

# A Comprehensive Analysis of Middle-European Molecular Sensitization Profiles to Pollen Allergens

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## Key Words

Allergen sensitization · Epidemiology · Specific immunoglobulin E antibodies · Microarray · Molecular diagnostics · Pollens

## Abstract

Molecular diagnosis of allergy and microarray technology have opened a completely new avenue of insight into sensitization profiles from both the clinical and the epidemiological point of view. We used this innovative tool in the description of sensitization patterns in pollen-sensitized patients in Middle Europe. Immunoglobulin E detection using 112 different allergenic molecules was carried out employing the ImmunoCAP ISAC microarray system. Sera from 826 patients sensitized to at least one pollen-derived molecule were subjected to analysis. The highest observed sensitization rate was 81.0% to grass-specific molecules (the most frequent being Phl p 1; 69.6%). The second most frequent sensitization was 54.8% to Betulaceae-specific molecules (Bet v 1; 54.2%). Together, grasses and Betulaceae components (and their cosensitizations with other components) comprised the vast majority of pollen sensitizations. Unexpectedly frequently observed sensitizations were those to Cupressaceae-specific molecules (14.1%), Oleaceae-specific molecules (10.8%), and the plane tree-derived molecule Pla a 2 (15.5%). The sensitization rates for all other molecules were within the expected range (Art v 1, 13.6%; Pla l 1, 9.6%;

Che a 1, 8.4%; Par j 2, 0.9%; Amb a 1, 0.8%, and Sal k 1, 0.5%). Cross-reacting molecule sensitization rates were found to be 12.4% for profilins, 5.0% for polcalcins, and 6.4% for lipid transfer proteins. Molecular diagnosis of allergy gives a more precise and comprehensive insight into pollen sensitization patterns than extract-based testing, allowing a better understanding of the sensitization process and regional differences. The data presented here may help to improve the diagnostic and allergen-specific treatment procedures in the respective region.

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## Introduction

Type I allergy is a major health problem affecting more than a quarter of the population in industrialized countries, and pollen is an important cause of respiratory allergy in countries all over the world. While the diagnosis of immunoglobulin E (IgE)-mediated pollen allergy is primarily based on clinical history and sensitization demonstrated through an allergy prick test and/or measurement of serum-specific IgE, this methodology has its limitations.

In vitro and in vivo allergy testing is based on sometimes insufficiently standardized allergen extracts that, owing to the natural variability of the allergen source or manufacturing procedure, can differ in terms of their al-

lergenic content [1–3]. An even more important disadvantage of allergenic extracts is that they are incapable of differentiating between primary sensitization and immunological cross-reactivity. Nonetheless, natural allergenic extracts were the cornerstone of pollen allergy diagnosis until several years ago, when molecular diagnosis was made possible after advances in molecular biology led to the development of a large spectrum of purified natural and recombinant allergenic molecules. Such presently routinely available reagents enable the use of the diagnostic approach commonly known as component-resolved diagnosis of allergy, and now allow the systematic study of the principal allergens and cross-reactivity processes involved in allergic sensitization.

IgE sensitization profiles to complex allergenic sources are highly heterogeneous within the population. It seems that sensitization to pollen starts in childhood as a weak mono- or oligomolecular response that evolves through so-called molecular spreading, becoming stronger and polymolecular and usually resulting in clinical allergy [4]. Multiple positivities are observed in many patients, but determining whether the sensitization is species specific or a result of cross-reactivity to proteins with similar protein structures was formerly frequently impossible. The introduction of microarrays with a much larger number of purified or recombinant molecules constituted a further development in the diagnosis of allergic diseases. Such microarrays now represent powerful tools in the screening of serum IgE reactivity, and they epidemiologically allow the definition of sensitization profiles: identification of sensitizations and cosensitizations to species-specific and cross-reacting allergen components may be especially important in decisions concerning specific immunotherapy [5].

Our study used this new diagnostic tool for precise detection of the primary sensitizing allergen sources in subjects showing a sensitization to pollen. The aim of this study was to assess the usefulness of molecular diagnosis in the description of the pollen sensitization patterns of allergic patients living in a Middle-European region, with a special focus on discriminating between cross-reactivities and multiple sensitizations to pollen allergens. Although not much data on the diagnostic accuracy of the microarray ISAC in pollen allergy is available, we decided to use this approach because of the possibility it provides to analyze a wide spectrum of pollen-derived component sensitizations. Furthermore, some recent studies have shown similar performances for component-based microarray ISAC and whole-allergen CAP system detection, reporting both high sensitivity and specificity for pollen allergens [6, 7].

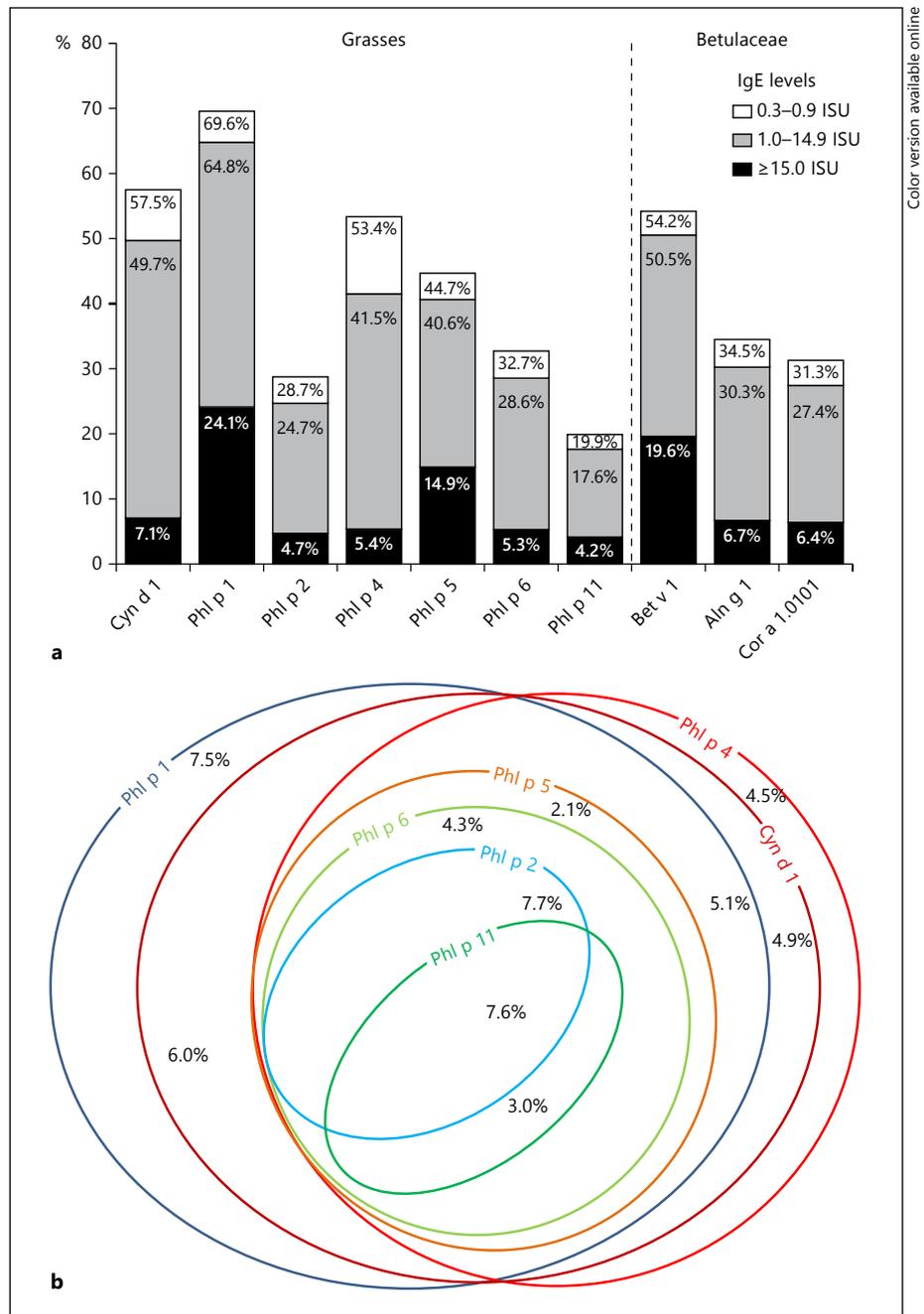
Specifically, we studied pollen-sensitized individuals in a population of patients living in the western part of the Czech Republic (presumably exposed to the same pollen allergens). To identify the dominating IgE-binding molecules and cross-reactivity patterns among them, we made use of a commercial microarray immunoassay of 112 purified natural and recombinant allergen molecules.

## Methods

This study was conducted using sera from patients from the region of Pilsen in the western part of the Czech Republic. We retrospectively analyzed sera from 1,331 patients who had been examined based on suspicion of allergy between December 2011 and June 2013 at the out-patient service of the Department of Immunology and Allergology of the University Hospital. Of the 956 samples positive to at least one allergen component, 826 samples were positive to at least one pollen-derived component and were subjected to further detailed analysis. This test group of 826 pollen-sensitive patients had at least one of the following diagnoses: chronic rhinitis (62%), bronchial asthma (32%), atopic dermatitis (27%), urticaria or edema (28%), and/or anaphylaxis (12%). Patient ages ranged from 2 to 68 years, with a mean age of 32.6 years. The sex ratio was 37.1% men to 62.9% women.

The detection of specific IgE to multiple allergen components was performed by means of the 112-component ImmunoCAP ISAC allergen microarray immunoassay (Thermo Fisher Scientific, Uppsala, Sweden). Briefly, microarray reaction sites were incubated with 20  $\mu$ l undiluted patient serum for 2 h to capture allergen-specific IgE antibodies by their corresponding allergen. Subsequently, the microarray slides were rinsed and washed to remove unbound sIgE. After drying, complexes of allergen-bound sIgE were stained with a secondary, fluorescence-labeled anti-human IgE for 1 h at room temperature while protected from light. After a second rinsing and washing procedure, the obtained fluorescence signals were scanned using a laser scanner (LuxScan 10K; CapitalBio, Beijing, China). Analysis of the corresponding digitized microarray images was performed using ImmunoCAP ISAC software, and image information was transformed into numerical data according to a reference serum of known IgE content. Results were expressed as ISAC standardized units (ISU). We did not consider the quantity of the detected specific IgE in further evaluations – results greater than or equal to 0.3 ISU/l were taken as positive [6].

The analysis was focused on pollen-specific allergen components and pollen-derived panallergens included in the ISAC system. Grass pollen allergy markers are represented in ISAC by the components nCyn d 1, rPhl p 1, rPhl p 2, nPhl p 4, rPhl p 5, rPhl p 6, and rPhl p 11. Betulaceae-pollen allergy markers are represented by rBet v 1, rAln g 1, and rCor a 1.0101. nCup a 1 and nCry j 1 represent Cupressaceae-pollen allergy markers; rOle e 1 and rOle e 9 represent Oleaceae-pollen allergy markers. Further species-specific allergy markers analyzed were rPla a 1 and nPla a 2 for plane tree, rPla l 1 for plantain, nAmb a 1 for ragweed, nArt v 1 for mugwort, rChe a 1 for goosefoot, rPar j 2 for wall pellitory, and nSal k 1 for saltwort. Finally, pollen-derived panallergens included profilins (rPhl p 12, rBet v 2, and rMer a 1), polcalcins (rPhl p 7 and rBet v 4), and lipid transfer proteins (LTPs) (nOle e 7, rPla a 3, and nArt v 3).



**Fig. 1. a** Sensitization rates to grass- and Betulaceae-specific molecules. **b** Venn diagram depicting mono- and cosensitizations to grass-specific molecules. Mono- and cosensitizations with a frequency of less than 2.0% are not shown.

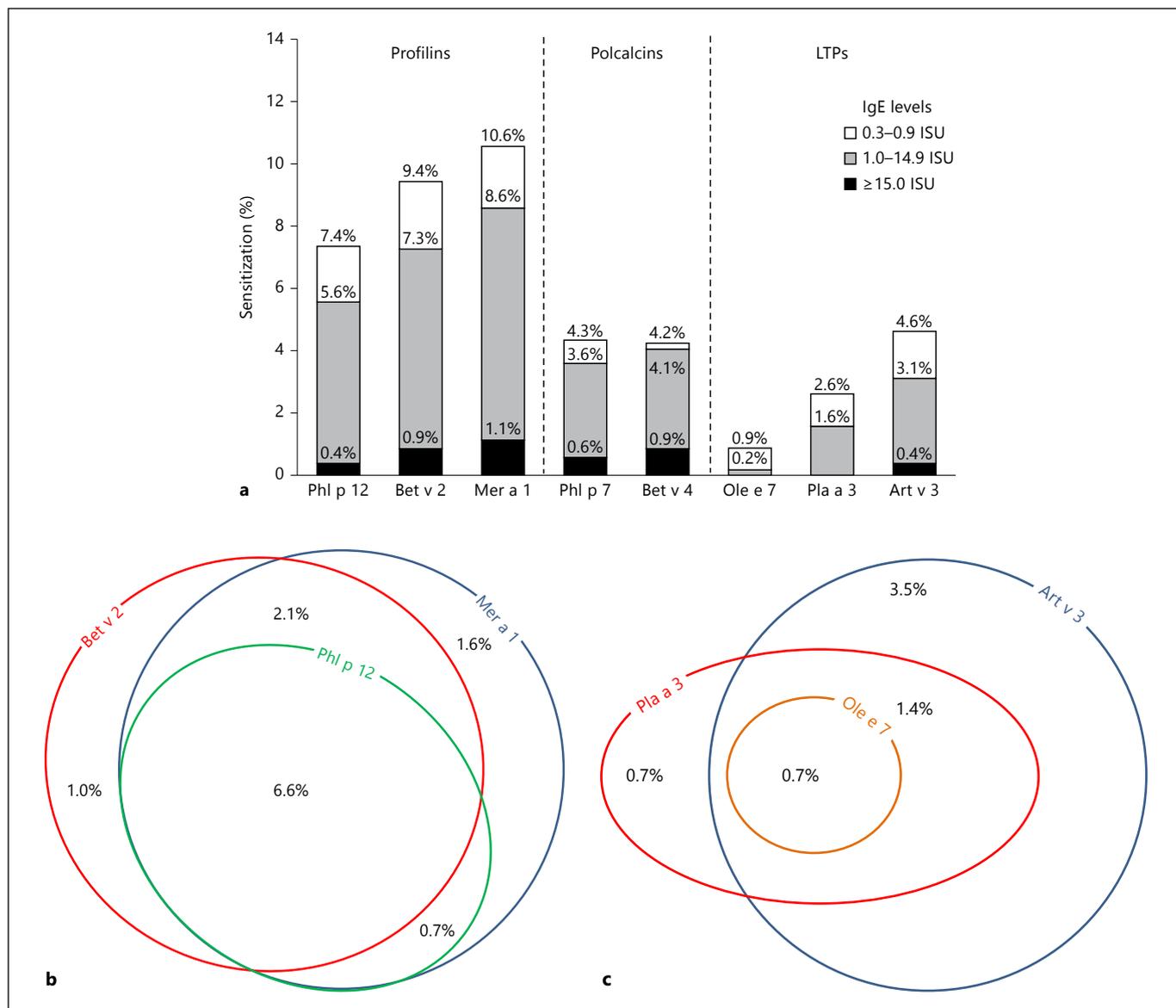
## Results

The results of the analysis describing the sensitization patterns in the group of 826 patients sensitized to at least one pollen-derived component in our region are listed below. Of these patients, 83.5% were also sensitized to at least one nonpollen allergen component. All percentages were calculated using the whole group of 826 patients.

### Grass Pollen Sensitization

Among all of the pollen-sensitized patients, the rate of sensitization to at least one grass-species-specific component reached 81.0%. The frequency of sensitization to individual grass-specific components and their combinations is shown in figure 1a and b.

The sensitization rate to profilin Phl p 12 was relatively low (fig. 2a) and was observed exclusively in patients reacting to at least one grass-specific component.



**Fig. 2.** **a** Sensitization rates to pollen-derived panallergens. **b** Venn diagram depicting mono- and cosensitizations to pollen-derived profilins. Mono- and co-sensitizations with a frequency of less

than 0.5% are not shown. **c** Venn diagram depicting mono- and cosensitizations to pollen-derived LTPs. Mono- and co-sensitizations with a frequency of less than 0.5% are not shown.

The sensitization rate to polcalcin Phl p 7 was also low (fig. 2a) and this sensitization without simultaneous co-sensitization to a grass-specific component was observed only exceptionally (in 0.4% of cases).

#### *Betulaceae* Pollen Sensitization

Among all pollen-sensitized patients in our cohort, the rate of sensitization to pollen-derived PR-10 proteins reached 54.8%. Figure 1a shows the observed frequencies of sensitization to individual *Betulaceae*-specific components.

The sensitization rates to profilin Bet v 2 and polcalcin Bet v 4 were relatively low (fig. 2a). In patients not sensitized to pollen-derived PR-10 proteins, sensitizations to profilin Bet v 2 or polcalcin Bet v 4 were observed only rarely, i.e. in 3.0 and 1.0% of cases, respectively.

Only a minority of patients sensitized to pollen-derived PR-10 proteins was not co-sensitized to at least one of the food-derived PR-10 proteins (4.8% of all pollen-sensitized patients, i.e. 8.7% of patients sensitized to Bet v 1 and/or Aln g 1 and/or Cor a 1.0101).



The sensitization rates to mugwort-specific components Art v 1 and LTP Art v 3 were 13.6 and 4.6%, respectively. Cosensitizations to Art v 1 and Art v 3 were present in 2.5% of the patients and no monosensitizations to Art v 3 were observed at all.

The sensitization rate to the plantain component Pla 1 was 9.6%, and that to the goosefoot component Che a 1 was 8.4%. Other pollen component sensitizations were more or less exceptional (Par j 2, 0.9%; Amb a 1, 0.8%, and Sal k 1, 0.5%).

The detected sensitization rate to the annual mercury profilin Mer a 1 was 10.6%, but no monosensitization to Mer a 1 was observed.

The global frequency of sensitization to pollen-derived profilins was 12.4%, to polcalcins it was 5.0%, and to LTPs it was 6.4%. Individual sensitization rates to pollen-derived panallergens and their combinations in pollen-sensitized patients are shown in fig. 2a–c. Cosensitization between polcalcins Phl p 7 and Bet v 4 was present in 3.5% of cases; sensitization to either Phl p 7 or Bet v 4 was present only in 0.8 and 0.7% of cases, respectively. Sensitization to either profilins or polcalcins or LTPs was observed in the majority of cases; cosensitizations among these groups of panallergens were rare (observed in 3.1% of cases).

The sensitization rate to the CCD component MUXF3 in pollen-sensitized patients was 6.5%, and this rate was similar to the result of another recent study [8]. Patients showing positivity to natural purified components were more frequently sensitized to MUXF3 than was the group as a whole – e.g. MUXF3 sensitivity was 2× higher in patients also sensitized to Cyn d 1, 1.4× higher for Phl p 4, 1.9× higher for Cup a 1, 1.9× higher for Pla a 2, and 1.7× higher for Art v 1. In any case, the proportion of MUXF3-positive patients among these subgroups was rather low, meaning that any possible false positivities due to CCD reactivity would not have considerably influenced the presented results.

A Venn diagram depicting mono- and cosensitizations to different groups of pollen-derived molecules is shown in figure 3b.

## Discussion

The sensitization rate to pollen-derived components observed in our patients is considerably higher than the rate of sensitization to non-pollen-derived components, underlining its clinical importance in the Middle-European region. It needs to be emphasized that in this paper we focus only on sensitization rates and not their

clinical relevance; carrying out the latter analysis without using specific provocation tests might become rather complicated due to the overlapping seasons of the certain pollens in our region.

Grasses (Poaceae) are the most common cause of hay fever in Europe, and it has been estimated that more than 40% of allergic patients are sensitized to their pollens [9, 10]. In our study, the rate was substantially higher – sensitization to at least one grass-pollen-specific component was observed in about three quarters of patients showing any positivity in the ImmunoCAP ISAC microarray. The sensitization rate to the first most frequent non-pollen-derived component Fel d 1 was 1.9× lower than to Phl p 1, and to the second most frequent, Der f 2, it was 2.4× lower than to Phl p 1, differing from data from other regions [11–13]. Among pollen sensitizations, the rate of reactivity to grass-derived components was also the highest, followed by sensitization to those derived from Betulaceae. Together, these two types of pollen components (and their cosensitizations with other components) represent the vast majority of pollen sensitizations in the Middle-European region.

The grass-pollen-sensitized patients in our study were usually cosensitized to several grass-specific components; monosensitization was markedly less frequent (fig. 1b). Phl p 1 ( $\beta$ -expansin) and Phl p 5 (ribonuclease) are generally assumed to be the specific components most commonly involved in grass pollen allergy [2, 5, 14–16]. We compared the grass pollen sensitization profiles of our patients with the results of studies from Italy [17–21] and confirmed the general finding that the most frequent targets for IgE binding are Phl p 1, Phl p 4, and Phl p 5 molecules; Phl p 5 sensitization without Phl p 1 sensitization was very exceptional, in concordance with other observations [22]. The observation of a nonnegligible rate of monosensitizations (in terms of grass pollen components) to Phl p 4 was a surprise – sensitization to this component is considered relatively unimportant in terms of eliciting clinically relevant allergy [23]. We have to emphasize, however, that it is probable that not all of our sensitized patients presented clinically relevant grass pollen allergy symptoms – clinical data were not analyzed in detail here. Surprisingly, a nonnegligible rate of isolated Cyn d 1 ( $\beta$ -expansin) + Phl p 4 (berberine bridge enzyme) cosensitization (without Phl p 1 sensitization and without CCD sensitization) – potentially indicating cosensitization to non-Pooideae grasses – was also observed; while this pattern is considered common in warmer climate areas [24], it was unexpected in our region. As Bermuda grass pollen is not present in our region, possible cross-

reactivity with  $\beta$ -expansins from other grasses could be the explanation.

In this context, it is necessary to stress the importance of the allergen content of grass pollen extracts used for diagnostic and therapeutic purposes. Several studies have focused on this issue and analyzed the composition of several commercially available preparations with respect to their qualitative and quantitative allergen compositions. One study analyzed only the Phl p 5 content [2], while other studies involved a wider spectrum of molecules including Phl p 1 and Phl p 4 [1, 3]. Unfortunately, considerable heterogeneity in the content of these major allergens was generally found. With regard to the sensitization patterns of Middle-European patients, it is necessary to point out the importance of quantifying not only the Phl p 5 content but also the Phl p 1 and possibly the Phl p 4 content of the preparations being used. Such information is crucial for effective diagnosis and treatment.

The most frequent sensitization to Betulaceae-derived components was, as expected, that to Bet v 1. The sensitization rates to Aln g 1 and Cor a 1.0101 were also rather high, but the vast majority of patients with these latter sensitivities cross-reacted to Bet v 1. Because of the known high cross-reactivity of the PR-10 components, the observed lower sensitization rates to Aln g 1 and Cor a 1.0101 in comparison to Bet v 1 might be only the result of a different sensitivity of the *in vitro* method for different allergen proteins. The cross-reactivity with plant-food-derived PR-10 proteins was also quite high in these same patients, presenting a pattern already generally known and described.

The relatively high sensitization rate for Cupressaceae-derived components found in our study was quite surprising for our region. The majority of patients were sensitized to the pectate lyase Cup a 1, and some were also cosensitized to Cry j 1, but monosensitization to Cry j 1 was exceptional. Cup a 1 is considered to be a diagnostic marker for primary sensitization to Cupressaceae pollen, which is not presumed to be frequent in the Middle-European region. Cross-sensitization with pectate lyases from other sources (e.g. mugwort or molds) may be questionable. The relatively high Cup a 1 sensitization rate suggests that its clinical relevance in Middle Europe should be reconsidered.

Ole e 1 is a specific marker for Oleaceae pollen sensitization. Positivity suggests authentic sensitization, although Ole e 1-like molecules are also present in grass (Phl p 11), goosefoot (Che a 1), plantain (Pla l 1), and saltwort pollen (Sal k 5). It seems, however, that the cross-reactivity of Ole e 1 with molecules outside the Oleaceae

family is limited and probably nonsignificant [25, 26]. The relatively high rate of sensitization to Oleaceae-specific components in our patients was due mostly to sensitization to Ole e 1, a finding concordant with other studies [27]. What was surprising, however, was the finding of a nonnegligible sensitization rate to  $\beta$ -glucanase Ole e 9, although the levels of sensitization were mostly low. Cosensitization between these two olive-related components (and also LTP Ole e 7) was only very exceptional, suggesting different sources of sensitization for each of them.

The source of sensitization for Ole e 1 in the Middle-European region is most probably cross-reactivity to ash due to Fra e 1, the homologous ash counterpart of Ole e 1. Ole e 7 (LTP) and Ole e 9 (1.3- $\beta$ -glucanase) are major olive allergens which commonly cause sensitivity in geographical areas exposed to high levels of olive pollen [25]; exceptionally, they can be the sole markers of sensitization to olive pollen seen in an individual [28]. Sensitization to LTP Ole e 7 may be associated with plant-derived food anaphylaxis especially to certain fruits [27], also suggesting a possible cross-sensitization. Finally, Ole e 9 shares some common epitopes with  $\beta$ -glucanases from birch and ash pollens, tomato, potato, pepper, banana, and latex [27, 29]. Exposure to such cross-reagents might be one possible explanation for the Ole e 9 sensitization in our patients, another being that the sensitization resulted from patients traveling to regions with high exposure to this pollen.

Positive specific IgE to Pla a 1 and Pla a 2 components may serve as a marker of primary sensitization to plane tree pollen [30, 31]. The finding of a relatively high sensitization rate to the plane tree pollen component Pla a 2 (polygalactouronase) in Middle Europe was also not expected. Cross-sensitization with polygalactouronases from other sources (e.g. grasses) may be questionable. The absence of sensitization to Pla a 1 is particularly surprising and as yet unexplained, although previous studies have revealed a markedly lower sensitivity to recombinant Pla a 1 compared to extract in skin prick and specific IgE testing [32]. Pla a 3 is a minor allergen that belongs to nonspecific LTPs with frequent cosensitization to plant-food LTPs [33], which is why sensitization to this component was not considered here as being specific for plane tree.

The nonnegligible sensitization rates to other species-specific pollen components from mugwort (Art v 1), plantain (Pla l 1), and goosefoot (Che a 1) were not surprising. We considered Art v 1 as a true major mugwort pollen allergen; it has previously been shown that it ac-

counts for IgE sensitization in approximately 80% of mugwort-pollen-allergic patients [34]. Due to conflicting data regarding the role of LTP Art v 3 as a primary sensitizer [24, 35, 36], this component was not taken into account here as a marker for mugwort sensitization even despite its nonnegligible sensitization rate (without Art v 1 cosensitization in about half of the cases).

The low sensitization rates to components from wall pellitory (Par j 2) and saltwort (Sal k 1), regarded as the most prevalent allergenic weeds in the Mediterranean, were expected in our region. The observed sensitization rate to ragweed component Amb a 1, though sometimes considered to be an emerging allergen in Middle Europe as well, was also low.

Our study showed that monosensitizations to all of the above mentioned components are rare. The majority of sensitizations occurred as cosensitizations with other pollen-derived components, of which grass pollen components were the most frequent, probably because their sensitization frequency is the highest.

Panallergens generally do not seem to represent very important pollen sensitization components in Middle Europe, somewhat in contrast to southern countries where the observed sensitization frequency is often higher [14, 28, 37] and the clinical relevance of this sensitization may also be more important [38, 39]. In our study, the highest observed sensitization rate was to profilins (fig. 2a). Previous studies have found no existing correlation between individual patients' levels of IgE response to specific profilins and the corresponding theoretical sensitizing source, suggesting that the result could be attributable to any profilin present in the patient's environment. This would support the use of most profilins as a common marker for polysensitization in component-resolved diagnosis [40, 41]. In contrast to these studies, regarding Phl p 12 and largely Bet v 2, our group of patients showed a certain relationship between sensitization to species-specific molecules and the respective profilin; the same

was also at least partially true for polcalcins Phl p 7 and Bet v 4. Cross-reactivity in the frame of groups of profilins, polcalcins, and LTPs was not complete (fig. 2b, c).

With regard to panallergens, the majority of sensitizations were cosensitizations with other components; monosensitizations to panallergens were very rare. Sensitization to other components from the same pollen source usually precede sensitization to profilins and/or polcalcins [17, 42] and it has been proposed that, at least for grass pollen allergy, panallergens are typically recognized at the late stage of molecular spreading. Such an assumption is in concordance with our observations of very low rates of monosensitization to profilins and polcalcins.

## Conclusion

Molecular diagnosis of allergy gives a more precise and comprehensive evaluation for an IgE-based epidemiology than does an extract-based approach. The geographical differences in sensitization patterns may reflect different ways of sensitization to the same allergen. The description of the pollen sensitization patterns of patients in a concrete region is important for estimating the frequency of clinically relevant allergy to various pollen species. The relatively high sensitization rate observed for Cupressaceae- and plane tree-derived molecules is surprising in the Middle-European region and calls for further analysis of its clinical relevance and possible cross-sensitization sources. The sensitization frequency to distinct components of each pollen species may be important when considering specific immunotherapy for allergy to this pollen and for optimizing the composition of the therapeutic allergen vaccines used in the respective region. Our data underline the necessity to focus not only on the Phl p 5 content but also on the Phl p 1 and possibly the Phl p 4 content in the diagnostic and therapeutic grass pollen extracts used in the Middle-European region.

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