

***In vitro* and *in vivo* allergenicity of recombinant Bet v 1 compared to the reactivity of natural birch pollen extract**

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Summary

Background Diagnostic procedures using natural extracts show only limited quantitative correlation between *in vivo* and *in vitro* results. Highly pure recombinant allergens might show more predictive findings.

Objective The aim of this study was to compare natural birch pollen extract (BPE) and recombinant *Betula verrucosa* (rBet v 1) for their diagnostic value comparing skin prick tests (SPTS) and nasal provocation tests (NPTS) with specific IgE in the serum.

Methods Thirty-four patients allergic to birch pollen and five healthy controls were investigated. SPT and NPT were performed with BPE and rBet v 1 at different concentrations. Specific serum IgE was measured by the Pharmacia CAP system.

Results Commercial BPE and rBet v 1 (10 µg/mL) were able to elicit similar allergenic reactions *in vivo* and IgE binding *in vitro*. SPT reflects immediate-type allergy as determined by NPT to a higher degree than specific IgE, for both reagents. To cause allergic reactions in NPT, higher amounts of rBet v 1 were needed than for skin tests and the sensitivity was lower than with BPE.

Conclusion rBet v 1 alone is sufficient for a reliable diagnosis of birch pollen allergy in most patients and induces comparable skin test reactivity as BPE, but less allergic reactions in nasal provocations.

Keywords allergy, birch pollen, hayfever, *in vitro*, recombinant allergens, skin prick test

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Introduction

Besides clinical history, diagnosis of allergy is based on skin tests, provocation tests and *in vitro* investigations with natural allergen extracts. These are complex mixtures that contain, besides the relevant allergenic proteins in unknown concentrations, many non-allergenic proteins and carbohydrates [1]. Studies using such extracts showed a low correlation between skin test reactivity and reactivity of specific serum IgE. In contrast to the more difficult to quantitate allergens present in extracts, recombinant allergens can be carefully quantified in terms of protein concentration and IgE reactivity [2, 3]. Therefore, these preparations can be used as a 'golden standard' for both *in vitro* and *in vivo* tests, allowing a direct comparison of the diagnostic results obtained with different methods [4].

Pollen from trees of the order Fagales are important allergen sources in many regions worldwide. Birch pollen play a central role as an allergenic source among trees due to a widespread growth of the tree. They are botanically related with most allergenic tree pollens and also show a high cross-reactivity with this pollen due to an also structural relationship. In addition, cross-reactivity of birch pollen with celery

and other foods is responsible for a considerable amount of pollen-related food allergy [5].

The major birch pollen allergen *Betula verrucosa* (Bet v 1) is responsible for a large part of birch-pollen-related allergies [6, 7]. Thus, this allergen source represents an ideal candidate for investigating the *in vivo* and *in vitro* allergenicity of a single component in comparison with the reactivity of a complex natural extract.

Bet v 1 is available as a pure recombinant protein for *in vivo* and *in vitro* use [8]. The Bet v 1 family consists of a considerable number of highly homologous pathogenesis-related proteins identified in species from the entire plant kingdom [9].

Previous studies have compared skin test reactivity of recombinant *B. verrucosa* (rBet v 1) with natural birch pollen extract (BPE) [8, 10]. However, no comparisons of the actual allergenicity of rBet v 1 and BPE by challenge tests are available. In this study, we performed nasal provocation test (NPT) and skin prick test (SPT) together with specific IgE determinations against BPE and rBet v1 to investigate the correlations between allergen extract reactivity and the reactivity to a single major allergen.

Patients, Materials and Methods

Patients

Thirty-four birch pollen allergic patients (mean age 39 years; 22 females/17 males) and five healthy controls (mean age 33.4

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years; two females/three males) were included in the study. The patients were recruited from our allergy unit. Diagnosis of pollen allergy was based on a typical clinical history of recurrent rhinitis (possibly also additional conjunctivitis and/or asthma) during the birch pollen season (April/May), a positive SPT to commercial birch extract (ALK[®], Horsholm, Denmark) and elevated specific IgE serum levels to birch pollen determined by the Pharmacia CAP System (Pharmacia, Uppsala, Sweden). All patients showed stable lung functions. Controls included five healthy non-allergic individuals as controlled by SPT and the CAP-RAST system.

During the study, patients were not allowed to use either antihistamines or topical or systemic steroids during at least 1 week before the investigations. Patients with other nasal diseases such as chronic allergic or non-allergic rhinitis and atopic dermatitis were excluded from the study.

The study was approved by the Ethical Committee of the University of Zürich. All patients and controls gave oral and written informed consent.

SPTs with extracts, natural and recombinant allergens

SPTs were performed with commercial extracts from birch pollen (ALK[®]) and the major allergen of birch as recombinant protein (rBet v 1a). rBet v 1a was diluted in 0.9% sterile sodium chloride solutions in concentrations of 1, 10 and 100 µg/mL. Sodium chloride (0.9%) and histamine hydrochloride (ALK) served as positive and negative controls, respectively. All SPTs were performed twice on the volar forearm and applied in the opposite directions. Twenty microlitres of the allergen solutions were used per test. A minimal distance of 3 cm between each test field was applied. SPTs were read after 20 min. Weal and flares were pen-marked and then transferred with scotch tape to a paper and the mean weal diameter was calculated by adding the maximal longitudinal (*d*1) and transversal (*d*2) diameter divided by two [(*d*1 + *d*2)/2]. SPT was considered positive when a mean diameter of ≥ 4 mm was achieved. Only patients with a SPT to negative control with 0.9% sodium chloride with a weal diameter below 1 mm were included in the study.

Determination of allergen-specific and total serum IgE

From all patients and controls, specific IgE to birch (t3), rBet v 1 (t215) and rBet v 2 (t216) were determined by the Immuno-CAP System[®] (Pharmacia). Total serum IgE levels were determined in all individuals by the Immuno CAP-System[®] as recommended by the manufacturer (Pharmacia).

NPTs

NPTs were performed with placebo (sterile sodium chloride), BPE and rBet v 1 used in the same concentrations as for SPT in 17 patients according to the recommendations of the EAACI and other expert groups [11, 12]. First, 0.9% sodium chloride solution was applied as a negative control. Thereafter, the patient received increasing doses of allergen (1, 10, 100 µg/mL) or placebo in 15-min intervals. Allergen solutions were administered into one nostril using a metred pump delivering 15 mL per puff. Evaluations were performed 15 min after each provocation by measuring objective and subjective parameters. Objective parameters were the number

of sneezes and the reduction in nasal airflow determined by active anterior rhinomanometry and rhinoscopy. Subjective parameters comprised sneezing, nasal secretion, nasal congestion and symptoms in other organs (e.g. itching of ears or eyes). For each of the subjective parameters, 0–2 points could be achieved after administration of each of the three allergen concentrations. The points achieved after testing all three allergen concentrations were added to yield the total score. When rhinomanometric flow was decreased for more than 30% (2 points) and a symptom score of more than 3 points was achieved, the test was regarded as positive and was stopped [11, 12].

Statistical analysis

Statistical analysis was performed by using the Mann–Whitney *U*-test and linear regression. A probability value of <0.05 was considered as statistically significant. Correlation analyses were carried out by linear regression and Spearman's rank test.

Results

SPTs

The whole group of birch pollen-allergic patients showed positive skin reaction to BPE; 31 of 34 patients showed positive SPT to 10 µg/mL and to 100 µg of rBet v 1. Two of the patients not reacting to rBet v 1 were sensitized to rBet v 2 as shown by specific serum IgE determinations (CAP t216). Only two of the sensitized individuals reacted positively in SPT with 1 µg/mL and 16 showed questionable results, whereas the remaining 16 patients were clearly negative. None of the control individuals had positive skin reaction to rBet v 1a in any concentration, or detectable Bet v 1-specific serum IgE.

All birch pollen-allergic subjects showed comparable reactions in SPT with natural BPE and rBet v 1 at 10 µg/mL concentration. SPT results to 100 µg/mL rBet v 1 were even stronger than to natural BPE on average. However, some individuals reacted stronger to the natural extract and, not surprisingly, these patients showed co-sensitization to rBet v 2.

Specific serum IgE to birch extract and rBet v 1

As shown by Immuno-CAP, the sera of the 34 allergic patients contained IgE antibodies raised against BPE (see Table 1 and Fig. 2). Thirty three of these patients showed IgE antibodies to rBet v 1, while none of the patients was monosensitized to rBet v 2. In total, in nine of 29 patients also IgE antibodies to Bet v 2 were detectable; in one of these nine patients, very low levels of specific IgE against birch (1.66 kU/L) and a higher level of Bet v 2 (2.85 kU/L) were measured.

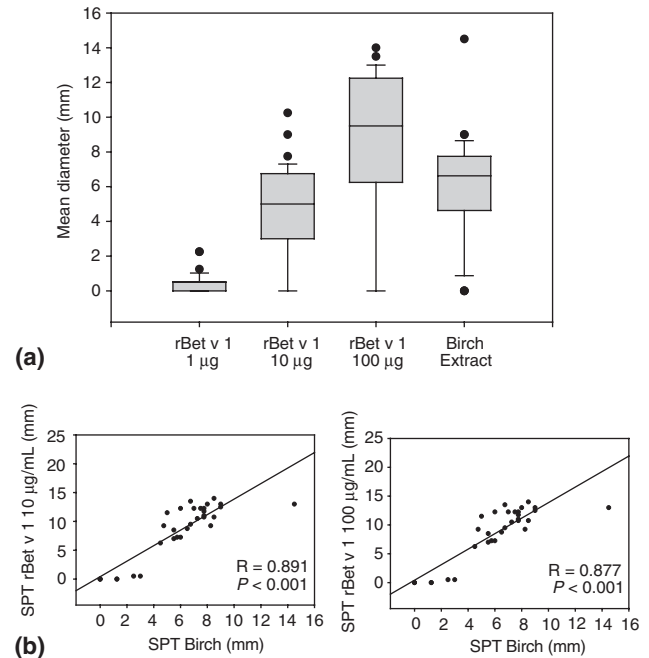
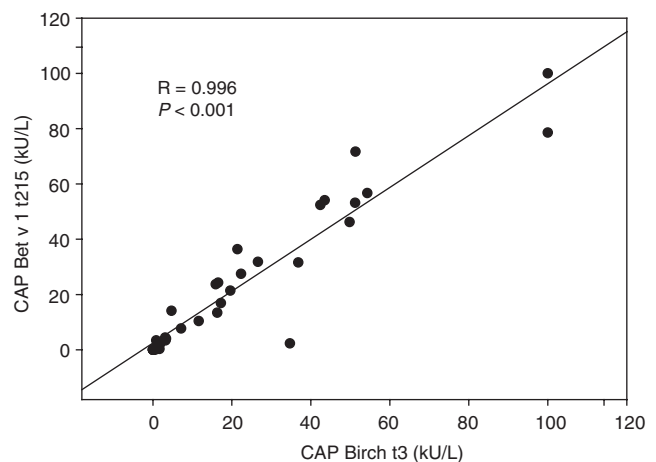
NPTs

In 23 patients and three healthy controls, were performed. Tests were negative in all controls. Twenty two of 23 patients responded positively to the BPE. The patient who did not

Table 1. *In vivo* and *in vitro* pattern of sensitization to birch, Bet v 1 and Bet v 2

Patient	History	SPT		Total IgE (kU/L)	CAP birch t3 (kU/L)	CAP rBet v 1 (kU/L)	CAP rBet v 2 (kU/L)
		birch pollen (mm)	rBet v 1 (100 µg/mL) (mm)				
1	RCA	5.5	6.5	182	7.14	7.69	<0.35
2	RCA	7.25	11.25	13882	>100	>100	<0.35
3	RCA	6.25	4	363	34.7	2.31	<0.35
4	RCA	4.5	5.5	117	11.6	10.4	<0.35
5	RCA	7.75	11	50.5	16.5	24.3	3.2
6	RCA	8.5	10.5	98.4	21.4	36.4	<0.35
7	RCA	9.25	12.5	183	42.4	52.4	<0.35
8	RCA	8.5	14.0	203	22.3	27.5	<0.35
9	RCA	9.0	13.0	174	51.3	71.7	<0.35
10	RA	6	7.5	107	3.33	4.01	<0.35
11	RA	4	0	771	1.66	<0.35	2.85
12	RA	8.5	9.5	162	43.5	54.1	<0.35
13	RA	8	12.25	395	26.6	31.8	<0.35
14	RA	8.5	9	104	49.8	46.2	9.4
15	RA	3.5	4.0	97.2	4.71	14.1	2.78
16	RA	7	8	202	17.2	16.9	2.7
17	RA	7.75	11.5	123	54.3	56.7	3.9
18	RA	7.75	11	93.2	16.5	24.3	3.2
19	RA	8.5	10.5	113	21.4	36.4	<0.35
20	RA	8.25	5.5	109	15.9	23.7	<0.35
21	RA	12.5	9.5	213	>100	78.6	9.16
22	RA	8.25	12.5	131	16.3	13.4	<0.35
23	RA	4.25	6.25	33.2	0.83	1.06	<0.35
24	RA	3.25	0	64.2	0.56	<0.35	0.68
25	RA	6.5	7.25	65.5	3.2	3.4	<0.35
26	RA	5.75	8.25	81.1	0.85	3.4	<0.35
27	RA	14.5	13.5	131	19.6	21.4	<0.35
28	RA	5.0	11.5	139	1.87	2.26	<0.35
29	RA	9.0	13.0	152	51.3	71.7	<0.35
30	RA	6.5	8.75	89.4	3.11	4.42	<0.35
31	RA	5.5	7	105	36.8	31.6	2.85
32	RA	6	7.25	71.6	3.33	4.01	<0.35
33	RA	7.75	11.0	113	24.3	29.4	<0.35
34	RA	6.75	8.25	45.4	7.56	7.84	<0.35
35	Healthy	0	0	5.56	<0.35	<0.35	<0.35
36	Healthy	0	0	50.5	<0.35	<0.35	<0.35
37	Healthy	0	0	11.9	<0.35	<0.35	<0.35
38	Healthy	0	0	65.1	<0.35	<0.35	<0.35
39	Healthy	0	0	117	<0.35	<0.35	<0.35

respond to BPE (no. 3) had applied a non-declared topical drug (so-called anti-allergic drops) as reported only after the challenge. Only nine of all patients reacted to a nasal challenge of 10 µg, and in two patients a positive reaction was elicited with only 1 µg of rBet v 1. (Fig. 2). With a maximum challenge of 100 µg rBet v 1, there was a clear positive reaction in 21 of the 22 patients reacting to BPE. Besides patient no. 3, patient no. 32 also did not react to nasal challenge with rBet v 1a. However, this patient showed very low levels of specific IgE to BPE and to Bet v 2, but was negative for rBet v 1-specific IgE determined by the highly sensitive Immuno-CAP System[®]. The difference in reactivity between 10 and 100 µg of rBet v 1 was significant, whereas rBet v 1 alone was able to elicit stronger reactions than BPE.

**Fig. 1.** (a) Findings for the SPT. Values are given as mean mm in diameter ($d_1 + d_2/2$). SPTs were performed with natural extract and three different concentrations of rBet v 1 (1, 10 and 100 µg/mL). (b) Relation between SPT with BPE and 10 and 100 µg/mL of rBet v 1.**Fig. 2.** Specific IgE values against birch and Bet v 1 detected by the Immuno-CAP System[®]. Values are given in kU/L.

Comparison between SPT, NPT and allergen-specific serum IgE

We found a weak, but significant correlation between SPT and allergen-specific IgE levels (Figs 4a and b) for the natural extract and rBet v 1 and also between NPT and specific serum IgE levels to both allergen preparations (Figs 4c and d). The correlation was in both cases slightly better for rBet v 1 than for the natural extract.

The correlation between SPT and NPT reactivity was more significant than the correlation between SPT, NPT and serology (Figs 4e and f). Interestingly, SPT and NPT

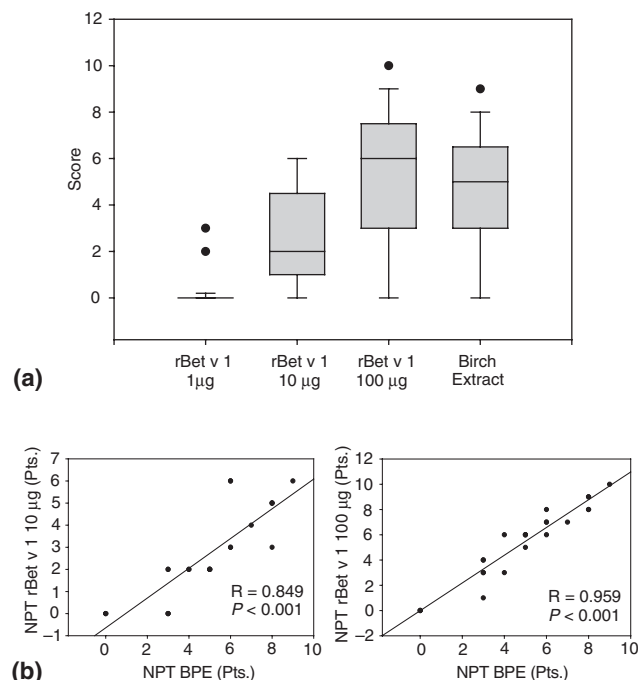


Fig. 3. (a) Findings for the NPT. Values are given in score points; a score ≥ 3 points was counted as a positive reaction. (b) Relation between NPT with BPE and 10 and 100 µg/mL of rBet v 1.

reactivity to natural extract was more significant than for rBet v 1.

Side-effects

In one patient, one tablet of cetirizine was given due to a very strong SPT reaction (> 13.5 mm) to decrease local itching. In all the subjects, no systemic symptoms were observed in either SPT or NPT.

Discussion

In this study we compared natural BPE and the single allergen Bet v 1 as recombinant protein. In addition to SPT and specific IgE, we therefore included NPT with increasing doses of rBet v 1 to study the allergenicity of Bet v 1, one of the best investigated allergens [13–15]. Concentrations of 1–100 µg of rBet v 1 were used to cover the range of Bet v 1 contained in BPE preparations.

First we demonstrated that, depending on the concentration of the recombinant allergen used the use of the single major allergen allows one to diagnose reliably birch pollen allergy in patients by *in vivo* and *in vitro* investigations (Figs 1–3). Already 10 µg/mL was enough to detect all but three patients reacting to BPE in SPT, while only one patient did not react with 100 µg/mL of rBet v 1. This patient did not show any specific IgE to Bet v 1 in serum, but was IgE-positive to Bet v 2. In comparison with BPE, a sensitivity of 94% and a specificity of 100% were achieved with rBet v 1 in SPT and serology. This is in accordance with the literature, where a specificity of 100% and a sensitivity between 87% and

100% is reported in studies using rBet v 1 in the same dose range [13, 15–17].

In concordance with many other studies describing the high reliability of rAllergens for IgE binding *in vitro*, a high sensitivity and specificity (96% and 100%, respectively) with a statistically significant correlation ($P < 0.001$) was found for rBet v 1 compared to BPE (Fig. 2).

On the other hand, provocation tests with rBet v 1 revealed the interesting finding that, in contrast to SPT, higher doses of the rAllergen were necessary to elicit an allergic reaction (Fig. 3). Only 91% of all patients reacting to natural BPE were responsive to a dose of 100 µg and just 39% were responsive to 10 µg of rBet v 1 in NPT. Therefore, the sensitivity of rBet v 1 is very low in nasal challenges compared to BPE. On the other hand, the specificity still reaches 100%. In comparison with natural BPE, the sensitivity in provocation tests is lowered, while the specificity remains very high. Factors such as minimal differences in folding or lack of post-translational modification of recombinant proteins may reduce the allergenicity of the recombinant protein for provocation tests, more than for skin tests [18, 19]. However, we cannot exclude the presence of unspecific, IgE-independent irritating agents in natural BPE being absent in pure recombinant allergen preparations.

In another study, nasal and bronchial challenges with natural Bet 1 and rBet v 1 were performed in 13 patients allergic to BPE, in addition to SPT [20]. Also, in this report slightly higher concentrations of the rAllergen were necessary to elicit positive challenge reactions as reported in this study.

In a further, recently published study, 24 patients were investigated with SPT, CAP and NPT to Bet v 1 and also to various grass allergens [18]. It was shown that 20 µg/mL for SPT and up to 40 µg/mL for NPT of the recombinant protein were sufficient to elicit positive reactions. Natural BPE was not included in this study setting.

From our results and from the findings of other groups, we can conclude that higher doses of rBet v 1 are needed to elicit *in vivo* allergic reactions by NPT challenges compared to the concentrations needed for positive SPT reactions.

Comparing the interdependence of SPT, NPT and specific serum IgE for both BPE and Bet v 1, we found a weak but significant correlation between SPT, NPT and *in vitro* detection of allergen-specific IgE (Fig. 4).

Taken together with the safety of the diagnostic use of rAllergens, demonstrated in many studies involving more than 2000 individuals as recently reviewed [4], this study shows that in the case of birch pollen allergy a single recombinant allergen (Bet v 1) is sufficient for a reliable, perfectly standardized diagnosis of the allergic condition with a sensitivity $> 90\%$ and a specificity of 100%. Although more complex allergenic sources like moulds or food mixtures of recombinant allergens will be required to reach a comparable diagnostic precision [19, 20], this study highlights the power of recombinant allergens for diagnostic applications. A precise patient-tailored diagnosis offered only by recombinant allergens is required for a specific immunotherapy and can now be performed for many allergenic sources. However, clinical studies will be required to document the therapeutic efficacy of recombinant allergens compared to classical specific immunotherapy performed with natural extracts.

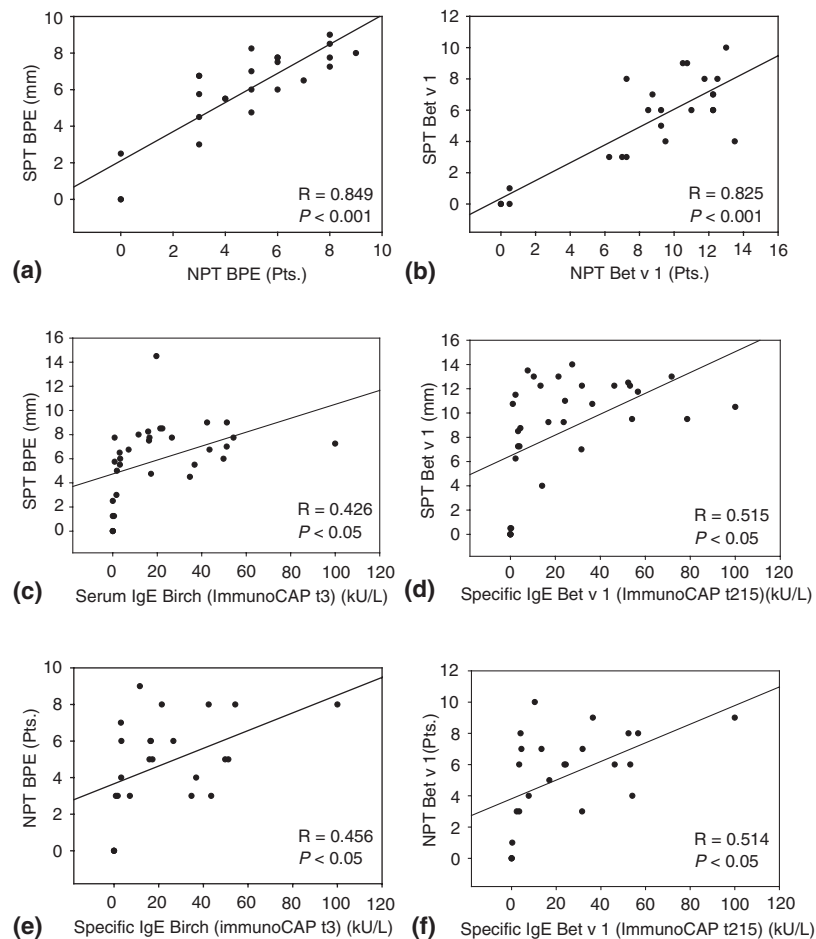


Fig. 4. Comparison between the skin tests, specific serum IgE and NPT are shown for both BPE (left column) and rBet v 1a (right column). (a, b) SPT and NPT. Results of SPT in relation with NPT with BPE (a) and SPT in relation with NPT with 100 µg/mL rBet v 1 (b) are shown. (c, d) SPT and specific serum IgE. (c) Results of SPT with BPE and specific serum IgE against birch. (d) Results of SPT with rBet v 1 (100 µg) and CAP Bet v 1. (e, f) NPT and specific serum IgE. Results from NPT with BPE vs. specific serum IgE against birch are shown in (e) while NPT with rBet v 1 vs. specific serum IgE against Bet v 1 are shown in (f).

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