

Fagales pollen sensitization in a birch-free area: a respiratory cohort survey using Fagales pollen extracts and birch recombinant allergens (rBet v 1, rBet v 2, rBet v 4)

A. Mari*, M. Wallner† and F. Ferreira†

*Allergy Unit, National Health Service, Rome, Italy, and †Department of Genetics and General Biology, University of Salzburg, Salzburg, Austria

Summary

Background Birch allergy is one of the most common pollinosis in areas where exposure to high levels of birch pollen is common. Little is known about birch sensitivity in areas without birch pollen exposure and reactivity to birch-related species within the Fagales order.

Objective the aim was to evaluate Fagales reactivity within a population not exposed to birch pollen using epidemiological, diagnostic, and laboratory approaches by means of extracts and allergenic molecules.

Methods A cohort of 5335 respiratory allergic patients was screened by means of skin testing birch, hazel, and oak pollen extracts. Patients were from a birch-free area, but exposed to other Fagales pollen species. A subset of patients was from an intensively cultivated hazel area. A sample of the Fagales allergic population was tested with other Fagales pollen extract (alder, hornbeam, beech, chestnut) and with apple and hazelnut. IgE detection was performed with birch, hazel, oak, apple, and hazelnut extracts, and with Bet v 1, Bet v 2, Bet v 4, and bromelain. IgE immunoblots were performed using birch and hazel extracts. Epidemiological, clinical, and laboratory data were analysed by stratifying the allergic population.

Results Twenty-five percent of the pollen allergic cohort was skin test positive to at least one of the three Fagales species. Combined reactivity to the three species was recorded in 80% of this cohort. Isolated hazel pollen reactivity was recorded in 13.5% of the Fagales allergic patients. Sixty-six percent of these subjects were from the intensively cultivated hazel area. Reactivity to apple and hazelnut was detected by skin test (40%) and IgE reactivity (60%), but only 19% of the positive patients reported symptoms related to at least one of the two foods. Reactivity to Bet v 1 was recorded in 84% of the birch/hazel/oak co-reactivity group, and in 28% of the subjects with the same co-reactivity, but associating a multiple pollen sensitization. IgE to Bet v 2 (50%) and Bet v 4 (23%) panallergens were recorded positive in the latter subset. Bet v 1 prevalence ranged between 48% and 21% among subgroups of patients coming from different areas. Furthermore, an IgE reactivity to hazel-restricted allergenic components was detected among subjects coming from the same area and having a hazel isolated reactivity.

Conclusion Fagales allergy can be found in birch-free areas caused by the exposure to other Fagales species. Birch allergens can be useful for mimicking the allergenic extract, but are also the exclusive tools for a fine diagnostic and epidemiological approach to Fagales pollen allergy. Allergenic molecules from the hazel family will increase the panel of available reagents for the molecule-based approach to allergy diagnosis and therapy.

Keywords allergens, epidemiology, Fagales allergy diagnosis, IgE detection, immunoblotting, prevalence, recombinant allergens, skin tests

Submitted 14 February 2003; revised 16 June 2003; accepted 15 July 2003

Introduction

Birch pollen allergy is one of the most common pollinosis in northern European countries [1] and a relevant allergenic source in northern Italy [2], but little is known about its relevance in other areas. The Fagales order includes three

families (Betulaceae, Corylaceae, Fagaceae), whose species (alder, birch, hazel, hornbeam, hop-hornbeam, beech, oak, chestnut) are widely present in the European environment as spontaneous, ornamental, or cultivated plants [1, 3].

Following the identification and cloning of Bet v 1, the major allergen from birch [4], several homologous allergenic molecules have been identified, characterized, and cloned in pollen from closely related species (Aln g 1 from alder [5], Car b 1 from hornbeam [5], Cas s 1 from chestnut [6], Cor a 1

Correspondence: Adriano Mari, MD, Via Malipiero 28, I-04100 Latina, Italy. E-mail: a.mari@panservice.it

from hazel [7], and Que a 1 from oak [5]). Moreover, several Bet v 1-homologous molecules causing food-related allergic disorders have been identified in plants (Mal d 1 in apple, Cor a 1 in hazelnut, Dau c 1 in carrot, and Api g 1 in celery) [8]. The close relationship between Bet v 1-related allergens has been demonstrated by means of nucleic acid cross-hybridization [9], sequence homology [5], monoclonal antibody [7] and antibody fragment reactivity [10], IgE [7, 11–14], and T cell epitopes cross-reactivity [15]. The increasing availability of purified native and recombinant molecules for environmental, epidemiological, diagnostic, and therapeutic studies is leading to a re-assessment of the approach to allergic diseases [14, 16–18].

A previous study, carried out in a birch-free area of central Italy [3], preliminarily showed how Bet v 1 sensitization can be recorded in the absence of the Bet v 1 natural source [19]. As reported by Niederberger et al. [13], the reactivity to rBet v 1 seems to identify a subset of patients having a co-reactivity to Fagales species (birch, hazel, and oak). Such a molecule-based approach also seems to be useful to identify Bet v 1-related sensitization within the multiple pollen-sensitized subset [19]. Patients belonging to this group showed a reactivity to two panallergens, profilins and calcium-binding proteins. Such a clustering of the reactivity to panallergens has been recently confirmed using allergenic molecules from grass pollen [20].

The aim of the present study was to evaluate, in a population not exposed to birch pollen [3], the following: (1) the pattern of sensitization to pollen of representative species of the three Fagales families (Betulaceae, Corylaceae, Fagaceae); to compare such reactivity with other closely related pollen species within the same genera (alder, hornbeam, beech, chestnut); to define the reactivity to two Fagales food-related sources (apple and hazelnut); (2) the relationship between the pattern of sensitization and the reactivity to birch allergenic molecules (Bet v 1, Bet v 2, and Bet v 4), and to define their clinical and epidemiological features. The comparative study of reactivity to allergenic extracts compared with allergenic molecules in different subsets of the studied population led to the identification of novel allergens within the hazel pollen extract that seems to be family restricted.

Materials and methods

Allergenic extracts, purified and recombinant molecule

Birch (*Betula verrucosa*), hazel (*Corylus avellana*), and oak (*Quercus alba*) pollen extracts (Allergopharma, Reinbek, Germany) were used for the selection of sensitized patients by means of the skin prick test (SPT). Other commercial Fagales extracts (alder, *Alnus glutinosa*, and beech, *Fagus sylvatica*, Allergopharma; chestnut, *Castanea sativa*, and hornbeam, *Carpinus betulus*, Stallergenes, Antony, France) were added. A complete panel of allergenic extracts from other pollen and non-pollen sources, already used in previous studies [19–21], was skin tested as well. Birch, hazel, oak, apple, and hazelnut extracts were used for IgE detection on the CAP system (Pharmacia, Uppsala, Sweden). In-house preparations of birch and hazel extracts [12, 22] were used for

IgE immunoblotting. *Escherichia coli*-produced recombinant Bet v 1, Bet v 2, Bet v 4, Phl p 1, and native bromelain were used for IgE detection (Pharmacia). Recombinant Cor a 1 was produced in *E. coli* as previously reported [22].

Patients, skin testing, and sera

Unselected consecutive subjects ($n = 5335$) presenting respiratory symptoms that suggested an allergic disease, and referred to the Allergy Unit were enrolled. Demographical (age and gender) and clinical data (respiratory and food ingestion-related symptoms) were recorded for each patient. Part of the studied population ($n = 717$, 13.4%) was from an intensively cultivated hazel area, north of Rome. In that area, about 6 000 000 plants are grown for hazelnut production (Dr Francesco Monzillo, Statistical Department CCIAA Viterbo, personal communication). The reactivity of such patients (high hazel pollen exposure, HHPE) was compared with that of subjects living in the Rome urban polluted area and in other suburban areas (eastern, hilly woody area, and southern plain area mainly devoted to cultivation), lacking such high exposure to hazel pollen (low hazel pollen exposure, LHPE). The population was further discriminated on the basis of their native condition within the four defined regional areas (Rome urban, and north, east, and south suburban; data from patients' records). Immigrant patients coming from other regional or national areas, or from abroad were excluded when the comparative epidemiological/environmental evaluation was carried out.

Patients underwent SPT, with a standardized procedure [19] for all the Fagales and non-Fagales extracts. A random-selected group of Fagales-reactive patients was also tested with a hazelnut extract and, by means of the prick-prick technique, with fresh apple (golden variety). A result was considered positive when a weal of at least 3 mm greater than the negative control was recorded 15 min after the application of the test. Patients matching the following three criteria were considered to have a multiple pollen sensitization (MPS): (a) positive SPT for more than 50% of all the tested pollen extracts; (b) at least three positive SPT for pollens either rare or absent in the studied area; (c) at least one positive SPT for one of the relevant pollen species (grasses, olive, pellitory, cypress, mugwort). Details of the criteria are reported in previous studies [19, 20]. Patients with an SPT reactivity to at least one pollen species were 2573 (73.7% of the whole allergic cohort), age ranging from 2 to 80 years, and the male/female ratio was 1.04. Patients with an SPT reactivity to at least one of the Fagales extract were 624 (25.5% of the whole pollen allergic cohort), age ranging from 3 to 71 years, and the male/female ratio was 1.26. None of the patients received specific immunotherapy.

Following SPT, sera were obtained from 475 and 904 patients who consented to blood sampling for an *in vitro* diagnostic procedure. Subjects belonging to the two groups had a positive SPT to Fagales (birch or hazel or oak) or grasses (timothy), respectively. Sampled patients were matched, for age and gender distribution, the original SPT positive cohorts. Sera were stored at -20°C until required.

Informed oral consent for skin testing and blood sampling was obtained by patients or caregivers during the allergy consult.

IgE detection and immunoblotting

IgE to birch, hazel, and oak pollen extracts, to apple and hazelnut extracts, to rBet v 1, rBet v 2, rBet v 4, rPhl p 1, and to native bromelain (nBRO) were detected by the ImmunoCAP system following the instructions of the manufacturer (Pharmacia). nBRO, a rarely allergenic glycoprotein, was used to determine the levels of IgE to cross-reactive carbohydrate determinants (CCD) [21]. Values equal to or greater than 0.4 kU_A/L were considered to be positive for all the CAP determinations. Birch and hazel extracts and rCor a 1 were tested by means of IgE immunoblotting and dotblot, respectively, and performed as previously reported [22].

Data and statistics

Data were stored by means of customized databases and retrieved by means of study-tailored query programs. Statistical analyses were carried out by means of the Prism package (Graphpad Software, version 3.0, San Diego, CA, USA). The Fisher's exact test, the Mann-Whitney test, and the Spearman's correlation test were applied where appropriate. Statistical significance was accepted at $P < 0.05$.

Results

Epidemiological and clinical data

Table 1 reports the overall prevalence for each of the Fagales extracts used for patient SPT selection and their grouping on the basis of isolated or associated reactivity. Among the pollen allergic population, 25.5% reacted to at least one of the tested Fagales pollen. Almost all the subjects reacting to birch or oak extracts had an associated reactivity to at least one of the other two species, whereas 13.5% of the hazel-positive SPT did not (Table 1a). Hazel monosensitized patients were 54.4% of all the hazel-sensitized subjects in the area with HHPE, with a statistical significant difference (Fisher's exact test, $P < 0.05$) when compared with LHPE areas (38.1%). Sixty-six percent of the hazel monosensitized subjects were from the HHPE area, although only 14.1% of the pollen allergic subjects were from the same area. The associated reactivity to the three pollen species (Table 1b) was the most frequent recorded combination ($n = 412$, 66%), representing the large majority (80%) of subjects within the MPS subset. Isolated or combined reactivity, other than to birch/hazel, were recorded in 57 subjects, but the number of

Table 1. Overall prevalence for each of the Fagales extracts

(a) Prevalence of Fagales allergy based on SPTing of the whole population with suspected respiratory allergy (total = 5335 subjects, at least one positive SPT = 3493, 65.5%, at least one positive SPT to pollen extracts = 2573, 73.7%)

Fagales	Total* n (%)		
At least one Fagales species	624 (25.5)		
	Total† n (%)	Isolated n (%)	Associated with other Fagales n (%)
Birch (<i>Betula verrucosa</i> , Betulaceae)	504 (80.8)	3 (0.6)	501 (99.4)
Hazel (<i>Corylus avellana</i> , Corylaceae)	608 (97.4)	82 (13.5)	526 (86.5)
Oak (<i>Quercus alba</i> , Fagaceae)	456 (73.1)	2 (0.4)	454 (99.6)

(b) Prevalence of different pattern of co-reactivity to birch, hazel, and oak extracts by means of SPT and IgE, within the cohort of subjects with a positive SPT to at least one Fagales species

Co-reactivity‡	MPS	SPT n (%)§	IgE n ¶	Birch IgE % ¶ (median/range)	Hazel IgE % ¶ (median /range)	Oak IgE % ¶ (median /range)
Birch+hazel+oak	No	107 (43.7)	81	93.8 (8.5/0.4–79)	91.0 (12.7/0.5–91.4)	76.6 (7.2/0.4–77.1)
Birch+hazel	No	37 (15.1)	22	72.7 (1.9/0.4–5)	80.0 (6.2/0.8–26.8)	12.5 (0.9/0.7–1.1)
Hazel	No	81 (33.1)	51	16.3 (2.1/0.9–3.1)	62.7 (5.6/0.4–88.1)	12.2 (2.5/0.9–6.7)
Birch+hazel+oak	Yes	305 (80.5)	227	89.0 (9.7/0.4–90.8)	86.9 (11.9/0.4–96.2)	89.8 (18.2/0.6–93.5)
Birch+hazel	Yes	37 (9.8)	27	59.3 (3.8/0.5–19.7)	71.4 (4.3/0.5–15)	0 (–)

SPT: skin prick test; MPS: multiple pollen sensitization.

*Percentage calculated among all the pollen-allergic patients. †Percentage calculated within the cohort of subject with a positive SPT to at least one Fagales species. ‡Co-reactivity: other combinations of positive SPT for the three Fagales pollens, within or outside the MPS condition, were very rare and not considered in the statistical analysis. §Percentage calculated within the MPS and No-MPS subsets of the cohort of subjects with a positive SPT to at least one Fagales species.

¶Percentage calculated within the MPS and no-MPS subsets of the cohort of subjects with a positive SPT to at least one Fagales species and tested for IgE to the pollen extracts. || kU_A/L.

observations was low and was not considered relevant in the statistical analysis. IgE reactivity almost paralleled SPT reactivity.

The reactivity to other Fagales extracts is reported in Table 2a. Patients were randomly selected among the three predefined subsets. Almost all the tested Fagales species were recorded positive when the three screening tests were positive, whereas in the hazel monosensitized group only the hornbeam, another species from the Corylaceae family, showed a positive SPT. Table 2 (b, c), reports the reactivity to apple and hazelnut by means of *in vivo* and *in vitro* tests. A reactivity was mainly recorded by IgE and SPT, but a minority of the patients reported symptoms following the ingestion of the two foods.

Table 3 reports IgE reactivity to recombinant birch allergens in the subsets defined by means of SPT to pollens. Profilin- and calcium-binding-protein-reactive sera (rBet v 2 and rBet v 4, respectively) were recorded only when an MPS was present. rBet v 1-reactive sera were mainly recorded when the associated reactivity to the three Fagales species was detected by means of SPT (83.7%). A lower prevalence (57.1%) was recorded in the birch/hazel SPT positive subset, and only in three cases with isolated hazel pollen reactivity (5.4%). Sera reactive with rBet v 1 recognized Cor a 1 in the hazel pollen IgE immunoblot (Fig. 5a), whereas none of the sera with isolated hazel pollen sensitivity reacted with the

major allergen from hazel pollen (hazel pollen IgE immunoblot, Fig. 4a).

Table 4 reports the prevalence of IgE reactivity to recombinant birch allergens and to rPhl p 1 in subsets of patients defined by means of positive SPT and IgE to birch or grass extracts, respectively. Native patients from the four predefined geographical areas were considered. No statistical differences were recorded for rBet v 2, rBet v 4, and rPhl p 1 among subsets of patients coming from different areas. Sera with IgE reactivity to rBet v 1 were more frequently recorded when patients coming from the HHPE area were tested (48%), whereas a statistically significant lower prevalence was recorded in the southern plain suburban area (21%), mainly devoted to extensive cultivations of other crops. Data from patients native from the eastern hilly woody area suggest a prevalence (46%) similar to that of the northern area, but no statistically significant value was reached because of the low number of observations.

Gender distribution varied when different subsets of the Fagales allergic cohort were evaluated. The male/female ratios were 1.58 (n.s.s.) and 0.86 (Fisher's exact test, $P < 0.01$) when a Fagales reactivity was recorded with or without the MPS condition, respectively. Ratios in the rBet v 1, 2, and 4 reactive subsets were 1.33 (n.s.s.), 1.86 (Fisher's exact test, $P < 0.02$), and 1.07 (n.s.s.), respectively. A mean age statistically significant difference (Mann-Whitney test,

Table 2. Reactivity to other Fagales extracts and food extracts

(a) Skin prick tests (SPT) with other Fagales extracts in randomly selected subjects from predefined Fagales-allergic subsets lacking a multiple pollen sensitization (MPS)

		Botanical families and species			
Co-reactivity*	<i>n</i>	Betulaceae Alder (<i>Alnus glutinosa</i>) <i>n</i> (%)	Corylaceae Hornbeam (<i>Carpinus betulus</i>) <i>n</i> (%)	Fagaceae Beech (<i>Fagus sylvatica</i>) <i>n</i> (%)	Fagaceae Chestnut (<i>Castanea sativa</i>) <i>n</i> (%)
Birch+hazel+oak	60	56 (93.3)	58 (96.7)	55 (91.7)	29 (48.3)
Birch+hazel	21	16 (76.2)	19 (90.5)	6 (28.6)	4 (19.0)
Hazel	32	0	25 (78.1)	0	0

(b) Apple: prick-prick test, IgE and symptoms in randomly selected subjects from predefined Fagales-allergic subsets lacking an MPS

Co-reactivity*	<i>n</i>	Prick-prick %	IgE % (median range)	Symptoms† %
Birch+hazel+oak	98	37.8	63.4 (5.8/0.4–27)	19.1
Birch+hazel	19	15.6	33.3 (1.0/0.8–1.2)	0
Hazel	19	5.2	5.2 (1.0)	0

(c) Hazelnut: SPT, IgE and symptoms in randomly selected subjects from predefined Fagales-allergic subsets lacking an MPS

Co-reactivity*	<i>n</i>	SPT %	IgE % (median range)	Symptoms† %
Birch+hazel+oak	96	45.8	56.7 (1.8/0.4–9.2)	12.9
Birch+hazel	19	15.8	12.5 (1.4/0.4–2.1)	0
Hazel	20	5.0	5.0 (1.4)	0

*Co-reactivity: other combinations of positive SPT for the three Fagales pollens were not considered in the statistical analysis.

†Symptoms: self-reported symptoms are reported. All the patients had an oral allergy syndrome, whereas two only reported urticaria and angioedema.

Table 3. Comparative IgE reactivity to rBet v 1, rBet v 2, rBet v 4 within the predefined subsets of Fagales pollen-sensitized patients.

Co-reactivity*	MPS	No.	rBet v 1 <i>n</i> (%) (median/range)	rBet v 2 <i>n</i> (%) (median /range)	rBet v 4 <i>n</i> (%) (median /range)
Birch+hazel+oak	No	80	67 (83.7) (9.5/0.4–53.2)	0	0
Birch+hazel+oak	Yes	270	76 (28.1) (10.2/0.4–91.2)	176 (65.2) (4.8/0.4–83.6)	83 (30.7) (16.4/0.4–136)
Birch+hazel	No	21	12 (57.1) (2.5/0.4–5.9)	0	0
Birch+hazel	Yes	31	3 (9.7) (1.8/1–2.9)	9 (29.0) (2.2/0.4–8.2)	2 (6.5) (4.4/0.4–8.4)
Hazel	No	55	3 (5.4) (3.0/0.7–5.3)	0	0

*Co-reactivity: other combinations of positive skin prick test for the three Fagales pollens were very rare and not considered in the statistical analysis.
MPS: multiple pollen sensitization.

Table 4. Prevalence of IgE reactivity to rBet v 1, rBet v 2, rBet v 4, and to rPhl p 1 in patients selected by means of positive SPT and IgE to birch or grass extracts, respectively; subsets were defined on the basis of the native condition of subjects within regional areas

	<i>n</i>	rBet v 1 <i>n</i> (%)	rBet v 2 <i>n</i> (%)	rBet v 4 <i>n</i> (%)	<i>n</i>	rPhl p 1 <i>n</i> (%)
Total	370	139 (37.6)	176 (47.6)	65 (17.6)	904	604 (66.8)
<i>Urban area</i>						
Rome (LHPE)	53	18 (34.0)	19 (35.8)	7 (13.2)	142	90 (63.4)
<i>Suburban areas</i>						
Northern (HHPE)	172	83 (48.2)*	79 (45.9)	34 (19.8)	276	199 (72.1)
Eastern (LHPE)	28	13 (46.4)	13 (46.4)	6 (21.4)	95	58 (61.0)
Southern (LHPE)	117	25 (21.4)**	65 (55.6)	18 (15.4)	391	257 (65.7)

The prevalence of each subset was compared with the total mean values.

SPT: skin prick test; HHPE: high hazel pollen exposure; LHPE: low hazel pollen exposure. For other area features, see text.
Statistics: Fisher's exact test, * $P < 0.02$, ** $P < 0.001$.

$P < 0.0001$) was recorded only comparing the rBet v 2 vs. rBet v 4 IgE positive subsets. The mean ages were 23.2 and 29.5, respectively, but positive results were recorded in a wide range of values (4–71 and 4–70).

Comparative IgE reactivity between birch extracts and recombinant birch allergens

The percentage of subjects with detectable IgE was 39.5%, 57.1%, and 26.1% when the rBet v 1, 2, and 4 isolated IgE reactivity was evaluated, and reached 89.3% when at least one of the three tested allergens was recorded positive (Fig. 1).

Figure 2 shows the correlation between IgE to Bet v E and the isolated or combined reactivity to recombinant molecules and CCD. The lower r_s value (Spearman's correlation test) was recorded for rBet v 4 ($r_s = 0.37$), whereas it reached 0.87 when the sum of the IgE values recorded for rBet v 1, 2, 4 plus the reactivity to CCD was compared with Bet v E IgE.

Figure 3 comparatively shows the IgE levels toward the birch extract and to the sum of the three recombinant allergens plus CCD-IgE reactivity. Statistically significant differences (Mann–Whitney test) were recorded comparing all the sera (Fig. 3, graph A) and sera in the CAP classes 1–4

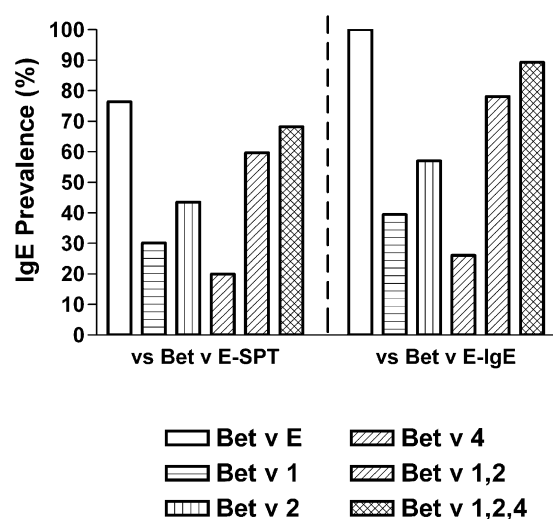


Fig. 1. Prevalence of IgE reactivity to a birch extract (Bet v E) compared with the isolated prevalence of rBet v 1, rBet v 2, and rBet v 4 reactivity and with three different combinations of three allergenic molecules (see graph legend) in a birch-allergic subject (skin prick test positive (SPT) to a birch extract) (sera = 491). Left part: different combinations compared with Bet v E SPT; right part: different combinations compared with Bet v E IgE.

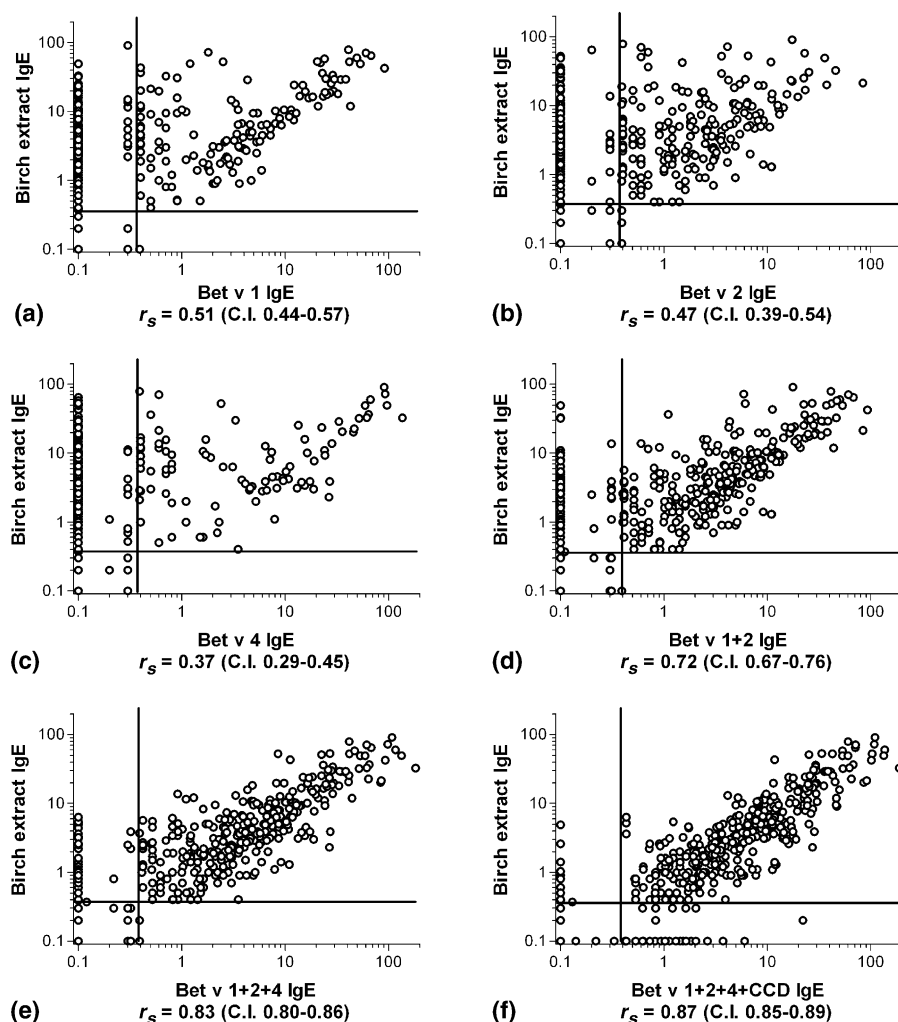


Fig. 2. Correlation of IgE values to a birch extract and different combinations of IgE reactivity to rBet v 1, rBet v 2, rBet v 4, and CCD. Sera ($n = 491$) were selected on the basis of the positive Bet v E IgE assay: A = rBet v 1 vs. birch extract; B = rBet v 2 vs. birch extract; C = rBet v 4 vs. birch extract; D = rBet v 1 + rBet v 2 vs. birch extract; E = rBet v 1 + rBet v 2 + rBet v 4 vs. birch extract; F = rBet v 1 + rBet v 2 + rBet v 4 + CCD vs. birch extract. Continuous lines indicate negative cut-off. Statistics: Spearman's correlation test ($P < 0.0001$).

(Fig. 3, graphs B–E). Sera showing IgE reactivity to the extract, but not to any of the tested molecules, were all in the class 1 and 2 range. None of the tested sera was in the class 6 range.

IgE immunoblot reactivity to hazel and birch extracts

Figure 4 comparatively shows IgE reactivity on hazel and birch extracts by means of the immunoblot assay. Ten sera were selected on the basis of an isolated SPT and IgE reactivity to hazel and on the level of exposure to hazel pollen. None of the sera recognized any bands in the birch extract. None of the sera recognized the Cor a 1 band in the hazel extract, whereas bands at 66, 46, and 14 kDa were detected by several sera. The 10 sera were tested on rCor a 1 with negative results (data not shown).

Figure 5 comparatively shows IgE immunoblot reactivity on hazel and birch extracts of 20 sera. Sera were selected on the basis of a positive birch/hazel/oak SPT and IgE reactivity and on the level of exposure to the hazel pollen. Serum 20 did not recognize rBet v 1 and reacted only to birch and hazel pollen extracts by means of SPT and IgE. All the sera, except serum 20, recognized the Cor a 1 band and the Bet v 1 band in hazel (Fig. 5a) and in birch (Fig. 5b) extracts, respectively.

The 66, 46, and 14 kDa bands were detected by several sera either from patients of the HHPE area or from the LHPE area. Sera 15, 17, and 19, lacking MPS and CCD reactivity, strongly recognized a 66-kDa band in the hazel extract, but not in the birch extract. Several bands, including the 66-kDa one, were recognized by sera from patients with MPS and CCD-IgE in both extracts. Reactivity to the 66-kDa band in the birch extract was generally weaker than that recorded in the hazel extract, and even more than that recorded with sera 15, 17, and 19.

Discussion

The present study was carried out to define the prevalence of Fagales pollen reactivity in a cohort of respiratory patients not exposed to birch pollen. Although birch pollen hypersensitivity is a very well-known allergy problem in northern Europe populations, little is known about the prevalence of co-reactivity to other Fagales pollen [23] as *in vivo* and *in vitro* data would suggest [13, 14]. Data from the present study confirm the preliminary report [19] about the clustered reactivity to the pollen of the Fagales order possibly induced by exposure to one or more of its pollen species [13].

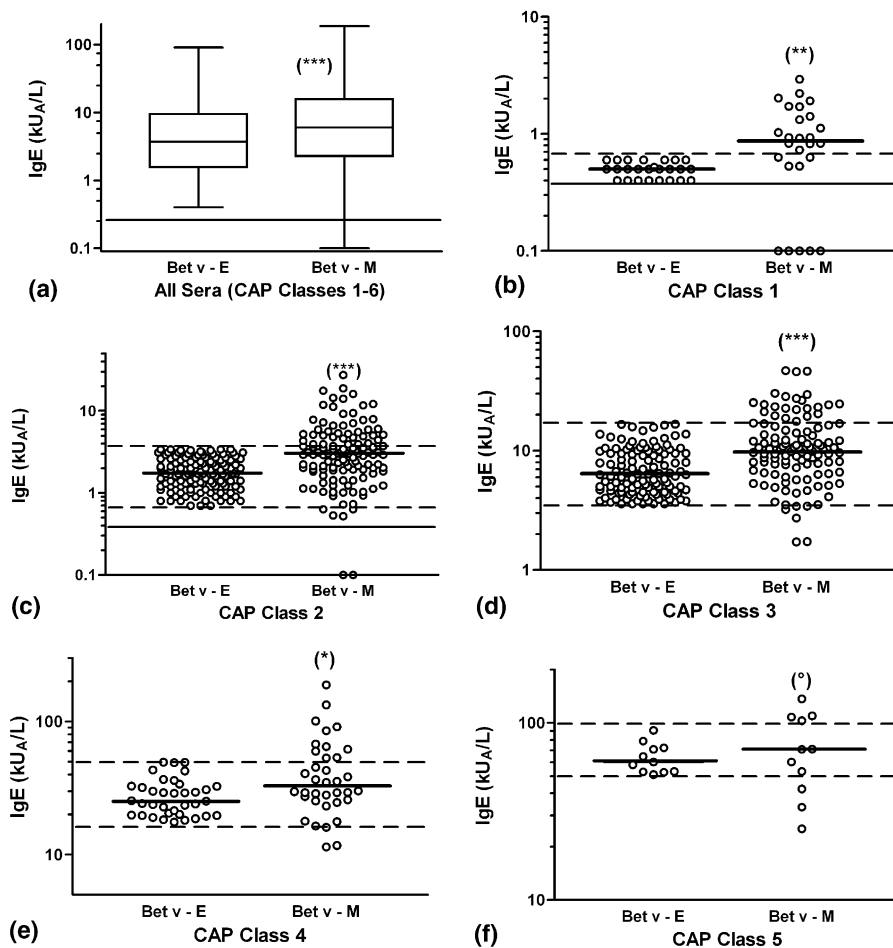


Fig. 3. Comparative evaluation of IgE to a birch extract (Bet v - E) and the sum of IgE reactivity to rBet v 1, rBet v 2, rBet v 4, and CCD (Bet v - M). Subjects ($n = 343$) are grouped on the basis of their CAP class values to birch extract. Graphs: A = CAP class 1-6 ($n = 310$); B = CAP class 1 - range values 0.35-0.7 ($n = 26$); C = CAP class 2 - range values 0.7-3.5 ($n = 123$); D = CAP class 3 - range values 3.5-17.5 ($n = 114$); E = CAP class 4 - range values 17.5-50 ($n = 36$); F = CAP class 5 - range values 50-100 ($n = 11$). No sera were in Bet v E class 6. Parallel dashed lines indicate class range. Continuous lines indicate negative cut-off. Statistics: Mann-Whitney test (°) n.s.s., (*) $P < 0.02$, (**) $P < 0.0004$, (***) $P < 0.0001$.

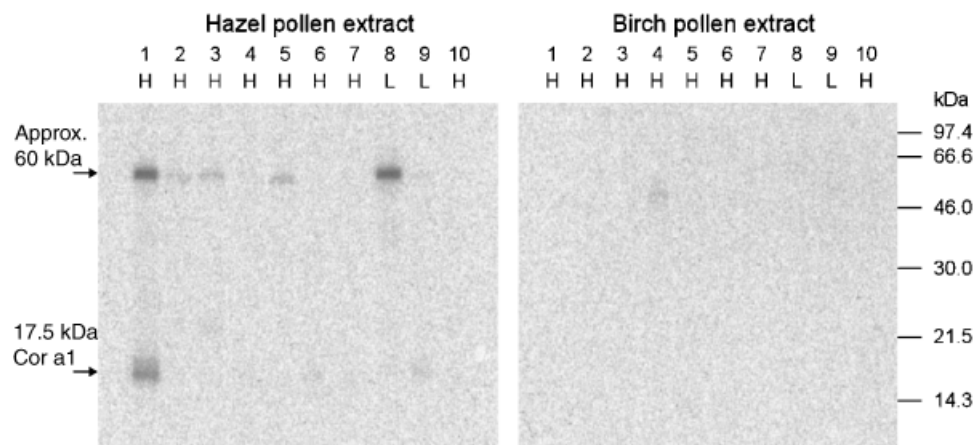


Fig. 4. IgE immunoblotting reactivity of 10 representative sera from Hazel monosensitized patients using hazel (left side) and birch (right side) extracts. Subjects from areas with high (H = HHPE) and low (L = LHPE) hazel pollen exposure were included.

Nevertheless, as reported by Niederberger et al. [13], the recorded reactivity does not always involve all the Fagales families. The prevalence of Fagales sensitization may vary among geographical areas, depending on the overall level of Fagales pollen exposure. This variability is also suggested by the differences recorded in the present study in the prevalence of Bet v 1-reactive sera among native patients. It cannot be

excluded that a similar phenomenon could characterize other European geographical areas where birch trees do not represent the only species from the Fagales order to which patients are exposed to [13, 24-27]. IgE reactivity to Bet v 1 seems to be a fine marker of the Fagales sensitization [13, 14], even for our cohort of patients lacking direct exposure to birch pollen. It is particularly useful in patients with MPS

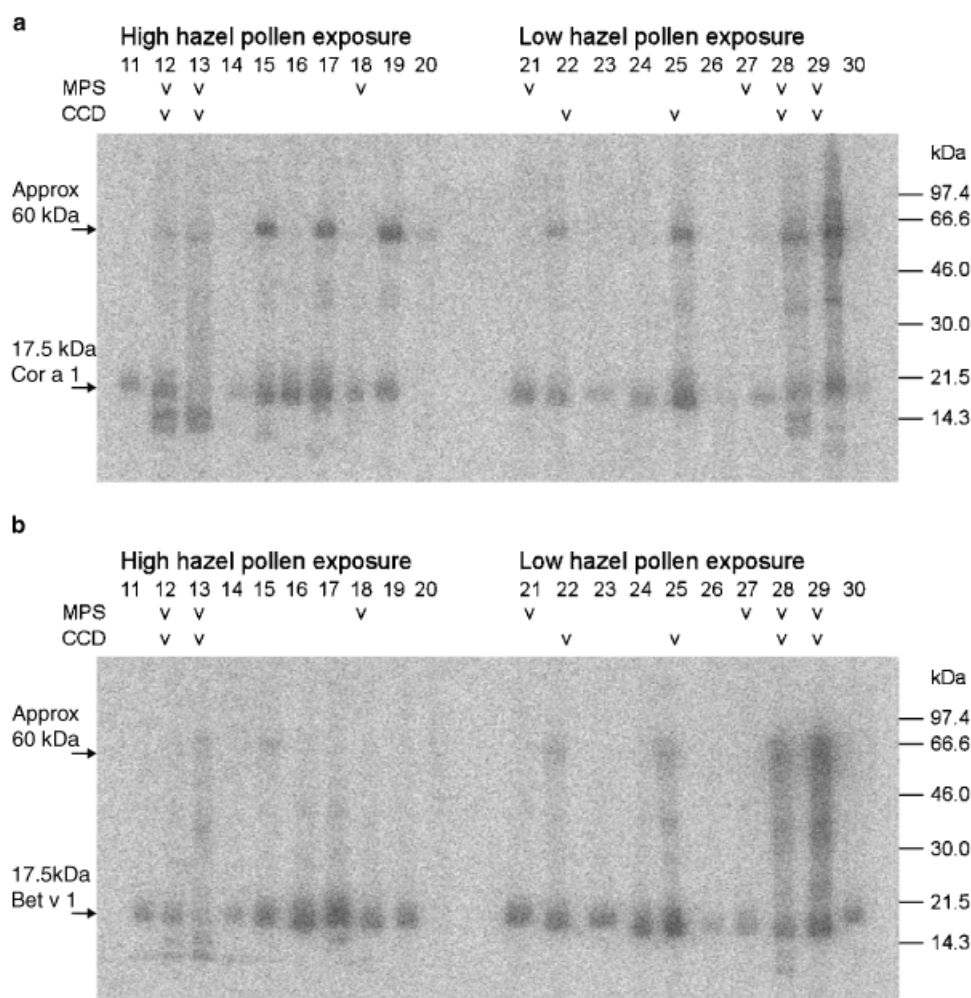


Fig. 5. IgE immunoblotting reactivity of 20 representative sera from Fagales allergic subjects. Nineteen patients (11–19, 21–30) were selected on the basis of their positive SPT and IgE to birch/hazel/oak pollen extracts and of a positive IgE assay to rBet v 1. Subject 20 was from a subject with birch/hazel positive SPT and IgE, and negative IgE to rBet v 1. Subjects from areas with high (H = HHPE) and low (L = LHPE) hazel pollen exposure were included. Sera from multiple pollen sensitization (MPS) and No-MPS subsets, either with or without IgE to CCD, were tested (markers are placed at the top of the lanes). (a) Hazel pollen extract; (b) birch pollen extract.

whose reactivity to a huge number of pollen hinders the identification of the Fagales order-restricted IgE sensitization. The prevalence of the IgE reactivity to Bet v 1 was confirmed to be lower in the MPS subset because of the common Bet v 2 and Bet v 4 IgE reactivity in this subset of patients [19]. Moreover, exposure to a high amount of hazel pollen in the HHPE area seems to lead to an IgE sensitization to allergenic molecules that are present only in hazel or hazel-related pollens. The isolated SPT and IgE reactivity to hazel pollen, the isolated parallel reactivity to the hornbeam pollen, the lack of IgE reactivity to Bet v 1 and Cor a 1, and the IgE recognition of protein bands in the hazel immunoblot and not in the birch one are in favour of such a hypothesis. The identification of one or more hazel pollen-restricted allergens may allow the identification of patients primarily sensitized by pollens from the Corylaceae family. Such patients would not be detected by Bet v 1 or Cor a 1. Although to a lesser extent, hazel monosensitized patients were also found in the LHPE areas. The reactivity to Corylaceae pollen-restricted allergens may also allow one to identify geographical areas

where exposure to Corylaceae pollen other than hazel may occur. As already shown for Bet v 1 [28], the measurement of the atmospheric dispersal of such molecules will allow us to determine the exposure to Corylaceae pollen. Similar information could parallel that obtained by means of a population survey using pollen extracts from different Fagales families [13].

Conversely, IgE reactivity to the two birch panallergens, Bet v 2 and Bet v 4, replicates those already reported in a previous study on MPS subjects [19], and parallels those reported for homologous molecules from the timothy grass pollen (Phl p 12 and Phl p 7) [20]. The widespread presence of highly homologous molecules documented in almost all the studied pollen sources [29, 30] seems to lead to homogeneous IgE reactivity prevalences between geographical areas, not dependent on the exposure to defined allergenic sources such as birch, grasses, olive, or pellitory.

Birch-associated food reactivity has been studied and reported by several authors [8, 31], mainly related to either Bet v 1 or Bet v 2 sensitization [32]. In a previous study, we

reported a higher prevalence of oral allergy syndrome in MPS patients sensitized to profilin when compared with those reactive to a calcium-binding protein [19]. In the present study, we focused the reactivity to apple and hazelnut in selected subsets of patients, excluding those having an MPS. Subjects displaying reactivity to all the tested Fagales and having IgE reactivity to Bet v 1 were more likely to have positive SPT and IgE reactivity to plant-derived foods, but only about 19% of this group reported allergy symptoms eating either apples or hazelnuts, whereas symptoms related to carrot or celery ingestion were never reported. Reactivity to hazelnuts was never reported among patients with hazel pollen monosensitization, suggesting no relationship between pollen and seed sensitization from the hazel plant in such a cohort of subjects. Hazelnut allergy was found rarely even within all the allergic patients in the HHPE area (data not shown).

The prevalence of IgE reactivity to Bet v 1 and Bet v 2 in an Italian cohort of patients has already been reported by Rossi et al. [31]. Forty-three percent of 68 subjects with positive SPT and IgE to birch reacted to Bet v 1, 31% reacted to Bet v 2, and 26% reacted to both the allergens. The authors found patients to be reactive to Bet v 1 among birch monosensitized patients, and such sera had IgE correlating with both birch and hazel extracts. Bet v 2-reactive sera were more likely to have multiple pollen sensitization. As part of a multi-centre study, the reactivity to Bet v 1, 2, and 4 has been recently reported for a 21-subject northern Italy cohort [33]. IgE reactivities to Bet v 1, 2, and 4 were 62%, 33%, and 27%, respectively. Higher values of Bet v 1 IgE reactivity were reported for almost all the other European cohorts, whereas reactivity to Bet v 2 and 4 varied from country to country. In the present study, the overall figures for Bet v 1, 2, and 4 IgE reactivity were 39.5%, 50.4%, and 22.7%, respectively, but it is also shown how greatly they vary when different criteria used to select and group the patients are applied. We recently showed how the prevalence of the IgE reactivity to grass pollen allergens [20] is influenced by several factors such as MPS, SPT reactivity, and total IgE levels. More homogeneous criteria should thus be adopted to compare prevalence data from different cohorts correctly.

The comparative reactivity of IgE to birch extract toward the reactivity to allergenic molecules has already been reported by several authors [14, 33]. The results are in favour of a complete or/ a slightly less efficient recognition of birch-reactive sera when a combination of birch allergens is used instead of the birch extract. Data from our study showed how about 89% of birch-reactive sera recognize at least one allergenic molecule. As reported for grass allergens [20], a more complete panel of birch allergens [34–36] would certainly enhance the sensitivity of the molecule-based birch pollen allergy diagnosis. Furthermore, the highest correlation between IgE values detected using the birch extract compared with the sum of IgE reactivity to the distinct allergenic molecules has been reached only by summing Bet v 1, 2, 4, and CCD. These results are in agreement with those reported by van Ree et al. [14] using Bet v 1, Bet v 2, and a protease-digested birch pollen extract. The use of nBRO in our study seems to lead to the identification of sera with CCD-IgE without birch extract reactivity. This finding has already been reported by us and could be due to the heterogeneity of CCD

reactivity [21]. The sum of the IgE values obtained using allergenic molecules plus CCD reactivity gave a significantly better estimation of the overall birch IgE concentration in sera. These findings are in agreement with the data recently reported for grass allergenic molecules [20]. A multiple band IgE recognition of birch pollen extract has already been reported by several authors [14, 33]. In our birch and hazel immunoblot study, sera from patients reactive to birch/hazel/oak either had a simple recognition of the 17-kDa band in both extracts (Bet v 1 and Cor a 1) or a more complex pattern of reactivity often associated with MPS sensitization or CCD-IgE. As for diagnostic sensitivity, the evaluation of IgE concentration using discrete allergenic molecules should be further enhanced by completing the panel of birch allergens [34–36].

In conclusion, the use of the most complete panel of allergenic molecules from birch may be used instead of the extract to study IgE reactivity to birch pollen, but new and useful information can be obtained by the use of discrete allergenic molecules. A correct epidemiology of Fagales allergy and a fine diagnostic procedure to the birch pollen allergic patient may result only from the use of such a molecule-based approach. Specific immunotherapy using allergenic extracts, characterized and standardized on the basis of the actual molecular knowledge, could lead to better results if the aforementioned diagnostic approach is adopted. Further studies are needed for the characterization of the hazel-restricted allergenic molecules.

Acknowledgements

We would like to thank Francesco Redi and Renato Carrozza for their technical assistance in IgE detection on the ImmunoCAP system, Enrico Mari for helpful advice in customized software development, and Paula Romagnoli Wright for manuscript language revision.

This study was supported by an internal funding of the Regional Branch of the Italian National Health Service and by the Joint Research Project S88-B01 (S8802-B01) of the Austrian 'Fonds zur Förderung der Wissenschaftlichen Forschung, FWF'.

References

- 1 D'Amato G, Spieksma FT, Bonini S. Allergenic pollen and pollenosis in Europe. Oxford: Blackwell Scientific Publications, 1991:1–226.
- 2 Corsico R, Falagiani P, Ariano R. et al. An epidemiological survey on the allergological importance of some emerging pollens in Italy. *J Invest Allergol Clin Immunol* 2000; 10:155–61.
- 3 Pignatti S. Flora d'Italia. Bologna: Edagricole, 1982.
- 4 Breiteneder H, Hassfeld W, Pettenburger K et al. Isolation and characterization of messenger RNA from male inflorescences and pollen of the white birch (*Betula verrucosa*). *Int Arch Allergy Appl Immunol* 1988; 87:19–24.
- 5 Ipsen H, Hansen OC. The NH2-terminal amino acid sequence of the immunochemically partial identical major allergens of Alder (*Alnus glutinosa*) Aln g I, birch (*Betula verrucosa*) Bet v I, hornbeam (*Carpinus betulus*) Car b I and oak (*Quercus alba*) Que a I pollens. *Mol Immunol* 1991; 28:1279–88.

- 6 Kos T, Hoffmann-Sommergruber K, Ferreira F et al. Purification, characterization and N-terminal amino acid sequence of a new major allergen from European chestnut pollen, Cas s 1. *Biochem Biophys Res Commun* 1993; 196:1086–92.
- 7 Rohac M, Birkner T, Reimitzer I et al. The immunological relationship of epitopes on major tree pollen allergens. *Mol Immunol* 1991; 28:897–906.
- 8 Breiteneder H, Ebner C. Molecular and biochemical classification of plant-derived food allergens. *J Allergy Clin Immunol* 2000; 106:27–36.
- 9 Valenta R, Breiteneder H, Petternburger K et al. Homology of the major birch-pollen allergen, Bet v I, with the major pollen allergens of alder, hazel, and hornbeam at the nucleic acid level as determined by cross-hybridization. *J Allergy Clin Immunol* 1991; 87:677–82.
- 10 Flicker S, Laffer S, Steinberger P et al. Engineering, purification and applications of His-tagged recombinant antibody fragments with specificity for the major birch pollen allergen, Bet v 1. *Biol Chem* 2000; 381:39–47.
- 11 Ipsen H, Wihl JA, Petersen BN, Lowenstein H. Specificity mapping of patients IgE response towards the tree pollen major allergens Aln g I, Bet v I and Cor a I. *Clin Exp Allergy* 1992; 22:391–9.
- 12 Hirschwehr R, Jager S, Horak F et al. Allergens from birch pollen and pollen of the European chestnut share common epitopes. *Clin Exp Allergy* 1993; 23:755–61.
- 13 Niederberger V, Pauli G, Gronlund H et al. Recombinant birch pollen allergens (rBet v 1 and rBet v 2) contain most of the IgE epitopes present in birch, alder, hornbeam, hazel, and oak pollen: a quantitative IgE inhibition study with sera from different populations. *J Allergy Clin Immunol* 1998; 102:579–91.
- 14 van Ree R, van Leeuwen WA, Akkerdaas JH, Aalberse RC. How far can we simplify *in vitro* diagnostics for Fagales tree pollen allergy? A study with three whole pollen extracts and purified natural and recombinant allergens. *Clin Exp Allergy* 1999; 29:848–55.
- 15 Ebner C, Ferreira F, Hoffmann K et al. T cell clones specific for Bet v I, the major birch pollen allergen, crossreact with the major allergens of hazel, Cor a I, and alder, Aln g I. *Mol Immunol* 1993; 30:1323–9.
- 16 Valenta R, Lidholm J, Niederberger V, Hayek B, Kraft D, Gronlund H. The recombinant allergen-based concept of component-resolved diagnostics and immunotherapy (CRD and CRIT). *Clin Exp Allergy* 1999; 29:896–904.
- 17 Kazemi-Shirazi L, Niederberger V, Linhart B, Lidholm J, Kraft D, Valenta R. Recombinant marker allergens: diagnostic gatekeepers for the treatment of allergy. *Int Arch Allergy Immunol* 2002; 127:259–68.
- 18 Ferreira F, Wallner M, Breiteneder H, Hartl A, Thalhamer J, Ebner C. Genetic engineering of allergens: future therapeutic products. *Int Arch Allergy Immunol* 2002; 128:171–8.
- 19 Mari A. Multiple pollen sensitization: a molecular approach to the diagnosis. *Int Arch Allergy Immunol* 2001; 125:57–65.
- 20 Mari A. Skin test with a timothy grass (*Phleum pratense*) pollen extract vs IgE to a timothy extract vs IgE to rPhl p 1, rPhl p 2, nPhl p 4, rPhl p 5, rPhl p 6, rPhl p 7, rPhl p 11, and rPhl p 12: epidemiological and diagnostic data. *Clin Exp Allergy* 2003; 33: 3–51.
- 21 Mari A. IgE to cross-reactive carbohydrate determinants: analysis of the distribution and appraisal of the *in vivo* and *in vitro* reactivity. *Int Arch Allergy Immunol* 2002; 129:286–95.
- 22 Breiteneder H, Ferreira F, Hoffmann-Sommergruber K et al. Four recombinant isoforms of Cor a I, the major allergen of hazel pollen, show different IgE-binding properties. *Eur J Biochem* 1993; 212:355–62.
- 23 Eriksson NE. Allergy to pollen from different deciduous trees in Sweden. An investigation with skin tests, provocation tests and the radioallergosorbent test (RAST) in springtime hay fever patients. *Allergy* 1978; 33:299–309.
- 24 Laurent J, Lafay M, Lattanzi B, Le Gall C, Sauvaget J. Evidence for chestnut pollinosis in Paris. *Clin Exp Allergy* 1993; 23:39–43.
- 25 Prados M, Aragon R, Carranco MI, Martinez A, Martinez J. Assessment of sensitization to holm oak (*Quercus ilex*) pollen in the Merida area (Spain). *Allergy* 1995; 50:456–9.
- 26 Ross AM, Corden JM, Fleming DM. The role of oak pollen in hay fever consultations in general practice and the factors influencing patients' decisions to consult. *Br J Gen Pract* 1996; 46:451–5.
- 27 Patriarca S, Voltolini S, Navone R et al. Biochemical and immunochemical characterization of hop-hornbeam (*Ostrya carpinifolia* Scop.) pollen. *Aerobiologia* 2000; 16:255–60.
- 28 Schappi GF, Suphioglu C, Taylor PE, Knox RB. Concentrations of the major birch tree allergen Bet v 1 in pollen and respirable fine particles in the atmosphere. *J Allergy Clin Immunol* 1997; 100:656–61.
- 29 Barletta B, Tinghino R, Iacovacci P et al. Profilins, calcium-binding proteins, and carbohydrate crossreacting determinants in 23 different pollen species. *J Allergy Clin Immunol* 2000; 105:S36.
- 30 Tinghino R, Twardosz A, Barletta B et al. Molecular, structural, and immunologic relationships between different families of recombinant calcium-binding pollen allergens. *J Allergy Clin Immunol* 2002; 109:314–20.
- 31 Rossi RE, Monasterolo G, Operti D, Corsi M. Evaluation of recombinant allergens Bet v 1 and Bet v 2 (profilin) by Pharmacia CAP system in patients with pollen-related allergy to birch and apple. *Allergy* 1996; 51:940–5.
- 32 Wensing M, Akkerdaas JH, van Leeuwen WA et al. IgE to Bet v 1 and profilin: cross-reactivity patterns and clinical relevance. *J Allergy Clin Immunol* 2002; 110:435–42.
- 33 Moverare R, Westritschnig K, Svensson M et al. Different IgE reactivity profiles in birch pollen-sensitive patients from six European populations revealed by recombinant allergens: an imprint of local sensitization. *Int Arch Allergy Immunol* 2002; 128:325–35.
- 34 Karamloo F, Schmitz N, Scheurer S et al. Molecular cloning and characterization of a birch pollen minor allergen, Bet v 5, belonging to a family of isoflavone reductase-related proteins. *J Allergy Clin Immunol* 1999; 104:991–9.
- 35 Cadot P, Diaz JF, Proost P et al. Purification and characterization of an 18-kd allergen of birch (*Betula verrucosa*) pollen: identification as a cyclophilin. *J Allergy Clin Immunol* 2000; 105:286–91.
- 36 Mahler V, Fischer S, Heiss S, Duchene M, Kraft D, Valenta R. cDNA cloning and characterization of a cross-reactive birch pollen allergen: identification as a pectin esterase. *Int Arch Allergy Immunol* 2001; 124:64–6.