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The clinical relevance of birch pollen profilin cross reactivity in sensitized patients

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Authors contributions

The article was written by FW and TB. JK, JF, FW, CG and WK acquired the data and performed data analysis and interpretation. TB developed the concept and design of the study. All authors critically revised the drafts of the article and approved the final version for publication.

Abstract

Background: Overlapping seasons and cross reactivity, especially to grass pollen profilin, can hamper the diagnosis of birch pollen allergy. To identify the primary sensitizing allergen and the clinical relevance of cross sensitization, we correlated sensitization profiles with *in vitro* and *in vivo* tests, symptom scores and pollen counts.

Methods: 433 patients with positive skin prick test (SPT) to birch pollen were analyzed regarding IgE to major birch and grass pollen allergens Bet v 1 and Phl p 1/p 5 and the profilins Bet v 2 and Phl p 12. Subgroups were analyzed by basophil activation test (BAT) and CAP-FEIA based cross- and self-inhibition tests.

Results: 349 patients were sensitized to Bet v 1, 44 to both Bet v 1 and Bet v 2 and 15 patients to Bet v 2 only. From Bet v 2 sensitized patients 40 were also sensitized to Phl p 12. *Ex vivo*, Bet v 2 and Phl p 12 induced dose dependent activation in basophils of these patients. Cross- and self-inhibition tests with both allergens confirmed cross reactivity. However, semi-quantitative analysis of SPTs demonstrated markedly increased reactivity to grass compared to birch pollen extract in Bet v 2 only sensitized patients. Accordingly, in most of those patients clinical symptoms precisely correlated with grass pollen counts.

Conclusion: Identification of the clinically relevant and sensitizing allergen needs correlation of actual pollen counts with clinical symptoms and sensitization status to major allergens. Semi-quantitative analysis of SPT or BAT and determining profilin-specific IgE can contribute to making the diagnosis.

Key words: basophil activation test, birch pollen, cross reactivity, grass pollen, profilin

Short title: Profilins in birch pollen sensitized patients.

Introduction

Birch pollen is next to grass pollen allergy the most common cause of pollen related allergic rhinoconjunctivitis (ARC) in northern and central Europe (1). Correlating with continuously increasing pollen counts the prevalence of both is still increasing. This is, like the tendency to an earlier start of the pollen season, most likely associated to climate change (2). Independent thereof pollen counts largely vary from year to year. Although birch pollen in central Europe sometimes can be detected already in January and February, counts reach a maximum in April and May and then decline from June to July or August. Although variation is high, there is a broad overlap with other pollen seasons. Most relevant is the grass pollen season starting as early as March with a maximum in July and August ending in October or November. Next to the seasonal overlap there is a high level of cross reactivity of IgE antibodies directed to grass and birch pollen profilins, both minor allergen (3). Profilins are ubiquitous proteins expressed in all eukaryotic cells. Especially the birch pollen profilin Bet v 2 and the grass pollen profilin Phl p 12 are well characterized (3-5). In birch pollen sensitized individuals 10% to 38% are also sensitized to Bet v 2, while 14% to 24% of patients sensitized to the major grass pollen allergens Phl p 1/5 are sensitized to Phl p 12 (6,7).

As a consequence of both the seasonal overlap and the cross reactivity of profilins there is a subgroup of patients who, despite symptoms matching the birch pollen season and positive SPT to birch pollen, actually are not sensitized to major birch pollen allergen Bet v 1. It is still unclear, if clinical symptoms in those patients are a consequence of profilin cross-sensitization or simply due to concomitant exposure to the primary sensitizing allergen. Although a few studies indicated that Bet v 2 sensitization under standardized conditions or in single patients might be able to mediate clinical reactions (8-14), the clinical relevance remains controversial (15).

To dissect the contribution of profilin cross reactivity in birch pollen sensitized patients, we investigated a cohort of birch sensitized pollinosis patients living in the south of Germany and finally integrated the results of comprehensive *in vitro*, *in vivo* and *ex vivo* diagnostic tests with subjective clinical symptom scores correlated to the objective pollen counts.

METHODS

Study protocol

433 outpatients with positive SPT to native birch pollen extract and symptoms of ARC between March and August were screened between June 2005 and December 2007.

Sensitization was proven by SPT and detection of specific IgE to Bet v 1, Bet v 2, Bet v 4, Bet v 6, Phl p 1, Phl p 5, Phl p 12 and birch and grass pollen extract. The study was performed with approval of the local ethics committee. All patients provided informed consent.

Determination of specific IgE antibodies

IgE to Bet v 1, Bet v 2, Bet v 4, Bet v 6, Phl p 1/Phl p 5, Phl p 12 and native birch and grass pollen extracts were determined using the ImmunoCAP system (ThermoFisher Scientific (Phadia), Freiburg, Germany) according to the manufacturer's instructions. The cut-off value for positive test results was 0.35 kUA/l.

Cross inhibition assay

CAP-FEIA based cross- and self-inhibition tests were used to detect cross-reactivity between Bet v 2 and Phl p 12 in 29 double sensitized patients (21 Bet v 1 and Bet v 2 sensitized patients and 8 Bet v 2 sensitized but Bet v 1 unsensitized patients). Bet v 2 and Phl p 12-reactive sera with a CAP of >2.0 kU/l were diluted to a concentration of approximately 1.0 kU/l. Sera were preincubated over night at 37°C with different Bet v 2 or Phl p 12 (Bühlmann, Schönenbuch, Switzerland) concentrations (1ng/ml; 10ng/ml; 100ng/ml; 1000ng/ml). The next day, remaining soluble antibodies were measured with the CAP-FEIA system (ThermoFisher Scientific (Phadia), Freiburg, Germany) using either Bet v 2 or Phl p 12 as solid phase as indicated. The inhibitory potential was calculated as the ratio of results of sera preincubated to sera left untreated on the basis of kU/l. Complete inhibition was defined as an absolute result of <0.35 kU/l.

Basophil activation test

For BAT 17 Bet v 2 and Phl p 12 double sensitized patients (11 Bet v 1 and Bet v 2 sensitized patients and 6 Bet v 2 sensitized but Bet v 1 unsensitized patients) and 9 healthy controls were recruited. The patients recruited for BAT additionally kept an ARC-symptom-diary. 'Flow CAST' reagents and protocol (Bühlmann, Schönenbuch, Switzerland) were used with slight modifications. EDTA-blood samples were incubated in stimulation buffer with either anti-FcεRI (0,5µg/ml; Bühlmann, Schönenbuch, Switzerland), fMLP (1µM; Bühlmann, Schönenbuch, Switzerland), different concentrations of birch pollen extract (IC-Test solution,

Allergopharma, Reinbek, Germany), Bet v 1, Bet v 2 or Phl p 12 (each Bühlmann, Schönenbuch, Switzerland) as indicated. For FACS analysis cells were stained with anti-IgE-FITC and anti-CD63-PE antibodies (both Bühlmann, Schönenbuch, Switzerland). The cut-off value for a positive test result was set as $\geq 15\%$ of CD63⁺ basophils. Data were analyzed using CellQuest and FACS Diva software (Becton Dickinson Deutschland, Heidelberg, Germany).

Pollen counts

The *Betulaceae* and *Poaceae* pollen counts registered in the region 'mittlerer Neckar' in 2008 were kindly provided by the foundation 'Deutscher Polleninformationsdienst' (<http://www.pollenstiftung.de>).

Statistics

Data are provided as means $\pm$ SEM; *n* represents the number of samples investigated. Data were tested for significance using the Student unpaired two-tailed *t* test or the Wilcoxon matched-pairs signed rank test, where applicable. A value of $p < 0.05$ was considered to indicate statistical significance.

Results

Profilin sensitization and distinct sensitization profiles

To better understand the clinical relevance of Bet v 2 sensitization we defined subgroups with distinct sensitization profiles for further analyses. To this end, 433 patients with a positive SPT to native birch pollen were screened for specific IgE to recombinant allergens. Of them 349 (80.6%) were exclusively sensitized to the birch pollen major allergen Bet v 1, 44 (10.2%) were sensitized to both Bet v 1 and the birch pollen profilin Bet v 2 while a small proportion of 15 (3.5%) patients was sensitized to Bet v 2 but not to Bet v 1 (Fig. 1).

Bet v 2 sensitization could be the primary event or detected based on cross reactivity, most probable to grass pollen profilins. Indeed, more detailed analysis of the group of Bet v 2 sensitized patients revealed, that except for two (one from the Bet v 1 and Bet v 2 sensitized group, the other from the Bet v 2 sensitized but Bet v 1 unsensitized group) all of them were

also sensitized to Phl p 1 and Phl p 5. Of them, 40 (70.2% of patients sensitized to Bet v 2 and grass pollen major allergens Phl p 1 and Phl p 5) were also sensitized to Phl p 12, the grass pollen profilin. From those, 29 patients were Bet v 1 and Bet v 2 sensitized while 11 belong to the Bet v 2 but not Bet v 1 sensitized group.

Bet v 2 and Phl p 12 are fully cross reactive in regard to IgE binding

We next determined cross reactivity between anti-profilin IgE antibodies for each individual patient. Detailed sensitization profiles of patients sensitized to Bet v 2 but not to Bet v 1 who were available for cross inhibition tests show (Table 1), that the units of IgE directed against native birch pollen extract and the units of IgE directed against Bet v 2 are comparable in most patients. In the majority of patients IgE-units detected against Bet v 2 are also similar to IgE-units directed against Phl p 12. This already suggests, that Bet v 2 reactivity could be due to cross reactive anti-Phl p 12 IgE. To determine and quantify cross reactivity, we performed inhibition experiments. Patient sera were incubated with different amounts of recombinant Bet v 2 or Phl p 12, respectively. For dose finding, three patient sera with very similar anti Bet v 2 and Phl p 12 IgE units were used. Figure 2a shows self- and cross-inhibition of the Bet v 2 CAP by recombinant profilin-allergens. Preincubation with Phl p 12 shows a clear cross inhibition. Results of the Phl p 12 CAP (Fig. 2b) are very similar. We could confirm those results performing the same experiment with a reduced approach limited to the range of 1ng/ml to 100ng/ml with all Bet v 1 non-reactive sera (Fig. 2c+d). This confirms complete cross reactivity of IgE antibodies directed to Bet v 2 and Phl p 12 in double sensitized patients.

Profilins are fully functional allergens activating basophils *ex vivo*

To prove, that polyclonal IgE antibodies reactive with profilins occurring *in vivo* are functional we performed BAT. Basophil activation status was determined by measuring cell surface CD63 expression. Basophils from Bet v 1 sensitized patients were strongly activated after contact with Bet v 1 or whole birch pollen extract. Basophil degranulation induced by Bet v 2 was only slightly less effective (Fig. 3a, lane 1; 3b). As expected, basophils from Bet v 2 but not Bet v 1 sensitized patients showed no reactivity to Bet v 1. However, Bet v 2 was a potent inducer of basophil activation (Fig. 3a, lane 2; 3b). Controls from unsensitized patients did not respond to birch pollen allergens (Fig. 3a, lane 3; 3b). Since both Bet v 2 and whole birch pollen extract induced a similar level of activation in Bet v 1 unsensitized

patients (Fig. 3b), in those the reactivity to birch profilin determines the reactivity to birch pollen allergens in general.

Activation of basophils by the profilins Phl p 12 and Bet v 2

To analyse if distinct levels of basophil activation following stimulation with different profilins might allow to draw conclusions in regard to the profilin of primary sensitization, we compared reactivity of basophils from Bet v 2 but not Bet v 1 sensitized patients to Bet v 2 and Phl p 12 in more detail. In 6/11 individuals 'titrated BATs' were performed. Since basophil reactivity in BAT differs widely between individual patients, the complete results of both Bet v 2 and Phl p 12 dose titrations are shown in figure 3c. All patients show a dose dependent activation of basophils by both allergens. Although the level of activation varies interindividually, Bet v 2 dependent activation and Phl p 12 dependent activation are very similar on an individual level. Consistent with the results from cross inhibition tests which had proven complete reciprocal inhibition, this shows that in patients with IgE to Bet v 2 and Phl p 12 both profilin allergens are able to induce basophil activation to the same extent.

SPT results differ depending on profilin only or additional major allergen sensitization

To determine profilin dependent cellular activation *in vivo*, we performed SPT. Similar to BAT results, Bet v 1 and Bet v 2 double sensitized patients developed intermediate to strong SPT reactions similarly to application of whole birch as well as to whole grass pollen extract (Fig. 4a). In contrast, patients sensitized to Bet v 2 but not to Bet v 1 developed strong reactions only to the grass pollen extract while reactivity to birch pollen extract was either absent, weak or intermediate (Fig. 4b). Most likely, as observed in BAT, this reflects a reduced potential of birch pollen profilin to induce mast cell and basophil degranulation in comparison to the birch pollen major allergen. In conclusion, like BAT, semi-quantitative interpretation of SPT already indicates if a patient is sensitized only to minor or also to the respective major allergens.

Profilin cross sensitization is usually not inducing ARC

IgE antibodies directed to birch and grass pollen profilin are fully cross reactive. Even results from BAT and cross inhibition tests don't allow to differentiate which of the profilins induced primary sensitization. In this regard only indirect conclusions from the sensitization pattern to

major allergens can be drawn. Irrespective of the origin of sensitization both BAT and SPT already point to an at least reduced potential of profilin allergens to induce allergic reactions. Table 2 shows the occurrence of birch and grass pollen season related ARC-symptoms in Bet v 2 sensitized patients in the seasons of 2008. Surprisingly and in contrast to the Bet v 1 and grass pollen sensitized patients, who suffered from symptoms of ARC throughout the birch- and grass pollen season, all except for one of the grass pollen and Bet v 2 but not Bet v 1 sensitized patients reported on symptoms of ARC strictly limited to the grass pollen season. Interestingly and in contrast to Bet v 1 sensitized patients, Bet v 2 but not Bet v 1 sensitized patients were not sensitized to the other important cross reactive birch pollen allergens Bet v 4 and Bet v 6 (Figure 4c) also indicating that the primary source of sensitization is probably not birch pollen in these patients.

The severity of ARC symptoms and their correlation with *Betulaceae* and *Poaceae* pollen counts actually measured is depicted in Figure 4d for a representative 29 years old male patient of this group living in the mid Neckar region. Despite high serum IgE to birch pollen of 7.5 kU/l (CAP class 3) he had no symptoms during the birch pollen season in his region comprising April and the first 10 days of May 2008. In contrast, the severity of his ARC symptoms nicely correlated with the grass pollen counts in May and June 2008, with both severity of his symptoms and pollen counts peaking at the end of May. In conclusion, the majority of patients sensitized to birch pollen profilin but not to birch pollen major allergen Bet v 1 do not suffer from birch pollen allergy. Bet v 2 sensitization rather reflects cross reactivity of Phl p 12 reactive IgE in heavily grass pollen sensitized patients. However, a minority of patients develop symptoms of ARC due to sensitization to Bet v 2.

Discussion

There is a long running debate on sensitization to birch pollen profilin possibly underlying ARC-symptoms and the source of profilin sensitization (15). Our data show, that only a small proportion of birch pollen reactive patients is sensitized to Bet v 2 only and that most of them are also sensitized to grass pollen major allergens Phl p 1/5 and grass pollen profilin Phl p 12, implying grass pollen to be the most important source of profilin sensitization and suggesting Bet v 2 reactivity in most cases to reflect cross reactivity (15,16,17). Indeed, IgE-based cross- and self-inhibition tests in Bet v 2 and Phl p 12 sensitized patients showed full cross reactivity and gave no evidence regarding the primary sensitizing pollen-profilin allergen. However, in BAT and in SPT the use of native birch pollen allergens resulted in weaker test responses in Bet v 2 sensitized/Bet v 1 unsensitized patients in comparison to patients sensitized to Bet v 1. Although this could be

caused by a lower Bet v 2 than Bet v 1 concentration in birch pollen extract, it is more likely that it actually indicates a lower potential of Bet v 2 to induce effector cell degranulation. Supporting this interpretation, Bet v 2 in BAT induces degranulation less efficiently than Bet v 1. However, even if the results of diagnostic tests induced by Bet v 2 are weak, it is striking that in 5 out of 6 patients sensitized to Bet v 2 but not to Bet v 1 exposure to birch pollen *in vivo*, under real life conditions, is not correlated with any symptoms of ARC at all. This confirms field studies (8,9,17) which failed to clearly prove an impact of sensitization to birch and other profilins on the occurrence of ARC related symptoms. However, and for the first time within the same study population, our results show that there indeed is a minority of Bet v 2 sensitized patients in whom the environmental exposure to birch pollen profilin is sufficient to mediate ARC-symptoms. Until now, there has only been anecdotal evidence of such patients. A case report by Asero *et al.* (10) describes a woman with positive SPT to birch pollen and symptoms of ARC starting in February which were considered to be due to a sensitization to profilins. A recent case report by Metz *et al.* (11) also reports on a patient with confirmed birch allergy due to Bet v 2 sensitization. In addition, other studies demonstrated the ability of Bet v 2 (12) and other profilins (13,14) to induce clinical symptoms at least in nasal, conjunctival and bronchial provocation tests. However, our findings show that patients with a clinically relevant sensitization to Bet v 2 are rare. Out of 433 birch pollen sensitized patients, of whom 15 were sensitized to Bet v 2 only, one patient reported on ARC-symptoms following exposure to birch pollen. The apparent mismatch between the ability of Bet v 2 to induce positive diagnostic tests in sensitized patients despite the absence of clinical symptoms under real-life conditions is most likely due to the lability of profilins and differences in the intensity of exposure (5). Consequently, in case of uncertainty about the relevance of birch pollen sensitization, the keeping of a patient-symptom-diary is essential. Semi-quantitative analysis of SPT or BAT should also be considered. Determining the units of IgE directed to birch pollen profilin Bet v 2 finally allows to identify the cause underlying birch pollen sensitization in such patients. However, it is important to keep in mind that in patients sensitized to Bet v 2 only, even if related to ARC-symptoms, standard birch pollen SIT most probably is not indicated. It would not only be ineffective but rather bear the risk to induce Bet v 1 sensitization. Thus, the diagnosis of birch pollen allergy must always be based upon a thorough diagnostic workup.

Figure legends

Figure 1. Characterization of sensitization profiles.

During the screening period from June 2005 to December 2007, 433 patients were tested positive to native birch pollen extract with CAP-FEIA. This population was set at 100%. Of those, 29 (6.7%) patients were Bet v 1/Bet v 2/Phl p 12 sensitized and 11 (2.5%) were Bet v 1 unsensitized but Bet v 2/Phl p 12 sensitized.

Figure 2. CAP-FEIA based self- and cross inhibition tests.

In sera of three patients with IgE specific to both Bet v 2 and Phl p 12 complete self- and cross inhibition of Bet v 2 **(A)** or Phl p 12 **(B)** reactive IgE antibodies could be obtained using Bet v 2 or Phl p 12 as inhibitors (results are shown as means $\pm$ SEM). The same approach with a reduced number of different Bet v 2 and Phl p 12 concentrations in eight Bet v 1 unsensitized but Bet v 2 sensitized patients confirmed complete self- and cross inhibition of Bet v 2 **(C)** or Phl p 12 **(D)** CAP-FEIA (n=8; results are shown as means $\pm$ sem).

Figure 3. Reactivity to native birch pollen, Bet v 1 and Bet v 2 in BAT.

(A) Percentage of CD63-positive basophils without stimulation (unstimulated), after anti-Fc ϵ RI challenge (as positive control) or after allergen specific challenge (birch, Bet v 1, Bet v 2) at a concentration of 50 ng/ml each. Lane 1 shows a representative dot plot of a patient sensitized to Bet v 1 and Bet v 2. Lane 2 shows a representative dot plot of a patient sensitized to Bet v 2 without sensitization to the major birch pollen allergen Bet v 1. Note the negative response to stimulation with Bet v 1. Lane 3 shows a dot plot of a sample of an unsensitized control person. **(B)** Mean percentage of CD63-positive basophils of all patients determined as described before. (Bet v 1/Bet v 2 sensitized n=11; Bet v 1 unsensitized/Bet v 2 sensitized n=6; unsensitized controls n=9; results are shown as means $\pm$ sem). **(C)** CD63 expression as marker of basophil activation after stimulation of basophils from Bet v 1 unsensitized patients with Phl p 12 and Bet v 2 in basophil activation test using allergen concentrations of 0.05; 0.5; 5 and 50 ng/ml; uc: unstimulated control. Data of ≥ 6 representative patients are shown; * * * = $p \leq 0,002$.

Figure 4. Clinical relevance and reactivity to profilin allergens *in vivo* in sensitized patients.

Comparison of skin prick test responses to grass and birch pollen extract within Bet v 1 and Bet v 2 sensitized **(A)** and Bet v 1 unsensitized but Bet v 2 sensitized **(B)** subgroups (from the lowest (0, negative) to the highest (++++) degree of skin reaction). Results of individual patients are represented by black dots (n = 10 (A); n = 6 (B); * = $p \leq 0,05$). **(C)** CAP classes of IgE directed to Bet v 4 or Bet v 6 of those Bet v 1 and Bet v 2 sensitized (left) and Bet v 1 unsensitized but Bet v 2 sensitized (right) patients shown in table 2. Missing sensitization to Bet v 4 and Bet v 6 of Bet v 1 unsensitized but Bet v 2 sensitized patients allows to ascribe their clinical symptoms in the birch pollen season to their sensitization to Bet v 2 **(D)** Mean decadal *Betulaceae* pollen concentrations don't align with the reported level of allergic symptoms in a representative Bet v 1 unsensitized/Bet v 2 sensitized 29 year-old male patient. The left vertical axis represents the counts of pollen grains per cubic meter of air, the right vertical axis represents the severity of allergic symptoms ranging from 0 (no symptoms of ARC) to 6 (severe symptoms of ARC).

Table 1: Detailed data of patients sensitized to birch- and grass pollen extract as well as Bet v 2 and Phl p 12 but not to Bet v 1 used for cross inhibition tests.

Table 2: Clinical symptoms as reported by Bet v 1 unsensitized but Bet v 2 sensitized (12-17) in comparison to Bet v 1 and Bet v 2 sensitized patients (1-11).

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Patient	Sex	Age (year)	Allergen tested	Specific IgE (kU/L)	CAP class	Allergen tested	Specific IgE (kU/L)	CAP class
1	M	32	timothy grass	>100	6	birch	7.5	3
			Phl p 1, Phl p 5	53.9	5	Bet v 1	0.11	0
			Phl p 12	2.9	2	Bet v 2	6.1	3
2	M	23	timothy grass	76.2	5	birch	9.2	3
			Phl p 1, Phl p 5	95.5	5	Bet v 1	0.01	0
			Phl p 12	6.6	3	Bet v 2	8.0	3
3	M	44	timothy grass	15.5	3	birch	1.3	2
			Phl p 1, Phl p 5	22.4	4	Bet v 1	0.02	0
			Phl p 12	0.59	1	Bet v 2	1.3	2
4	F	48	timothy grass	0.37	1	birch	0.74	2
			Phl p 1, Phl p 5	0.00	0	Bet v 1	0.09	0
			Phl p 12	0.59	1	Bet v 2	1.1	2
5	M	29	timothy grass	75.3	5	birch	1.9	2
			Phl p 1, Phl p 5	78.4	5	Bet v 1	0.06	0
			Phl p 12	1.5	2	Bet v 2	1.2	2
6	F	24	timothy grass	>100	6	birch	1.8	2
			Phl p 1, Phl p 5	>100	6	Bet v 1	0.05	0
			Phl p 12	3.2	2	Bet v 2	1.2	2
7	F	15	timothy grass	>100	6	birch	3.7	3
			Phl p 1, Phl p 5	>100	6	Bet v 1	0.05	0
			Phl p 12	3.8	3	Bet v 2	3.1	2
8	M	29	timothy grass	16.5	3	birch	2.3	2
			Phl p 1, Phl p 5	1.7	2	Bet v 1	0.08	0
			Phl p 12	17.9	4	Bet v 2	3.0	2

Table 1

No	sex	age	anti Bet v 1 IgE	ARC during birch pollen season	ARC during grass pollen season	SPT to birch pollen	SPT to grass pollen
1	f	33	+	Yes	yes	+++	+++
2	m	18	+	yes	yes	++	++
3	f	37	+	yes	yes	+++	+++
4	m	25	+	yes	yes	++	+++
5	f	45	+	<i>n.s.</i>	<i>n.s.</i>	<i>n.s.</i>	<i>n.s.</i>
6	m	45	+	yes	yes	++	+++
7	f	15	+	yes	yes	+++	+++
8	f	23	+	yes	yes	+++	+++
9	f	25	+	yes	yes	+++	+++
10	m	35	+	yes	yes	++	+++
11	f	43	+	<i>n.s.</i>	<i>n.s.</i>	++	++
12	f	21	-	<i>no</i>	yes	+	+++
13	m	41	-	<i>no</i>	yes	+	+++
14	m	20	-	<i>no</i>	yes	+	+++
15	m	26	-	yes	yes	++	+++
16	m	29	-	<i>no</i>	yes	++	+++
17	f	45	-	<i>no</i>	<i>no</i>	0	++

Table 2

Figure 1

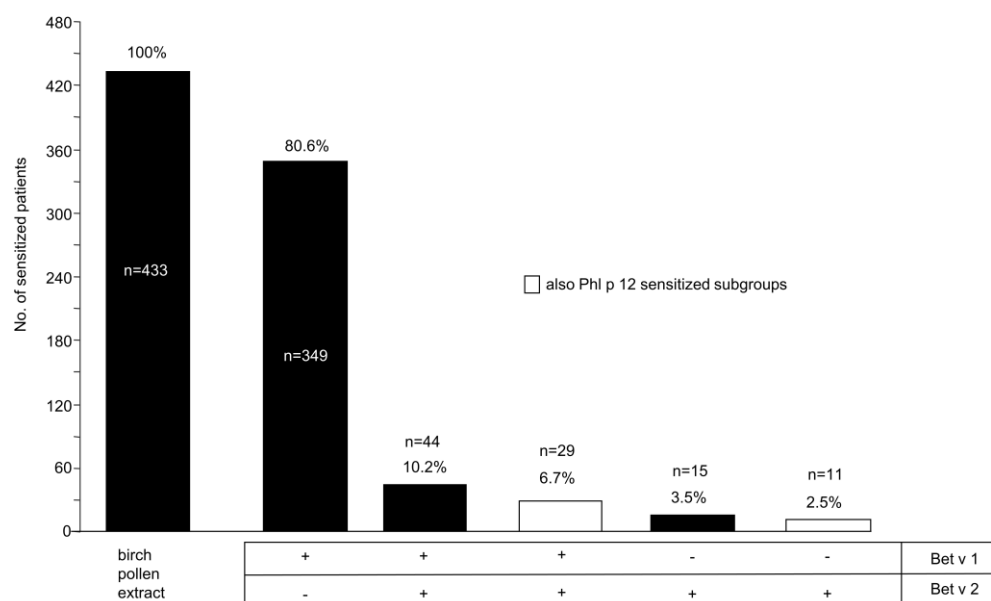


Figure 2

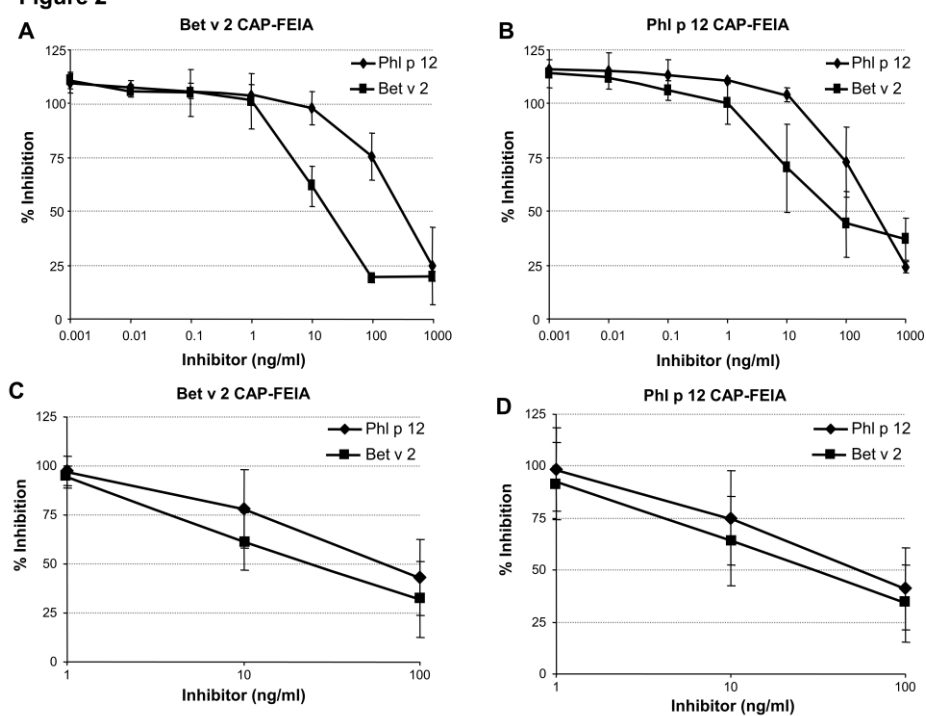


Figure 3

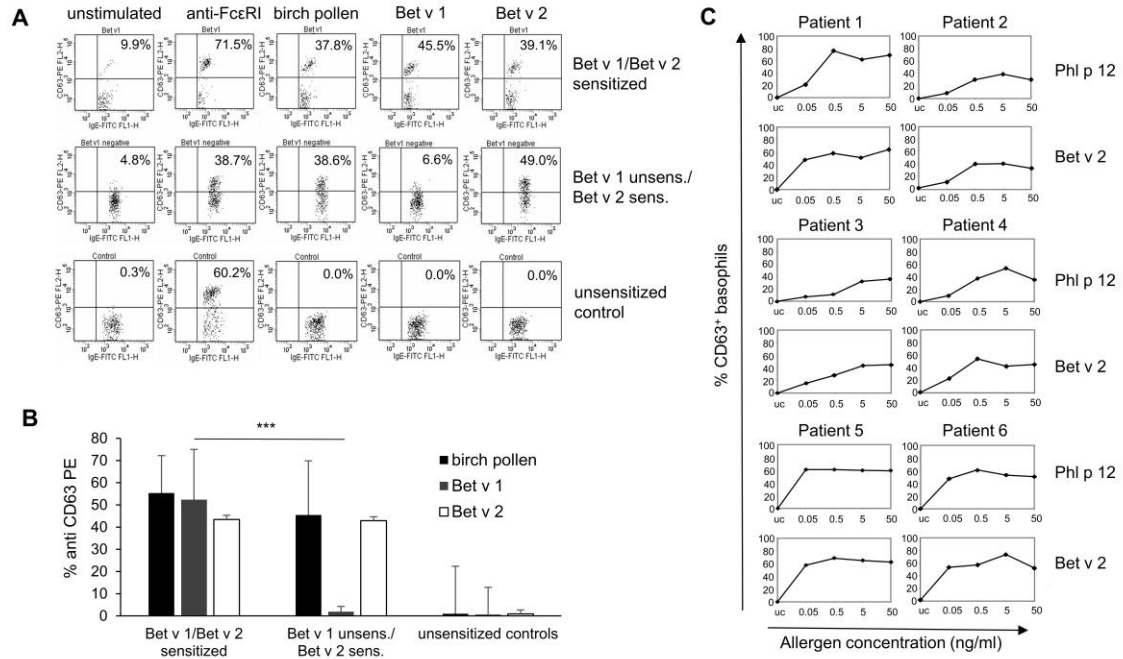


Figure 4

