

Sensitization to cross-reactive carbohydrate determinants and the ubiquitous protein profilin: mimickers of allergy

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Summary

Background During the last decade, evidence has been provided for profilins and cross-reactive carbohydrate determinants (CCDs) to be capable of inducing cross-reactive IgE antibodies with little clinical relevance.

Objective To investigate the prevalence of sensitization to CCD and profilin in isolated allergies (birch, timothy grass, house dust mite, pets (cat and/or dog), natural rubber latex (NRL) and hymenoptera venom). To study the contribution of anti-CCD and anti-profilin IgE antibodies as a cause of clinically irrelevant IgE for NRL and apple.

Methods For the first part of the study, 100 patients with inhalant allergy, 17 patients with NRL allergy and 40 patients with venom anaphylaxis were enrolled. Diagnosis was based on a questionnaire and a positive IgE determination and skin test for relevant allergen. Patients were identified as sensitized to CCD if they had a negative prick test and positive IgE for the glycoprotein bromelain. Sensitization to profilin was assessed by IgE for rBet v 2 (recombinant birch profilin). For the second part of the study, sera containing IgE against apple ($n = 82$) or NRL ($n = 38$) were classified as true-negative or false-positive according to the presence or absence of an oral allergy syndrome (OAS) or NRL-induced anaphylaxis. In these patients, sensitization to CCD and profilin was evaluated as described above.

Results No sensitization to bromelain-type CCD and profilin was found in isolated birch pollen or NRL allergy. In contrast, sensitization to bromelain-type CCD was found in 4/17 patients with isolated grass pollinosis, 5/24 patients with combined pollinosis (birch, timothy, mugwort) and 7/33 patients with venom anaphylaxis. Sensitization to profilin was almost restricted to patients with combined pollen allergy (5/24). In pollen-allergic individuals with a false-positive IgE against NRL the prevalence of sensitization to bromelain-type CCD and profilin IgE was higher than in NRL-allergic patients ($P < 0.00001$ and $P = 0.0006$, respectively). In pollen-allergic individuals with a false-positive IgE to apple, the frequency of sensitization to bromelain-type CCD was higher than in OAS patients ($P = 0.004$). Clinically irrelevant NRL and apple were also found in four and five out of the seven patients sensitized to venom CCD, respectively. In pollinosis, clinically irrelevant NRL and apple IgE antibodies were inhibited by bromelain and recombinant birch profilin, whereas in isolated venom anaphylaxis these antibodies were inhibited by bromelain.

Conclusions Patients monoallergic to NRL or birch pollen showed no sensitization to bromelain-type CCD or profilin. Sensitization to profilin and/or bromelain-type CCD, caused by pollen (timothy grass, mugwort) or hymenoptera venom allergens, can elicit false-positive IgE antibodies against NRL and apple.

Keywords carbohydrate determinants, cross-reactivity, food, hymenoptera venom, IgE, latex, profilin
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Introduction

Simultaneous IgE reactivity in the same patient to multiple allergens has been a major issue in IgE serology almost since the development of the radioallergosorbent test. The basis for multiple reactivity remains incompletely elucidated, but

several explanations have been proposed. First, true independent sensitization for different allergens may account for some of the results. Second, non-specific absorption of IgE to the allergosorbent, a phenomenon that is particularly relevant when total serum IgE is extremely elevated, can be responsible. Third, immunochemical cross-reactivity due to sequence homologies between allergens from different sources is possible [1].

Bet v 1 homologues belong to the pathogenesis-related proteins (PR 10) and account for most of *in vitro* (serologic)

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and *in vivo* (clinical) cross-reactivity between birch pollen on the one hand and *Rosaceae* fruits (e.g. apple, pear, plum) as well as *Umbelliferae* vegetables (e.g. celery, fennel, carrot) on the other hand [2–4]. Profilins, a family of actin-binding proteins, are ubiquitous in eukaryotic cells and probably function as key mediators of membrane–cytoskeleton communication. Profilins have been demonstrated to be present in pollen (e.g. birch, mugwort, timothy grass), fruit (e.g. apple, cherry), vegetables (e.g. celery, peanut, carrot) and natural rubber latex (NRL) [4–6]. Unlike Bet v 1, the clinical role of profilins is less clear. It has been demonstrated that birch profilin is a highly efficient sensitizer, but sensitization to birch profilin is rarely associated with symptoms [7, 8].

Cross-reactivity, however, extends beyond the protein level. Characteristic $\alpha(1,3)$ -fucose (linked to the proximal *N*-acetyl glucosamine) and/or $\beta(1,2)$ -xylose (linked to the core mannose) bearing carbohydrate moieties of pollen and plant food glycoproteins, generally referred to as cross-reactive carbohydrate determinants (CCDs), have also been identified as highly cross-reactive IgE-binding structures [1, 4, 9–14]. The prevalence of anti-CCD IgE has been estimated to be 10–15% in patients with grass pollen allergy [15] and increases up to 60% in patients with concomitant sensitization to pollen from trees, grasses and weeds [16]. However, invertebrate glycoproteins, as present in hymenoptera venom, also bear IgE-binding $\alpha(1,3)$ -fucose-containing CCD [17]. About one in four honeybee–bumblebee venom and one in 10 yellow jacket venom allergics have been demonstrated to be anti-CCD IgE positive [18]. Whether these anti-CCD IgE antibodies have clinical relevance remains controversial [19–21]. Anyhow, it is obvious that the limitations, difficulties and pitfalls of serologic allergy diagnosis are magnified when dealing with issues of CCD [15, 16, 21–23]. Table 1 summarizes the cross-reactive structures relevant to this study.

In the first part of this study, we investigated the prevalence of sensitization against bromelain as a representative of $\alpha(1,3)$ -fucose and/or $\beta(1,2)$ -xylose bearing CCD in a group of patients allergic to different allergens (birch pollen, timothy grass pollen, house dust mite (HDM), cat, dog, NRL and hymenoptera venom anaphylaxis). Patients were considered as anti-CCD IgE positive when they had a negative skin prick test (SPT) and positive IgE for bromelain [16]. Bromelain is a protease isolated from pineapple that rarely induces allergic symptoms [10, 24–26]. In pollinosis, NRL anaphylaxis and hymenoptera venom allergy, the prevalence of anti-bromelain-type CCD IgE was compared with the sensitization rate for profilin assessed by IgE quantification for rBet v 2 (recombinant birch profilin). In NRL anaphylaxis and in

patients with triple pollen allergy (birch, timothy grass, mugwort), sensitization for rHev b 8 (recombinant *Hevea* profilin) was also studied.

In the second part of this study, the possible contribution of bromelain-type CCD, Bet v 1 (major birch pollen allergen) and profilin as a cause of false-positive, i.e. clinically irrelevant, specific IgE for NRL and apple was assessed.

Study population and methods

Study population

In order to investigate the prevalence and origin of anti-CCD and anti-profilin antibodies, the first part of the study was performed in patients allergic to pollen (isolated birch pollinosis ($n = 33$), isolated timothy grass pollinosis ($n = 17$), combined pollinosis ($n = 24$)), NRL ($n = 17$), HDM ($n = 19$) or pets (cat and/or dog) ($n = 7$). Patients were selected from a population of approximately 1000 patients. Diagnosis was based on a compelling history confirmed by a positive specific IgE and SPT for the relevant allergens. Forty patients with a clear history of isolated hymenoptera venom anaphylaxis corroborated by a positive venom IgE and skin test were also studied.

Anti-CCD and anti-profilin IgE antibodies containing sera demonstrate a broad spectrum of cross-reactivity including pollen, fruit, vegetables and hymenoptera venom. Therefore, in the second part of this study, we investigated the possible contribution of anti-bromelain-type CCD and anti-profilin antibodies as a cause for positivity of apple IgE and latex IgE. Apple IgE positive sera ($n = 82$) were classified as true-positive or false-positive according to the presence or absence of symptoms of an oral allergy syndrome (OAS) due to the consumption of the fruit. Latex IgE positive sera ($n = 38$) were considered as true-positive or false-positive according to the presence or absence of symptoms of NRL-induced anaphylaxis confirmed by a positive SPT. The presence of false-positive IgE for apple and latex in isolated hymenoptera anaphylaxis was studied separately.

Total and specific IgE

Quantification of total IgE and specific IgE to inhalant allergens (HDM, cat, dog, *Cladosporium herbarum*, *Phleum pratense*, *Artemisia vulgaris*, *Betula verrucosa*), *Hevea* latex, honeybee venom and yellow jacket venom, apple, rBet v 1 (recombinant major birch pollen allergen), rBet v 2 (recombinant birch profilin), rHev b 8 (recombinant *Hevea* profilin)

Table 1. Cross-reactive structures involved in the 'latex-fruit' syndrome and/or oral allergy syndrome

Type	Prototype	Examples
Peptidic	Major birch pollen allergen Profilin	Birch pollen (Bet v 1), apple (Mal d 1) Birch pollen (Bet v 2), timothy grass pollen (Phl p 12), mugwort pollen (Art v 4), <i>Hevea</i> latex (Hev b 8), apple (*)
CCD	$\beta(1,2)$ -xylose and $\alpha(1,3)$ -fucose residues $\alpha(1,3)$ -fucose residues	Ubiquitous in pollen and plant food (e.g. bromelain from pineapple) Phospholipase in hymenoptera venom (e.g. Api m 1 from honeybee)

*Not defined in IUIS Allergen Nomenclature. CCD, cross-reactive carbohydrate determinant.

and bromelain as a representative for CCD was performed according to the manufacturer's instructions (Immuno-CAP, Pharmacia Diagnostics, Brussels, Belgium). Due to insufficient serum, IgE for rBet v 2 could not be quantified in one patient with isolated venom anaphylaxis. Values of specific IgE below 0.35 kUa/L were considered to be negative.

IgE inhibition assays

IgE inhibition tests were carried out by incubating 50 µL serum of the patient with three different concentrations of bromelain (1, 10 and 100 µg; Sigma-Aldrich, St Louis, MO, USA), recombinant birch profilin (1, 10 and 100 µg; Biomay, Vienna, Austria) or buffer during 1 h at room temperature, followed by determination of allergen-specific IgE using the Immuno-CAP system.

Skin tests

Skin tests included HDM, cat, dog, *Cladosporium cladosporioides*, timothy grass, mugwort, birch (HAL, Haarlem Allergen Laboratories, Haarlem, The Netherlands), *Hevea* latex (Stallergènes, Fresnes, France), hymenoptera venoms (ALK-Abellø, Zaventem, Belgium) and bromelain (Sigma-Aldrich Chemic GmbH, Steinheim, Germany). The bromelain extract was adjusted to a protein concentration of 500 µg/mL in a phenol-containing buffer (HAL) and was prepared on a weekly basis. SPT were considered to be positive when weal reaction equalled or exceeded a diameter of 3 mm [27].

Statistical analysis

Results were expressed as median (range). The Mann-Whitney *U*-test was applied where appropriate. Pearson's χ^2 with Yates' continuity correction and Fischer's exact test were used to compare frequencies. Differences were considered significant at a *P*-value less than 0.05.

Results

Total serum IgE

Plant-allergic patients with a false-positive IgE for apple had a significantly higher total IgE compared with patients with a true-positive IgE for apple (respectively, 304 kU/L (26–7600) vs. 130 kU/L (13–2886), *P* = 0.04). No significant differences for median (range) of total IgE were observed between the different patient groups (data not shown).

Plant allergy: pollinosis and NRL anaphylaxis

As shown in Fig. 1, no bromelain IgE was found in patients with isolated NRL anaphylaxis or isolated birch pollinosis. In contrast, anti-bromelain IgE was present in patients with isolated timothy grass pollinosis and combined pollinosis (birch + timothy grass; birch + timothy grass + mugwort).

In patients sensitized to plant bromelain-type CCD, false-positive IgE for honeybee and/or yellow jacket venom was significantly more frequent as compared with plant-allergic

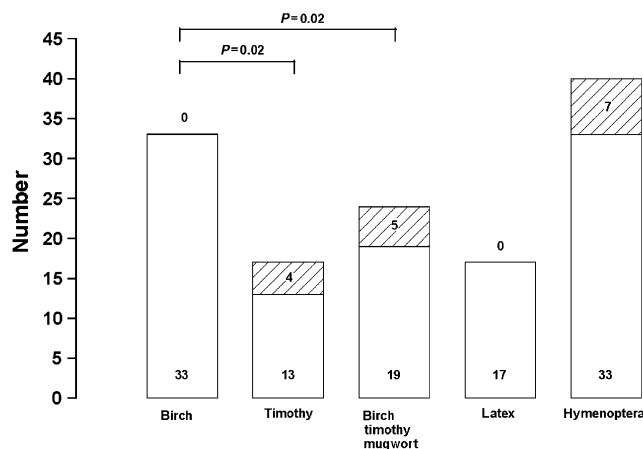


Fig. 1. Prevalence of sensitization to bromelain-type cross-reactive carbohydrate determinant (CCD) in isolated birch allergy (*n* = 33), timothy grass pollinosis (*n* = 17), combined pollinosis (*n* = 24), isolated natural rubber latex (*n* = 17) and isolated hymenoptera venom allergy (*n* = 40). Open bars denote CCD-negative patients, and hatched bars denote CCD-positive patients. Pearson's χ^2 with Yates' continuity correction and Fischer's exact test was performed and *P* < 0.05 was regarded as significant.

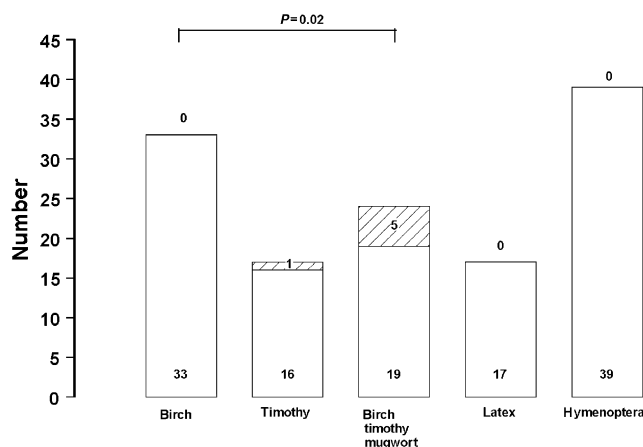


Fig. 2. Prevalence of sensitization to profilin (rBet v 2) in isolated birch allergy (*n* = 33), timothy grass pollinosis (*n* = 17), combined pollinosis (*n* = 24), isolated natural rubber latex (*n* = 17) and isolated hymenoptera venom allergy (*n* = 39). Open bars denote profilin-negative patients, and hatched bars denote profilin-positive patients. Pearson's χ^2 with Yates' continuity correction and Fischer's exact test was performed and *P* < 0.05 was regarded as significant.

patients without sensitization to plant CCD (respectively, 4/6 vs. 2/38, *P* = 0.003).

Sensitization to rBet v 1 (recombinant major birch pollen allergen) was restricted to the patients with birch pollinosis. No sensitization to this allergen was found in patients with isolated timothy grass pollinosis or NRL allergy.

As shown in Fig. 2, sensitization to rBet v 2 (recombinant birch profilin) was absent in isolated birch pollinosis, patients monoallergic to NRL, and restricted to 1/17 patients with isolated timothy grass pollinosis. In contrast, IgE for rBet v 2 was positive in 5/24 patients with combined pollen allergy (birch + timothy grass (1/16); birch + timothy grass + mugwort (4/7)). No sensitization to rHev b 8 (recombinant *Hevea* profilin) was found in isolated NRL anaphylaxis. However, the IgE for recombinant *Hevea* profilin was

Table 2. Numbers of positive IgE results (rBet v 1, rBet v 2 and bromelain) in patients with true- and false-positive IgE for apple

	Recombinant major birch pollen allergen (rBet v 1)	Birch profiling (rBet v 2)	Bromelain (BRO)	rBet v 1 + rBet v 2	rBet v 1 + BRO	rBet v 1 + rBet v 2 + BRO	None	Total positive
True positive (<i>n</i> = 51)	42	1	0	1	3	3	1	Bet v 1: 49; Bet v 2: 5; BRO: 6
False positive (<i>n</i> = 31)	14	1	3	2	4	6	1	Bet v 1: 26; Bet v 2: 9; BRO: 13

Remark: The combination rBet v 2 + BRO was not observed.

Table 3. Numbers of positive IgE results (rBet v 2 and bromelain) in patients with true- and false-positive IgE for NRL

	Bromelain (BRO)	Birch profiling (rBet v 2)	BRO + rBet v 2	None	Total positive
True positive (<i>n</i> = 17)	0	0	0	17	rBet v 2: 0; BRO: 0
False positive (<i>n</i> = 21)	8	2	9	2	rBet v 2: 11; BRO: 17

NRL, natural rubber latex.

positive in 4/7 patients with a triple pollen allergy, and values of the rHev b 8 and rBet v 2 IgE were almost comparable (data not shown).

In plant-allergic individuals with false-positive IgE for apple, the frequency of anti-bromelain-type CCD IgE was significantly higher than in patients with apple-mediated OAS (13/31 vs. 6/51, $P = 0.004$, Table 2).

In plant-allergic individuals with false-positive IgE for apple, the frequency of sensitization to rBet v 1 was comparable to patients with apple-mediated OAS (26/31 vs. 49/51, $P = 0.1$), whereas the frequency of anti-birch profilin IgE tended to be higher than in patients with apple-mediated OAS (9/31 vs. 5/51, $P = 0.05$, Table 2).

Plant-allergic patients with false-positive IgE for latex had a significant higher prevalence of anti-bromelain-type CCD IgE than NRL-allergic patients (17/21 vs. 0/17, $P < 0.00001$, Table 3).

Plant-allergic patients with false-positive IgE for latex had a significant higher prevalence of anti-birch profilin IgE than NRL allergic patients (11/21 vs. 0/17, $P = 0.0006$, Table 3).

Hymenoptera venom anaphylaxis

As shown in Fig. 1, anti-bromelain IgE was not restricted to plant allergy but was also observed in 7/40 patients with isolated venom anaphylaxis.

In patients sensitized to hymenoptera CCD false-positive IgE for at least one of the investigated pollen species was significantly more prevalent as compared with patients with hymenoptera anaphylaxis without sensitization to venom CCD (respectively, 5/7 vs. 0/33, $P = 0.00006$). Double positivity for honeybee and yellow jacket venom IgE was significantly more frequent in patients sensitized to venom CCD as compared with venom-allergic patients without sensitization for venom CCD (respectively, 6/7 vs. 4/33, $P = 0.0007$).

False-positive IgE values for apple or latex were not restricted to patients suffering from plant allergy (see above), but were also found in respectively four and five out of the seven patients sensitized to venom CCD. No false-positive IgE results for apple or latex were observed in hymenoptera anaphylaxis without sensitization to venom CCD.

HDM and pet allergy

No anti-bromelain IgE was found in patients allergic to HDM or pet allergens (data not shown).

Serologic cross-reactivity studied by means of IgE inhibition

IgE inhibition assays were performed with 11 sera with different recognition profiles of the two cross-reactive structures under investigation. NRL IgE inhibition experiments were performed with the sera of two patients with an isolated NRL allergy and seven patients without NRL allergy (five due to pollinosis, two due to isolated hymenoptera venom anaphylaxis). Apple IgE inhibition studies comprised three sera from patients with an OAS due to apple and six sera from patients without apple allergy (four due to pollinosis, two due to hymenoptera venom anaphylaxis). Inhibition exceeding 20% was considered to be positive. Bromelain inhibited clinically irrelevant NRL IgE results due to pollinosis in three out of five sera and in both sera from patients with isolated venom anaphylaxis (Fig. 3a). Bromelain also inhibited clinically irrelevant apple IgE results due to pollinosis in three out of four sera and both sera obtained from patients with isolated venom allergy (Fig. 3c).

Recombinant birch profilin inhibited clinically irrelevant NRL IgE results due to pollinosis in four out of five sera (Fig. 3b) as well as clinically irrelevant IgE apple IgE results due to pollinosis in two out of four sera (Fig. 3d). Recombinant birch profilin did not inhibit clinically irrelevant IgE results

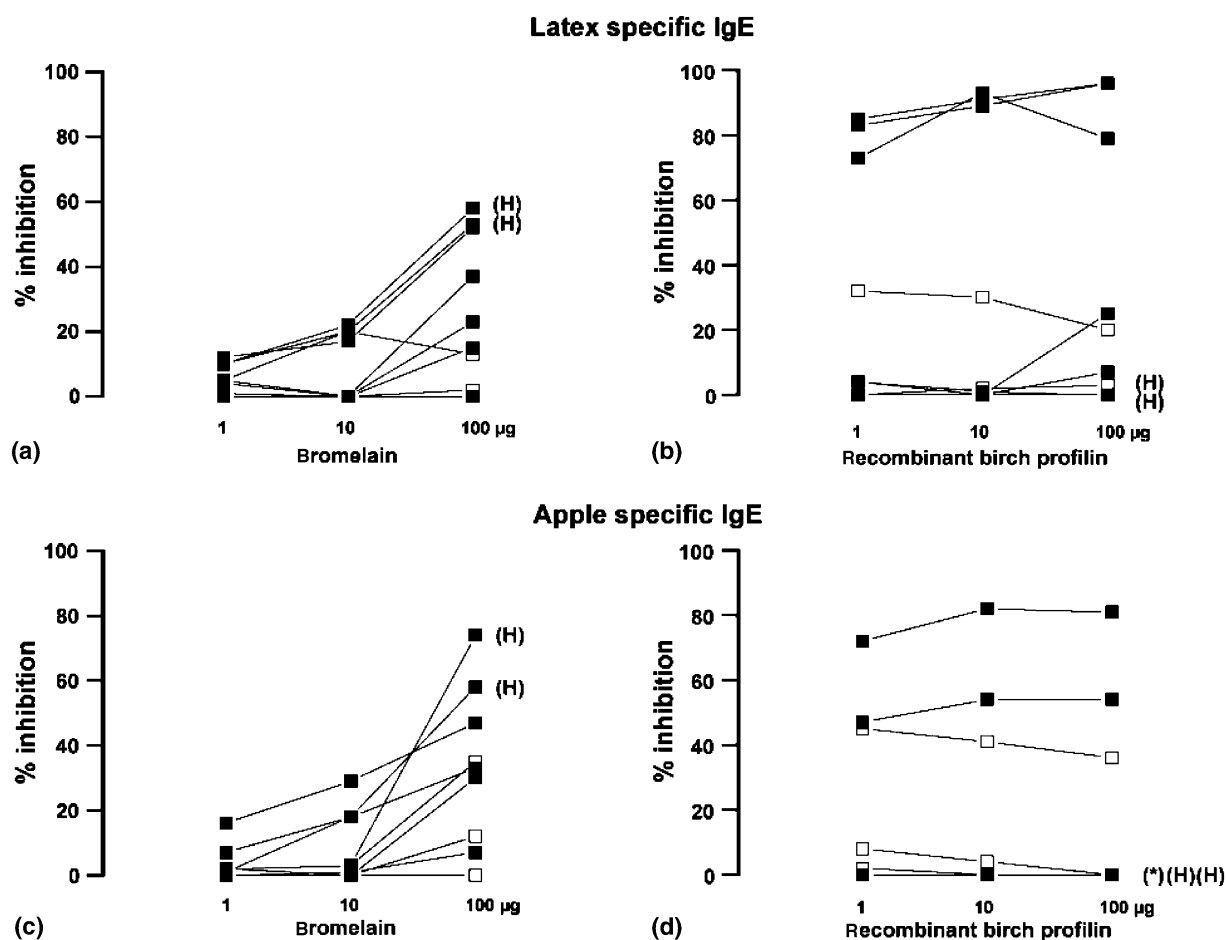


Fig. 3. Percentages of IgE inhibition. Inhibition of nine sera with natural rubber latex (NRL) IgE (clinically irrelevant (■), $n = 7$; clinically relevant (□), $n = 2$) with bromelain (panel a) and recombinant birch profilin (panel b). Inhibition of nine sera with apple IgE (clinically irrelevant (■), $n = 6$; clinically relevant (□), $n = 3$) with bromelain (panel c) and recombinant birch profilin (panel d). (H) denotes the samples containing clinically irrelevant NRL and apple IgE obtained from patients with hymenoptera venom anaphylaxis, whereas the remaining samples were drawn from patients with timothy grass and/or mugwort pollinosis. (*) Four samples that showed no inhibition are summarized by one curve.

for NRL or apple in the two patients sensitized to hymenoptera venom (Figs 3b and d).

Cat IgE was not inhibited by bromelain and recombinant birch pollen profilin (data not shown).

Discussion

Physicians frequently rely on *in vitro* quantification of specific IgE to confirm the diagnosis of IgE-mediated allergy. However, a positive specific IgE cannot always be translated in clinical allergy, and immunochemical cross-reactivity between homologous epitopes expressed in different allergens has been identified as one of the main reasons for clinical irrelevant IgE. The most likely explanation for the lack or poor clinical relevance of some of these cross-reactive IgE antibodies has to be sought in terms of epitope valency and low affinity. Ubiquitous structures such as profilins and CCDs present on glycoproteins of plants and hymenoptera venom have been recognized to elicit the syntheses of IgE antibodies that lack biologic activity and to hamper serologic diagnosis of allergic diseases. Moreover, as a result of their

widespread occurrence, CCDs are believed to elicit a very broad spectrum of cross-reactive IgE, comparable to, if not exceeding, those induced by profilins. Particularly carbohydrate determinants characterized by an $\alpha(1,3)$ -fucose attached to the proximal glucosamine residue and/or $\beta(1,2)$ -xylose residue attached to the β -mannose of the trisaccharide core are highly immunogenic in man. The primary aim of this study was to investigate the prevalence and origin of such bromelain-type CCD and profilin sensitization in patients monoallergic to different indoor and outdoor allergens, NRL and hymenoptera anaphylaxis. As no unique marker for bromelain-type CCD reactivity is available, a discrepancy between a negative SPT and positive IgE for the glycoprotein bromelain identified CCD sensitization. It should, however, be kept in mind that this assay does not cover the entire spectrum of anti-CCD antibodies [23, 28].

Our data confirm that sensitization against bromelain-type CCD generally originates from weed pollen, grass pollen or venom allergy. In fact, sensitization to timothy grass and mugwort pollen was found to elicit false-positive IgE for hymenoptera venom, and *vice versa*. Moreover, sensitization to pollen or venom CCD frequently resulted in triple positivity for birch, timothy grass and mugwort pollen IgE

and double positivity for honeybee and yellow jacket venom IgE. This might explain the considerable number of clinically irrelevant venom IgE antibodies observed in pollinosis [29]. Because clinically irrelevant IgE results may be misleading and negatively influence the patients' quality of life (e.g. avoidance measures, immunotherapy), prudence is called for the isolated use of IgE serology to identify the causative venom for specific immunotherapy. First, venom IgE might simply reflect sensitization to carbohydrate moieties of pollen glycoproteins, some of the patients not remembering ever been stung. Second, it should be kept in mind that true coexisting honeybee and yellow jacket is rare [18, 23].

Although birch pollen and NRL allergens bear potential cross-reactive $\alpha(1,3)$ -fucose and/or $\beta(1,2)$ -xylose containing CCD [30–32], no bromelain-type CCD sensitization could be detected in isolated birch pollen and NRL allergy. This probably reflects the fact that the major birch pollen allergen and the most relevant allergens in patients with occupational NRL allergy (Hev b 5 and Hev b 6) lack or have less abundant sugar moieties than the major allergens in grass and weed pollen and hymenoptera venom [10, 17, 33, 34].

In accordance with others [7, 35], our study confirms that IgE to recombinant birch profilin was absent in patients with isolated birch pollinosis. IgE to recombinant birch profilin was also rare in patients with isolated timothy grass pollinosis. These data correlate well with the observation of Wensing et al. [8], who recently found sensitization to natural profilin in only 2/11 patients monosensitized to birch or grass pollen. Although we could not define a group of patients monoallergic to weed pollen and the triple pollen group was too small for definite conclusions, our data seem to confirm that mugwort frequently induces sensitization to profilin [7, 8, 36, 37]. This presumption is further supported by the prevalence of profilin sensitization in patients suffering from pollinosis and a concomitant allergy for indoor allergens (HDM and/or pets). In this population, only 4/50 (8%) patients with birch and/or timothy grass pollinosis and 6/13 (46%) of the patients with triple pollen allergy had a positive IgE for recombinant birch profilin, respectively.

Sensitization to profilin was also absent in isolated NRL allergy. Both specific IgE for rBet v 2 (recombinant birch) and rHev b 8 (recombinant *Hevea* profilin) were negative in all patients with isolated NRL anaphylaxis. On the other hand, in triple pollen allergy, IgE values for recombinant *Hevea* profilin correlated well with IgE values for recombinant birch profilin. Taken together, these data support the assumption that profilin is an allergen of only minor clinical importance in occupational NRL allergy [38–41]. Moreover, sensitization to *Hevea* profilin rather reflects cross-reactivity to other plant profilins, e.g. weed pollen profilins.

Generally, sera containing anti-carbohydrate and anti-profilin IgE antibodies display a broad spectrum of cross-reactivity not restricted to pollen and venoms, but also involves fruit and vegetables [21, 22]. In addition, Fuchs et al. [31] found CCD to be a possible explanation for cross-sensitization between NRL and pollen. Moreover, recently carbohydrate epitopes in Hev b 2 (*Hevea* β -1,3-glucanase) have been suggested to participate in binding IgE antibodies from atopic patients without NRL allergy [32, 42]. Obviously, CCD must, to some extent, have contributed in the induction of clinically irrelevant plant food IgE in pollinosis [43] and in

the over-estimated prevalence of NRL allergy in some epidemiological studies [44].

Our study confirms that in our regions both the apple-mediated OAS and also clinical irrelevant IgE for apple are generally associated with birch pollinosis, with Bet v 1 as a dominant cross-reactive structure. Nevertheless, according to our inhibition experiments we believe that clinical irrelevant IgE for apple might, to some extent, also result from sensitization to pollen CCD and profilin.

In pollinosis, a significant majority of the clinical irrelevant NRL sera was confirmed to be positive for the bromelain and/or recombinant birch profilin IgE [45, 46]. It has to be stressed that one of these studies [45] was performed in central Spain, where birch trees are almost absent and pollinosis is mainly due to simultaneous sensitization to grass and olive tree (*Olea europaea*) [47]. In these regions, a higher prevalence of sensitization to profilin has been described [47, 48] and sensitization to CCD is more likely to occur, as the major olive pollen allergen (Ole e 1) is glycosylated, in contrast to the major birch pollen allergen [49]. Anyhow, in our population it seems that timothy grass through sensitization against $\alpha(1,3)$ -fucose and/or $\beta(1,2)$ -xylose carrying CCD, and mugwort pollinosis through sensitization against bromelain-type CCD and/or profilin, can be mimickers of NRL allergy, a presumption that is supported by inhibition of clinically irrelevant NRL IgE in patients suffering from pollinosis. From this observation, joined to the negative profilin and CCD IgE results in isolated NRL allergy, we anticipate that *in vitro* diagnosis of NRL allergy might improve by using extracts that lack bromelain-type CCD and profilin.

It is noteworthy that clinical irrelevant serology for apple and NRL was not restricted to patients with grass or weed pollinosis, but was also observed in patients with isolated hymenoptera venom anaphylaxis. In these patients, sensitization to venom CCD was frequently associated with a clinical irrelevant positive IgE for different pollen species (birch, timothy grass and mugwort), whereas quantification of specific IgE for the non-glycosylated recombinant birch pollen allergens remained negative (data not shown). As almost 95% of our patients with birch pollinosis are sensitized to Bet v 1, it is reasonable to believe that these data suggest recognition of carbohydrate epitopes present in the whole birch pollen extract but absent on the non-glycosylated recombinant allergens produced in *Escherichia coli*.

Obviously, clinical irrelevant IgE antibodies should preferably go undetected in serologic tests. Recently, addition of a protease-digested pollen extract as a soluble inhibitor during the serum incubation step of an IgE assay was proven to prevent IgE binding to carbohydrate moieties of solid phase-immobilized allergen [50]. Application of non-glycosylated recombinant proteins produced in *E. coli* and more functional tests, e.g. flow cytometric analysis of *in vitro* activated basophils, are alternative approaches to avoid the detection of anti-CCD IgE [46]. Routine application of such techniques, however, still awaits firm proof that anti-CCD IgE is indeed of negligible clinical relevance.

In summary, this study confirms that sensitization to non-mammalian $\alpha(1,3)$ -fucose and/or $\beta(1,2)$ -xylose bearing CCD and/or profilin should be considered in grass and weed pollinosis, as well as in hymenoptera venom anaphylaxis. In

addition, it was demonstrated that isolated birch pollinosis or NRL allergy did not induce sensitization to CCD and profilin, probably as their major allergens lack or have less abundant sugar moieties. *In vitro* diagnosis of apple and NRL allergy might benefit from extracts without profilin and CCD.

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