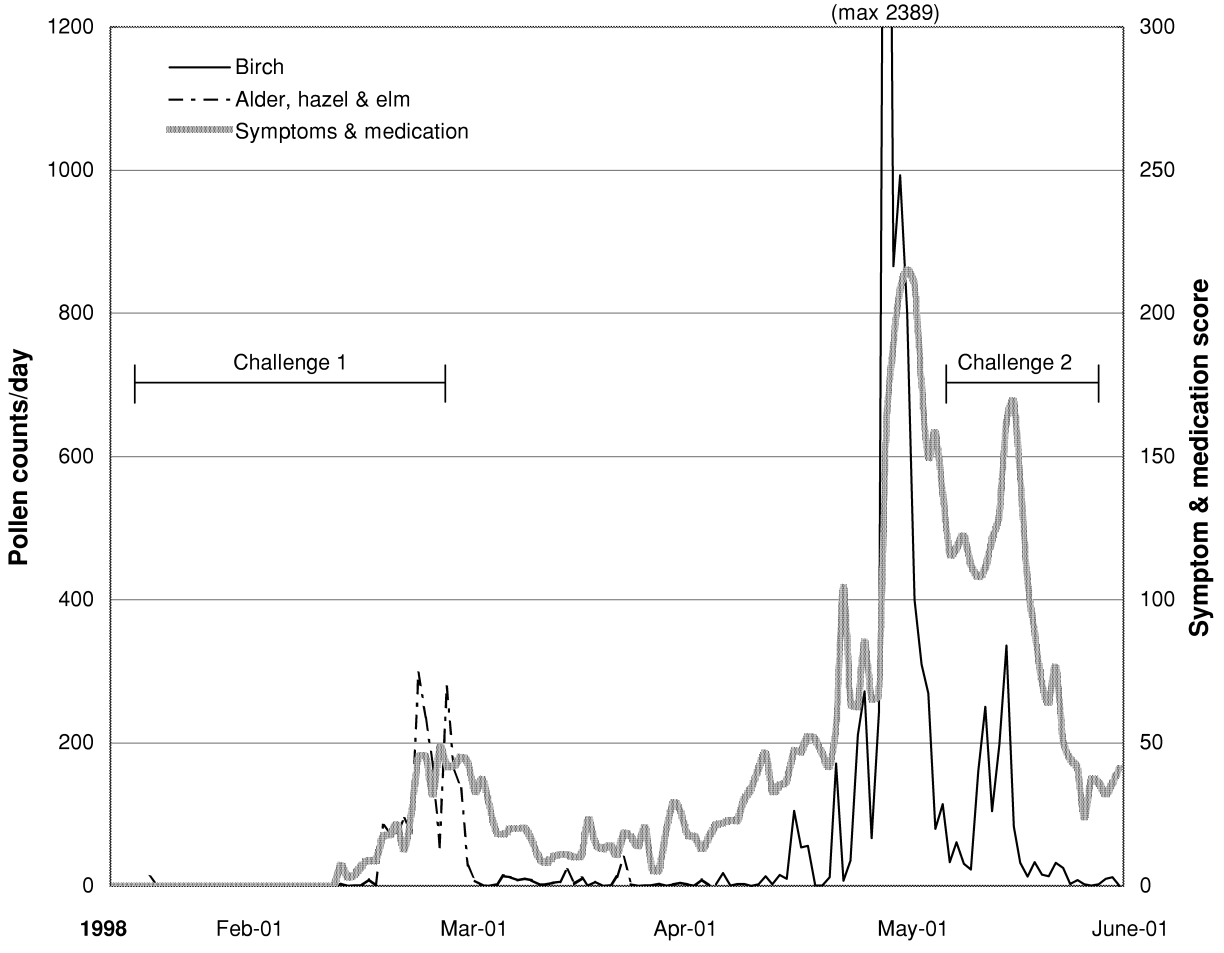

Fig. 1. The pollen counts and the combined symptom and medication scores for 21 patients. The intervals of the challenge sessions are marked. Between 1 April and 31 May, 99% of the birch pollen was counted and 88% of the symptoms occurred. All in-season challenges took place after the maximum peak of birch pollen of the season.

medication score in the period from 1 April to 31 May of 3.9 (0.6–7.9). The mean (range) eosinophil blood count rose from 0.18 (0.03–0.49)$\times 10^9$ cells/l before season to 0.28×10^9 cells/l (0.06–0.56) ($P=$ 0.0006) in season, while the total IgE level did not change significantly.

3.1. Challenges

Before the pollen season, 14/26 blinded challenges were positive, against 19/27 open challenges. One patient was subjected to open challenge only. During the pollen season, 16/26 blinded and 20/26 open challenges were positive. One placebo reaction was registered both during the challenges before and in season, but not in the same patient. In neither of the cases did the patient react to the test dose, to the active challenge or during the open challenges. Using the result of the open challenge as the true diagnosis, the sensitivity of the DBPCFC was 0.74 (14/19) and 0.80 (16/20) before season and in season, respectively. The specificity of the DBPCFC was one, since no positive blinded challenges were seen among patients with negative open challenges.

Fig. 2 shows the challenge results for the 20 patients with at least one positive challenge, who were tested both before and during season. The two patients with a placebo reaction were excluded from the figure. The patient scores of the first positive challenges at each challenge session were significantly higher during the pollen season [$P<0.05$ (Wilcoxon $P=0.049$, t-test $P=0.035$)]. Moreover,

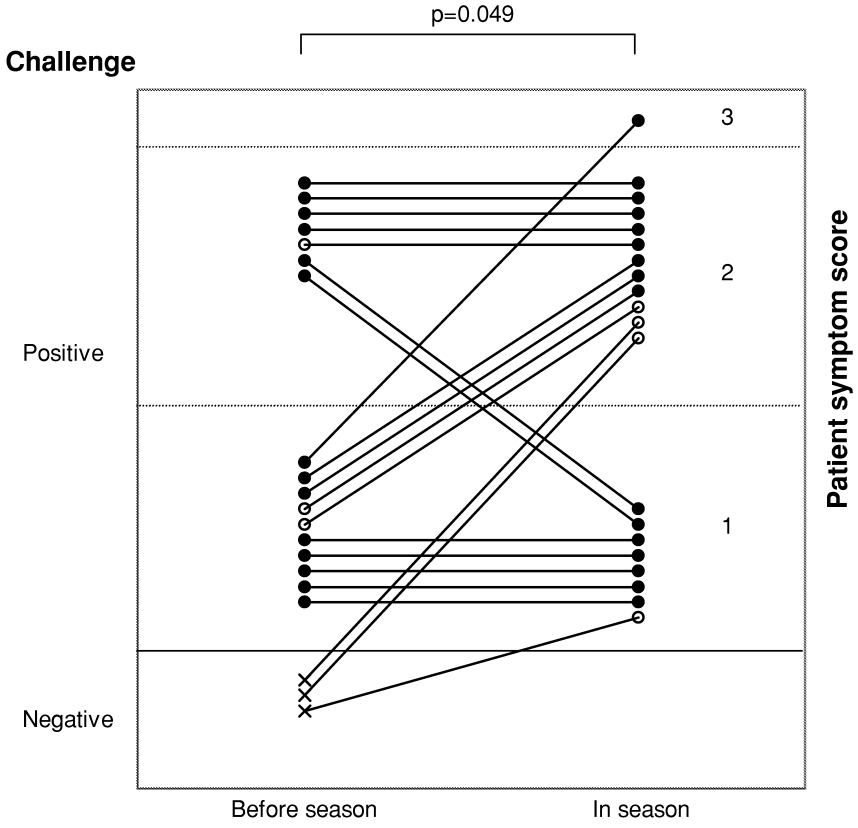

Fig. 2. The course of the oral challenges for the 20 patients with a positive challenge either before or during the pollen season or both. The filled symbols indicate that the DBPCFC was positive and the open symbols indicate that the DBPCFC was negative, but the subsequent open challenge was positive. The x marks the negative result of the challenges before the season for three patients, who turned out to be positive, when they were tested during the season. The symptoms were graded by the patients (1–3).

the maximal scores of each combined blinded and open challenge session (not shown), evaluated by the patients, were significantly higher during the season (Wilcoxon $P=0.039$, t-test $P=0.029$).

The symptoms recorded during the challenges were generally mild and all patients had OAS. Objective signs were recorded during 12 challenges before season and 12 challenges in the season. The objective symptoms were rhinitis, conjunctivitis, and local urticaria on the mouth. Two patients had a fall in PEF of 60 l during the challenges before season, but not combined with a significant change in FEV1. One patient was very nervous at the first visit before season and felt uneasy during the open challenge procedure, but there were no objective signs of an allergic reaction. Lung function, heart rate, blood pressure, and serum tryptase (Pharmacia-Upjohn) were within the normal range.

The sums of the challenge scores are illustrated in Fig. 3. Both the scores given by the patients and by the investigators are shown. Before the season, the sum of the scores of the second open challenge was significantly higher than the scores of the DBPCFC (patient/investigator, $P=0.01/0.003$) and also higher than the score of the first open challenge ($P=0.03/0.004$). The same pattern was seen during the

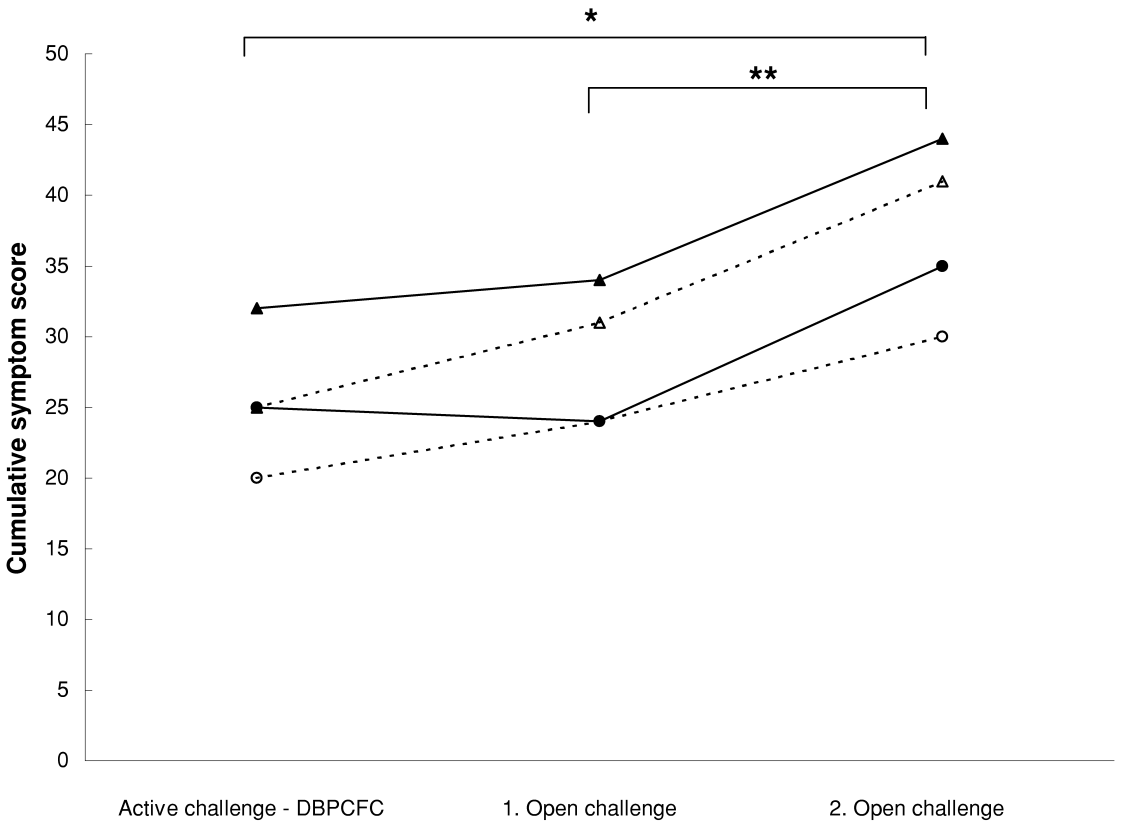

Fig. 3. Symptom scores during DBPCFC, 1., and 2. open challenge before (open symbols & dotted line) and during season (filled symbols & full line). The symptoms were evaluated by both the patients (circles) and the investigators (triangles). The sums of the scores for the 20 challenge positive patients are illustrated. The scores of the second open challenge were significantly higher than the scores of the active challenge and the first open challenge, evaluated by both the patients and the investigators (*before season $P=0.01$ ptt./$P=0.003$ invest., in season $P=0.01/0.007$; **before season $P=0.03/0.004$, in season $P=0.007/0.007$).

in-season challenges (second open challenge> DBPCFC, $P=0.01/0.02$; second open challenge> first open challenge, $P=0.007/0.007$). The two patients with a reaction to placebo were excluded from Fig. 3.

3.2. Skin prick test

Skin prick results in mean values for all patients ($n=26$) and the challenge-positive ($n=20$) patients, respectively, are shown in Table 1. In absolute values, skin wheal areas to both allergen preparations, fresh apples, and positive control (histamine 10 mg/ml) diminished significantly during season ($P=0.001$–0.04). But when the results were adjusted for the area of the positive control at the actual visit, only the Gloster whole-apple extract gave significantly smaller areas during season than before season ($P=0.02$). But since none of the patients had skin wheal areas ≥ 7 mm^2 to the Gloster extract either before or during season, this result is clinically irrelevant. The challenge-positive patients did not differ significantly from the challenge-negative patients.

3.3. Specific IgE

As shown in Table 2, the level of specific IgE was significantly higher during season for birch pollen measured by the CAP system ($P=0.001/0.0002$) and for the Golden Delicious whole-apple extract ($P=0.0006/0.0001$) and rMal d 1 ($P=0.02/0.002$) measured by EAST. The challenge-positive patients did not differ significantly. None of the patients had positive specific IgE against apple measured by the Magic Lite system and therefore the data are not included in the table. Both values in kU/l (mean) and classes (median) for all patients ($n=26$) and for the challenge-positive ($n=20$) patients are listed in the table.

Table 1
SPT results

			Before season		In season		Difference (P)	
			All	Pos	All	Pos	All	Pos
Fresh fruit								
GD	mm^2	(mean)	24.4	28.9	17.0	20.2	0.03	0.04
	/H10	(mean)	0.8	0.9	1.0	1.1	ns	ns
Gloster	mm^2	(mean)	20.4	24.5	11.3	13.3	0.001	0.001
	/H10	(mean)	0.7	0.8	0.6	0.8	ns	ns
Extracts								
GD	mm^2	(mean)	6.4	8.0	4.3	5.4	0.03	0.03
	/H10	(mean)	0.3	0.3	0.2	0.3	ns	ns
Gloster[a]	mm^2	(mean)	1.1	1.4	0.3	0.3	0.01	0.01
	/H10	(mean)	0.04	0.05	0.01	0.02	0.02	0.02
Birch	mm^2	(mean)	35.6	41.0	31.3	30.3	0.01	0.02
	/H10	(mean)	1.2	1.4	1.7	1.7	ns	ns
Mal d 1	mm^2	(mean)	7.2	9.1				
	/H10	(mean)	0.3	0.4				
Bet v 1	mm^2	(mean)	29.7	33.2				
	/H10	(mean)	1.4	1.6				

The 26 patients (All) tested both before and during season are included and the results of the 20 challenge-positive (Pos) patients is specified. The results from the visits before and in season and the difference (P) for each group are shown.

[a] SPT with the Gloster extract resulted in skin wheal area <7 mm^2 in all patients.

Table 2
Specific IgE results

			Before season		In season		Difference (*P*)	
			All	Pos	All	Pos	All	Pos
CAP								
Apple	Class	(median)	2	2	2	2	ns	ns
	kU/l	(mean)	3.0	3.6	3.2	4.0	ns	ns
Birch	Class	(median)	3	4	4	4	ns	ns
	kU/l	(mean)	21.4	25.7	33.2	41.1	0.001	0.0002
EAST								
GD	Class	(median)	1.5	2	1.5	2	ns	0.016
	kU/l	(mean)	1.1	1.3	2.6	3.3	0.0006	0.0001
rMal d 1	Class	(median)	0	0.5	0.5	1.5	ns	ns
	kU/l	(mean)	0.5	0.6	1.0	1.3	0.02	0.002

The 26 patients (All) tested both before and during season are included and the results of the 20 challenge-positive (Pos) patients is specified. The results from the visits before and in season and the difference (*P*) for each group are shown.

3.4. Leukocyte histamine release

HR results are shown in Table 3 as maximal release (mean) and 1/2 max HR (median). The only significant increase was seen for the whole-apple Gloster extract: 1/2 max increased during season ($P=0.008$), but only when the results for all 26 patients were compared. With the exception of one patient, all included had positive HR to Bet v 1. The HR results from the visit before and during the birch pollen season were inconclusive in five and three patients, respectively.

Table 3
HR results

				Before season		In season		Difference (*P*)	
				All	Pos	All	Pos	All	Pos
Fresh fruit									
GD	1/2 max	No	(median)	5	6	6	6	ns	ns
	Max	ng/ml	(mean)	42.8	47.7	44.8	47.4	ns	ns
Gloster	1/2 max	No	(median)	5	5	5	5	ns	ns
	Max	ng/ml	(mean)	32.4	35.7	33.7	36.3	ns	ns
Extracts									
GD	1/2 max	No	(median)	2	2	1	1.5	ns	ns
	Max	ng/ml	(mean)	39.2	42.0	26.1	30.0	ns	ns
Gloster	1/2 max	No	(median)	0	0	0	0	0.008	ns
	Max	ng/ml	(mean)	2.4	3.2	10.8	12.1	ns	ns
Mal d 1	1/2 max	No	(median)	3	4				
	Max	ng/ml	(mean)	40.9	44.5				
Bet v 1	1/2 max	No	(median)	7.5	8				
	Max	ng/ml	(mean)	54.8	56.0				

The 26 patients (All) tested both before and during season are included and the results of the 20 challenge-positive (Pos) patients is specified. The results from the visits before and in season and the difference (*P*) for each group are shown.

3.5. Immunoblot

The immunoblotting results are shown in Fig. 4. The patient withdrawn before the in-season visits was not included in this part of the study. In all, serum from 23/26 patients exhibited binding to a band with a molecular mass around 18 000 (except Nos. 12, 15, 25), but the binding was very weak in seven patients (Nos. 1, 4, 9, 14, 18, 23, 24). In the patients with the most pronounced binding the band is dual, corresponding to the double band seen at the India ink-stained strip of the NC membrane. Besides the M_r 18 000 band, other bands were seen, primarily in the upper molecular area around M_r 30 000 and 43 000 (Nos. 3, 8, 13, 20, 22, 23, 24). Only one patient (No. 6) showed binding to a band below the M_r 14 000 marker. The non-allergic control and the buffer control showed week, unspecific binding at M_r 30 000 and also some distinct double bands in the upper molecular area. There was no unspecific binding at the bands around M_r 14 000, 18 000, or 40 000. The binding to the M_r 18 000 band was more pronounced during season in four of the patients (Nos. 6, 7, 16, 19) whereas one patients (No. 5) showed a diminished binding during season. These five patients were all challenge-positive.

4. Discussion

It is generally agreed that the diagnosis of food allergy must rely on the outcome of DBPCFCs [37]. Regarding birch pollen allergy and concomitant food allergy to fruits, nuts and vegetables, the anamnestic information seems to be of greater diagnostic value than in classical food allergy to, e.g., milk or egg [5,38,39]. However, only a few studies of clinical aspects of allergenic cross-reactions have included controlled challenges and even fewer blinded challenges, and the issue is therefore not well described. Some patients have experienced severe symptoms and in fear of reiteration, they have kept a diet without the offending food for a long time, which may influence the liability of the anamnestic information. According to the history of the patients, 20 of the 26 patients tested both before and in season had symptoms to apple. The reactions were confirmed by open challenges in 18 of the patients before and in season. Two patients, who claimed to be tolerant to apple, actually developed mild OAS during the in-season challenges. These findings support the need for controlled challenges at least for scientific use.

The results of the challenge tests confirmed a slight increase in reactivity during season. Not all parameters increased significantly but the trend was clear: both the symptom scores of the first positive challenge and the maximal score at each challenge session increased during season. The fact that the individual scores of the challenges did not change significantly, even though the sums of the scores increased, could be explained if the scores of the three challenges were not entirely independent. The reason why it was chosen to perform both blinded and open challenge at the same day, was an aim to keep the period of antihistamine abstinence during the pollen season as short as possible. Further, we wanted to compare the different challenge procedures. If the challenges had been performed on different days during the season the outcome might have been influenced by the actual pollen counts. It seemed as if the patients were more sure of the symptoms during the in-season challenges (observations by the investigators), but there is a possibility of some kind of learning effect. As described, the symptoms were generally mild and often milder than remembered by the patients. The scores showed a modified ''dose–response'' effect: The sum of the scores of the second open challenge (30–190 g apple) was significantly higher than both the scores of the active challenge of the DBPCFC (20 g) and of the first open challenge (10 g). The sum of the scores of the first open challenges and of the active challenge was not significantly different, and for most of the patients, the symptoms during the second open challenge seemed to reach a plateau, where an additional bite of apple did not add to the severity symptoms. This could mean that tachyphylaxis developed during the challenge procedure and hence, it would be favorably to be able to increase the dose of apple from challenge to challenge with at least a factor of 10.

The sensitivity of the DBPCFC was between 0.74 and 0.80. This study was not a true diagnostic trial, since the patients were highly selected and no real control group was included. To improve the sen-

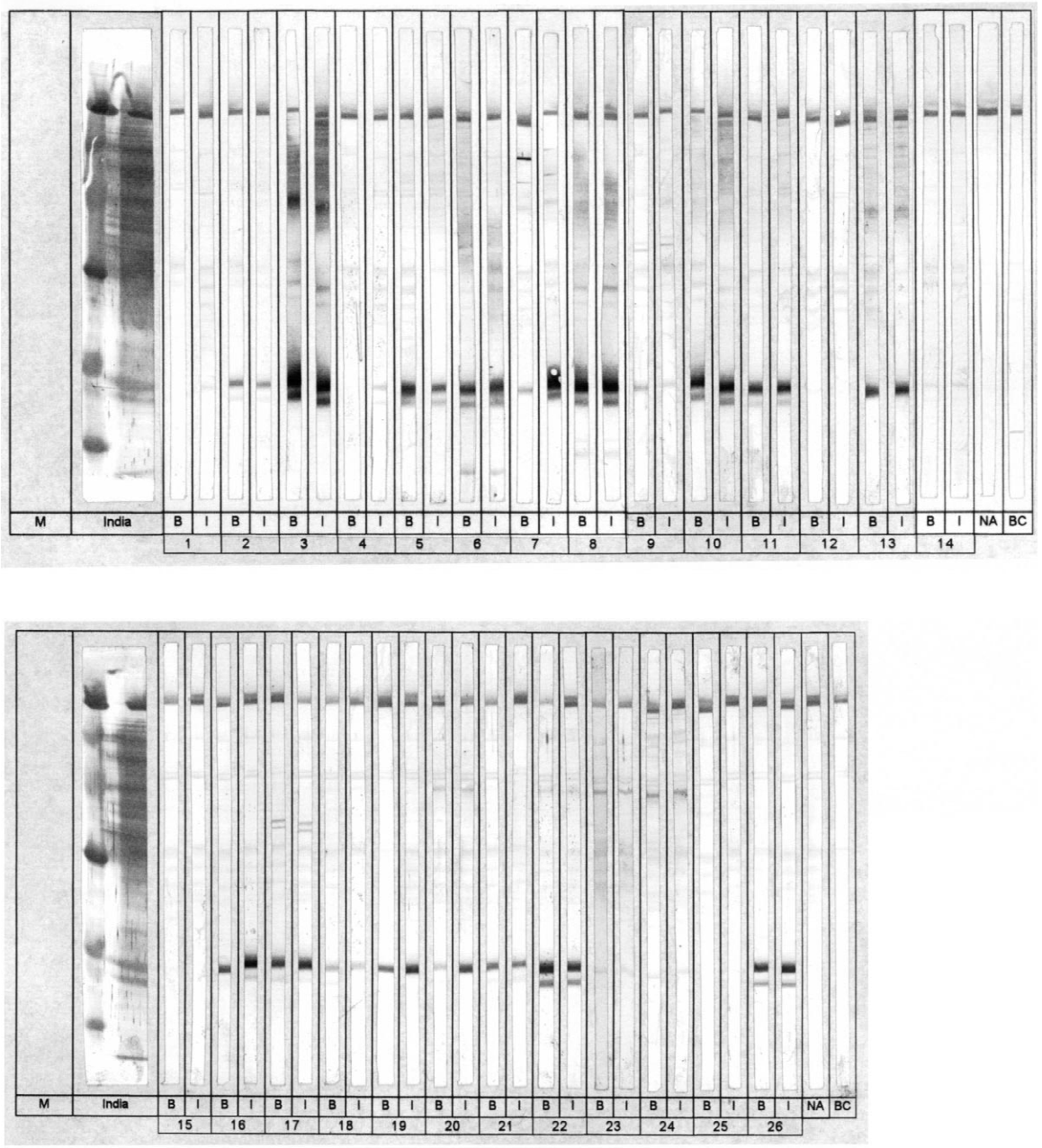

Fig. 4. Immunoblotting experiments with the Golden Delicious whole-apple extract. Sera from the 26 patients tested both before and during season, a non-atopic control (NA) and a buffer control (BC) were included. The marker and the India ink stained part of the NC membraneare shown to the left. The letter B indicates sera from the visit before the season and | marks the in-season sample.

sitivity it would be necessary to increase the relative amount of apple, but in this DBPCFC model it would be impossible to blind more apple without additional adding of flavour. It is a question whether it would be possible to make a blinded challenge with raw apple as sensitive as an open challenge with

raw unprocessed fruit. Moreover, the way most of us devour a raw apple is difficult to mimic in a blinded model. Finally, the sensitivity of some of the patients is probably so low that addition of other compounds in order to blind the challenge, leads to loss of symptoms due to the diminished concentration of apple.

The allergenicity of apples changes during ripening [22], a fact that may have influenced the results of this study. The French Golden Delicious apples used for oral challenges in this study were harvested during summer 1997, meaning that they were stored for about 6 months before the first part of this study and about 9 months before the second part. Storage of apples in controlled atmosphere (CA) with a low oxygen tension suppresses the expression of Mal d 1, resulting in unchanged Mal d 1 concentrations during storage [23]. Neither the HR nor SPT with fresh Golden Delicious were significantly increased from the February to May 1998, despite an increase in specific IgE to the Golden Delicious extract and rMal d 1 measured by EAST. Immunoblotting results showed an increase in the IgE binding to apple in four patients during season and a decrease in one.

SPT and HR with the fresh Danish Gloster apples and the extract from German Gloster apples gave conflicting results. According to literature this apple strain contains low amounts of Mal d 1 [20,21] and yet SPT and HR with the fresh fruit presented a reactivity comparable to that of the fresh Golden Delicious apples and the Golden Delicious extract. The Gloster extract on the other hand, gave significantly lower SPT and HR results than both the fresh apples and the Golden Delicious extract. Immunoblotting experiments confirmed a high Mal d 1 content of the Danish Gloster apples compared to the German strain (data not shown). The observed divergence could be explained by differences at the genomic level, climate, or growing conditions in the two countries, or the Danish apples could have been stored in a non-controlled atmosphere resulting in a high expression of Mal d 1 not seen in fresh or CA-stored apples. The result calls for circumspection regarding dietary advice on these matters.

The results for the different allergen preparations showed an intra-assay correlation. But there was no significant correlation between the results of the different methods or between the clinical reactivity of the patients and either the SPT results, the level of specific IgE, or the HR (data not shown).

The reasons for the general decrease in skin reactivity to both histamine 10 mg/ml, fresh fruit, and allergen preparations during the in-season visits remain uncertain. The same investigator performed all skin tests and the SPT solutions were handled as suggested by the manufactures. The patients were instructed to stop antihistamine (acrivastine) intake at least 48 h before the tests. The plasma half-life of this drug is 1.5 h (inc. metabolites 2.5 h) and in a single-dose study the skin wheal reaction was close to baseline after 24 h [40,41]. Some patients could have used antihistamine against the instructions within the last 48 h before the visit, a notion supported by the fact that SPT with grass also diminished during season (data not shown). Another possibility is a decrease in skin reactivity due to an exhaustion at the receptor level during the peak pollen season, even though a Finish study found an increase in skin reactivity to grass after the grass pollen season compared to pre-season values [42] and an Italian study showed parallel results for birch [25].

The peak in Alder and Hazel pollen in February and March poses a possibility of a priming effect even before the pollination of birch. And even though none of the patients registered symptoms or use of medication in February before the first challenge session this early pollen season could be a bias for this study by adding an in-season effect to the first part of the study and thereby diminishing possible differences.

In conclusion, the results showed that DBPCFC with apple was possible. The sensitivity of the DBPCFC method used in this study was 0.74/0.80 compared with the outcome of open challenges, and the symptom scores of the DBPCFC were significantly lower than of the open challenge procedure. The results of the oral challenge tests indicated an increase in reactivity to apples during the birch pollen season. The symptom scores of the first positive challenge and the maximal symptom score during each challenge session were increased during season.

The symptoms during the challenges were generally mild, but a possible clinical implication of the results may be that the risk of severe reactions to

cross-reacting fruits, nuts, and vegetables in highly sensitized individuals increases during the birch pollen season. A fact that should be taken into account during the investigation of severe reactions and in the advice to patients with a history of serious systemic reactions.

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References

[1] M. Hannuksela, A. Lahti, Contact Dermatitis 3 (1977) 79.
[2] N.E. Eriksson, Allergy 33 (1978) 189.
[3] K.E. Andersen, H. Lowenstein, Contact Dermatitis 4 (1978) 73.
[4] A. Lahti, F. Bjorksten, M. Hannuksela, Allergy 35 (1980) 297.
[5] S. Dreborg, T. Foucard, Allergy 38 (1983) 167.
[6] L. Halmepuro, H. Lowenstein, Allergy 40 (1985) 264.
[7] P.G. Calkhoven, M. Aalbers, V.L. Koshte, O. Pos, H.D. Oei, R.C. Aalberse, Allergy 42 (1987) 382.
[8] R. Hirschwehr, R. Valenta, C. Ebner, F. Ferreira, W.R. Sperr, P. Valent, M. Rohac, H. Rumpold, O. Scheiner, D. Kraft, J.Allergy Clin. Immunol. 90 (1992) 927.
[9] C. Ortolani, M. Ispano, E. Pastorello, A. Bigi, R. Ansaloni, Ann. Allergy 61 (1988) 47.
[10] C. Ortolani, E.A. Pastorello, L. Farioli, M. Ispano, V. Pravettoni, C. Berti, C. Incorvaia, C. Zanussi, Ann. Allergy 71 (1993) 470.
[11] M. Fernandez-Rivas, R. van Ree, M. Cuevas, J. Allergy Clin. Immunol. 100 (1997) 728.
[12] R. Asero, Ann. Allergy Asthma Immunol. 83 (1999) 377.
[13] C. Ebner, T. Birkner, R. Valenta, H. Rumpold, M. Breitenbach, O. Scheiner, D. Kraft, J. Allergy Clin. Immunol. 88 (1991) 588.
[14] S. Vieths, B. Schoning, A. Petersen, Int. Arch. Allergy Immunol. 104 (1994) 399.
[15] S. Vieths, K. Janek, H. Aulepp, A. Petersen, Allergy 50 (1995) 421.
[16] B. Fahlbusch, O. Rudeschko, W.D. Muller, G. Schlenvoigt, S. Vettermann, L. Jager, Int. Arch. Allergy Immunol. 108 (1995) 119.
[17] M. Vanek Krebitz, K. Hoffmann Sommergruber, M. Laimer da Camara Machado, M. Susani, C. Ebner, D. Kraft, O. Scheiner, H. Breiteneder, Biochem. Biophys. Res. Commun. 214 (1995) 538.
[18] B. Schoning, W.H. Ziegler, S. Vieths, W. Baltes, J. Sci. Food Agric. 71 (1996) 475.
[19] R. Fritsch, B. Bohle, U. Vollmann, U. Wiedermann, B. Jahn-Schmid, M. Krebitz, H. Breiteneder, D. Kraft, C. Ebner, J. Allergy Clin. Immunol. 102 (1998) 679.
[20] S. Vieths, A. Jankiewicz, B. Schoning, H. Aulepp, Allergy 49 (1994) 262.
[21] D.Y. Son, S. Scheurer, A. Hoffmann, D. Haustein, S. Vieths, Eur. J. Nutr. 38 (1999) 201.
[22] S. Vieths, B. Schoning, A. Petersen, Food Agric. Immunol. 5 (1993) 93.
[23] L.S. Hsieh, M.J. Moos, Y. Lin, J. Allergy Clin. Immunol. 96 (1995) 960.
[24] M. Wachs, D. Proud, L.M. Lichtenstein, A. Kagey-Sobotka, P.S. Norman, R.M. Naclerio, J. Allergy Clin. Immunol. 84 (1989) 492.
[25] E. Crimi, S. Voltolini, P. Gianiorio, G. Orengo, C. Troise, V. Brusasco, P. Crimi, A.C. Negrini, J. Allergy Clin. Immunol. 85 (1990) 1014.
[26] J. Bousquet, A. Hejjaoui, W.M. Becker, P. Cour, I. Chanal, B. Lebel, H. Dhivert, F.B. Michel, J. Allergy Clin. Immunol. 87 (1991) 737.
[27] R.G. Van Wijk, Clin. Exp. Allergy 21 (1991) 661.
[28] EAACI Subcommittee on Skin Tests, Allergy 48 (1993) 48.
[29] C. Ortolani, M. Ispano, E.A. Pastorello, R. Ansaloni, G.C. Magri, J. Allergy Clin. Immunol. 83 (1989) 683.
[30] L.K. Poulsen, C. Liisberg, C. Bindslev Jensen, H.J. Malling, Clin. Exp. Allergy 23 (1993) 61.
[31] C. Moller, B. Bjorksten, G. Nilsson, S. Dreborg, Allergy 39 (1984) 37.
[32] M. Ceska, U. Lundkvist, Immunochemistry 9 (1972) 1021.
[33] P.S. Skov, H. Mosbech, S. Norn, B. Weeke, Allergy 40 (1985) 213.
[34] U.K. Laemmli, Nature 227 (1970) 680.
[35] S. Dreborg, The skin prick test, Thesis, Lindköbing University (1987).
[36] C. Bindslev-Jensen, L.K. Poulsen, in: D.D. Metcalfe, H.A. Sampson, R.A. Simon (Eds.), Food Allergy: Adverse Reactions To Food and Food Additives, Blackwell Science, Cambridge, 1997, p. 137.
[37] C. Bruijnzeel Koomen, C. Ortolani, K. Aas, C. Bindslev Jensen, B. Bjorksten, D. Moneret Vautrin, B. Wuthrich, Allergy 50 (1995) 623.
[38] H.A. Sampson, in: D.D. Metcalfe, H.A. Sampson, R.A. Simon (Eds.), Food Allergy: Adverse Reactions To Food and Food Additives, Blackwell Science, Cambridge, 1991, p. 113.
[39] A. Norgaard, C. Bindslev Jensen, Allergy 47 (1992) 503.
[40] F. Leynadier, M. Murrieta, J. Dry, J.N. Colin, C. Gillotin, D. Steru, Ann. Allergy 72 (1994) 520.
[41] D. Bayramgurler, N. Bilen, R. Apaydyn, L. Altintas, G. Sal, S. Dokmeci, T. Utkan, Clin. Exp. Dermatol. 24 (1999) 407.
[42] T. Haahtela, H. Jokela, Allergy 35 (1980) 15.