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**ALLERGENICITY AT COMPONENT LEVEL OF SUBPOLLENS PARTICLES FROM DIFFERENT SOURCES
OBTAINED BY OSMOLAR SHOCK: A MOLECULAR APPROACH TO THUNDERSTORM-RELATED ASTHMA
OUTBREAKS**

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Key messages

1 Sub pollen particles obtained from pollens after osmotic shock retain allergenicity consistently with their involvement in thunderstorm asthma outbreaks

2 Multiplex ELISA inhibition test represents a new and simple tool for assessing the major allergens content of sub pollen particles

3 Phl p 2 and Phl p 5 are the allergens more concentrated in the grass SPPs and therefore are the candidate allergens for identifying patients at risk of thunderstorm asthma attacks

Abbreviations

TA: thunderstorm asthma

SPP = sub-pollen particle

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Ethical approval statement

The research was conducted ethically following the World Medical Association Declaration of Helsinki. All subjects have given their written informed consent and that the study protocol was approved by the institute's committee on human research. (CE: 495-1).

Availability of data declaration

The data that support the findings of this study are available from the corresponding author upon reasonable request.

CONFLICT OF INTEREST DECLARATION

Enrico SCALA has received consultant arrangements and speakers' bureau participation from Stallergenes and Thermo Fisher Scientific.

All other herewith declare the absence of any Conflict of Interest regarding the present study

ABSTRACT

Background

The so-called “thunderstorm asthma” (TA) is an uncommon but dramatic outbreak of asthma attacks occurring during a thunderstorm in the pollen and molds season. Mechanisms which make the pollen able to enter the deeper airways and provoke severe asthma symptoms are still unclear.

Objective

To test the hypothesis that sub pollen particles (SPPs) originated from the rupture by an osmotic shock of pollen associated with TA contain allergens

Methods

After hydration, SPPs released from pollen grains of grass, pellitory, olive, cypress, ragweed and birch were isolated and determined by microscopy. Allergens were determined by in vitro ELISA inhibition tests indirectly using the sera from 10 polyreactive patients. An inhibition < 50% was considered as negative, 50-75% moderate, > 75% complete.

Results

The inhibition experiments showed that the SPPs from birch and cypress were unable to inhibit serum IgE reactivity to Bet v 1 and Cup a 1, respectively. Ragweed SPPs inhibited ragweed pollen extract and Amb a 1 by $75.8 \pm 0.11\%$ and $81.2 \pm 0.15\%$ respectively. Olive and pellitory SPPs retained almost the whole IgE binding capability in all cases tested. Grass SPPs inhibited $32 \pm 0.06\%$ of *Lolium perenne* Lol p 1 and 65% of *Phleum pratense* extracts, but results were highly variable for individual allergens ($97.5 \pm 0.03\%$ for Phl p 2, $45.3 \pm 0.12\%$ for Phl p 5, $24.7 \pm 0.22\%$ for Phl p 6, and $38.3 \pm 0.2\%$ for Phl p 1).

Conclusions

Inhibition experiments confirm the hypothesis that SSPs obtained after the osmotic shock of pollen involved in TA, namely grass, pellitory and olive tree pollen, contain allergens and therefore they can induce severe asthma attacks during thunderstorms.

INTRODUCTION

The link between heavy springtime thunderstorms and severe asthma attacks in pollen-hypersensitive patients has been known for over three decades. After the first report from Birmingham, UK (1), episodes of thunderstorm-related asthma outbreaks have been reported from different parts of the world including Australia (2,3), USA and Canada (4, 5), Iran (6), and Europe (7-9). In most reported cases, grass pollen hypersensitivity has been associated with this condition (10,11), although more recently also hypersensitivity to other pollen sources including pellitory (*Parietaria spp*) (12) and olive (8) has been reported as a risk factor. Even hypersensitivity to fungal spores such as *Alternaria* or *Cladosporium* can be at the basis of thunderstorm-associated asthma outbreaks (13,14).

Generally, severe asthma attacks during thunderstorms occur at the beginning of the thunderstorm in patients that are outdoors and are not taking anti-allergic therapy (2,9). In fact, during the very first phases of a severe thunderstorm that eventually leads to an abatement of pollen levels in the atmosphere, pollen grains absorb water particles getting more and more hydrated and undergoing an osmotic shock leading to the release of allergenically active sub-pollen particles (SPPs) (0.5-5 μm in diameter) that are perfectly fitted to penetrate the small airways of allergic patients (15). Thus, the essential conditions favoring a possible asthma attack during thunderstorms include: (a) hypersensitivity to an airborne allergen source; (b) elevated pollen (or spores) counts at the beginning of the thunderstorm; (c) outdoor exposure and (d) lack of anti-allergic therapy (16).

In temperate climate areas the global warming that was recorded during the last 30 years, and more dramatically during the last decade, has led to conditions that favor thunderstorms to occur the whole year round and not only during springtime or summer as was the case in the last century (17-19). This causes a much larger spectrum of pollen species to be potentially exposed to the osmotic shock that characterises the formation of the sub-pollen allergenic particles responsible for thunderstorm asthma epidemics. Although it has been hypothesized that all allergenic pollens are potentially able to induce thunderstorm-related asthma attacks (9), to the best of our knowledge this has not been demonstrated scientifically, and so far data for only grass, pellitory, and olive pollen, and some moulds are available. In the present study, we

investigated at the molecular level the allergenicity of SPPs obtained by an osmotic shock from a spectrum of allergenic pollens to better understand which allergens are carried by SPPs and which groups of hypersensitive patients are really at risk for severe asthma attacks during thunderstorms.

MATERIALS AND METHODS

Isolation and analysis of sub-pollen particles

Subpollen particles (SPPs) released from pollen grains were isolated according to the protocol of Bacsi et al. (20) with minor modifications. The following allergenic species were considered: timothy grass (*Phleum pratense* L.), pellitory (*Parietaria officinalis*, L.), olive tree (*Olea europaea* L.), common ragweed (*Ambrosia artemisiifolia* L.), giant ragweed (*Ambrosia trifida* L.), birch (*Betula pendula* L.) and cypress (*Cupressus sempervirens* L.). In order to minimize the variability linked to pollen's age and geographical origin, for all plant species we used pollen collected by us in one single region (Lombardy) during the 2019 season. All pollens were stored under the same conditions (at room temperature with silica gel) until use, prepared and analyzed all together at the start of the study.

To allow the release of SPPs, pollen (0,1 g) was suspended in 2 ml of sterile bi-distilled water containing protease inhibitors (Protease Inhibitor Cocktail for general use, Sigma–Aldrich, St. Louis, MO, USA). After 10 minutes from hydration, intact grains were removed from the suspension by low-speed centrifugation (1500 g) for 5 minutes at room temperature. The supernatant was then filtered through a 6 µm mesh filter.

SPPs were collected from the pollen grain-free supernatant using centrifugation at 9000g for 15 minutes at room temperature and the pellet containing SPPs was re-suspended in 100 µl Milli-Q water containing protease inhibitors. The total protein concentration was assayed according to Bradford assay. In the final SPPs-containing solutions, the protein concentration ranged from 0.3 µg µl⁻¹ to 0.45 µg µl⁻¹ for all the taxa considered. The final solutions were then adjusted to a concentration of 0.3 µg µl⁻¹ for all the tested plants.

Each step was checked using microscopy and flow cytometry. In order to minimize the possible influence of pollen variability on inhibition tests, at least five independent samples were prepared for each pollen species; three of these were pooled and used for the incubation with allergic sera.

The percentage of SPPs-releasing pollen grains at successive times after hydration (5, 10, 15 and 20 minutes) and the size of the isolated SPPs for each allergenic species were estimated through of a Zeiss Axioplan light microscope connected to a Leica DC 300F camera for photo documentation.

To better define the size of SPPs, in addition to the measurements directly obtained by the microscope, image analysis was applied. The acquired digital images were processed by using the Image-Pro Plus program (Media Cybernetics, Silver Spring, MD, USA). The area of at least 300 SPPs for each of the five prepared samples for each species was measured and then converted to

the equivalent diameter $\left(Diameter = 2 \sqrt{\frac{Area}{\pi}} \right)$. (Table S1). SPPs were visualized both at 40X and 100X magnification, calibrating the procedure in both cases.

In vitro ELISA inhibition test

The residual allergenicity of the sub-pollen particles obtained by hydration was indirectly detected in vitro by ELISA inhibition tests using the sera from eight well-characterized allergic patients selected out from a database including about 70,000 samples previously evaluated for IgE reactivity to molecular allergens, representing the serum bank of the IDI-IRCCS institute. The specific IgE content of these sera was re-measured in three independent experiments by the first version (Experiment #1) and the second version (Experiment #2 and #3) of the commercially available Allergy Explorer-ALEX® (Macro-ArrayDX Wien, Austria)(21, 22), a CE certified platform containing both different allergen extracts and single components that are spotted onto a nitrocellulose membrane in a cartridge chip, before and after absorption with the SPPs obtained from the respective allergen sources. Briefly, 100 µl of serum were incubated overnight with an equal volume of PBS or of a solution containing the SPPs (at total protein concentration: 0.3 µg

μl^{-1}) from *Cupressus spp*, *Parietaria judaica*, *Betula pendula*, *Phleum pratense*, *Olea spp*, *Ambrosia trifida*, or *Ambrosia artemisiifolia*, respectively.

Samples and controls were then diluted 1:5 with the diluent containing a CCD (cross-reactive carbohydrate determinants) inhibitor provided by the manufacturer, able to guarantee a CCD inhibition of 85%. An amount of 0.5 mL of each sample was subsequently incubated on the chip under agitation. After two hours, the chips were washed 5 times, and a pre-titred dilution of anti-human IgE labelled with alkaline phosphatase was added. After 30 min of incubation and another cycle of extensive washing, the enzyme-substrate was added. After eight minutes, the reaction was stopped, the membranes dried, and a couple-charged device camera measured the intensity of the color reaction for each allergen spot. A dedicated software digitalized the images, preparing a report listing the allergen extracts and single molecules and their score in kUA/mL. All previous steps were carried out following carefully manufacturer's instructions.

We evaluated IgE reactivity to the following allergens:

- (i) *Olea europaea* (olive tree) extract and the respective major component Ole e 1; European ash (*Fraxinus excelsior*) extract and the respective major component Fra e 1; common privet (*Ligustrum vulgare*) extract and lilac (*Syringa vulgaris*) extract for the sample inhibited with *Olea spp* SPPs.
- (ii) *Phleum pratense* (timothy grass) extract and the respective components Phl p 1, Phl p 2, Phl p 5 0101, Phl p 6; *Lolium perenne* component Lol p 1, and the other grass extracts provided in the chip (*Zea mays*, *Secale cereale*, *Paspalum notatum*, *Phragmites communis* and *Sorghum halepense*) for the sample inhibited with *Phleum pratense* SPPs.
- (iii) *Ambrosia artemisiifolia* (short ragweed) extract and its respective components Amb a 1 and Amb a 4 for the sample inhibited with *Ambrosia trifida* or *Ambrosia artemisiifolia* SPPs.
- (iv) *Alnus glutinosa* (alder) extract and its respective components Aln g 1 and Aln g 4; *Betula* (birch) extract and its respective components Bet v 1, Bet v 2, and Bet v 6; *Corylus* (hazel tree) extract and its respective component Cor a 1 0103, ; *Fagus sylvatica* (beech) extract and its respective component Fag s 1, and *Quercus robur* (oak) extract for the sample inhibited with *Betula pendula* SPPs.

(v) Extracts from *Juniperus ashei*, *Cryptomeria japonica*, *Cupressus sempervirens*, and the *Cupressus arizonica* component Cup a 1 for the sample inhibited with *Cupressus* SPPs.

(vi) *Parietaria judaica* (pellitory) extract and its respective component Par j 2 for the sample inhibited with *Parietaria officinalis* SPPs.

The inhibition of profilin (Phl p 12 and Bet v 2) and polcalcin (Phl p 7 and Aln g 4) IgE reactivity by the SPPs was assessed for all sera as well.

To check the specificity of IgE inhibitions obtained, IgE reactivity of study sera to different allergen sources provided on the chip was used as control; in other words, the absence of any inhibition to allergens other than those directly involved in the specific experiment by SPPs was used to validate the experiment.

An inhibition < 50% was considered as negative, 50-75% moderate, > 75% complete.

RESULTS

Fig. 1 shows the mean percentage of SPPs-releasing-pollen grains upon hydration (T0) and after 5, 10, 15 and 20 minutes. On average, in all the considered species, the percentage of SPPs-releasing grains increased during the first 10 minutes and then remained constant (increases were not statistically significant, Fig.1A). The comparison of percentages after 10 minutes of hydration among the different species is reported in Fig.1B. *Betula pendula* showed the highest percentage of releasing grains (87%) followed by *Cupressus sp.* (51), *Phleum pratense* (40), *Olea sp.* (24), *Ambrosia artemisiifolia* (15). *Ambrosia trifida* and *Parietaria judaica* showed the lowest, not statistically different, values (9% and 8%, respectively).

SPPs isolated from the pollen of the different allergenic species (figure S1) after 10 minutes of hydration were analyzed for their size by image analysis and their allergenic potential by Macro-Array inhibition test. Table S1 shows the mean diameter of SPPs from different species that ranged from $1.9 \pm 1.1 \mu\text{m}$ to $3.8 \pm 1.1 \mu\text{m}$ whereas table 1 shows the IgE reactivity of six individual patients before and after inhibitions with SPPs from the different allergen sources.

Grass: grass sub-pollen particles retained an allergenic activity that varied from one allergenic component to another. While pre-absorption of sera with the osmotically obtained particles was able to inhibit IgE reactivity to the whole *Phleum pratense* pollen by 65%, for the single components Phl p 1, Phl p 2, Phl p 5, and Phl p 6 such proportions were $38,3\pm0,2\%$, $97,5\pm0,03\%$, $45,3\pm0,12\%$, and $24,7\pm0,22\%$, respectively (Table 1). Also, extracts from *Zea mays* (maize), *Secale cereale* (rye), *Paspalum notatum* (Bahia grass), *Phragmites communis*, *Sorghum halepense* (Johnson grass) and Lol p 1 from *Lolium perenne* (rye grass) were inhibited on average by $49,1\pm0,58\%$, $79,6\pm0,2\%$, $52,0\pm0,47\%$, $60,0\pm0,17\%$, 100%, and $31,8\pm0,06\%$, respectively (Table 1).

Parietaria: parietaria SPPs were able to inhibit IgE reactivity to both the whole pollen and the major allergen, Par j 2, by $94,1\pm0,05$ and $89,6\pm0,1$, respectively%. (Table 1)

Olive: olive SPPs were able to inhibit IgE reactivity to both whole pollen and major allergen, Ole e 1, by 100% and $91,2\pm0,1\%$, respectively. Interestingly, similar finding was obtained using other members of the Oleaceae family (namely, ash, privet, and syringe) as substrate. (Table 1).

Birch: birch pollen SPPs did not induce any significant inhibition of IgE reactivity to both whole pollen or the major allergen, Bet v 1, (19% and $12,6\pm0,22\%$, respectively) (Table 1). Similar results were found using whole hazel pollen and the major allergen Cor a 1, *Fagus sylvatica* and the major allergen Fag s 1, and *Alnus glutinosa* and the major allergen Aln g 1, as substrate (Table 1)

Ragweed: short ragweed SPPs exerted a strong inhibition of IgE reactivity to whole pollen and the major allergen, Amb a 1 ($75,8\pm0,1$ and $81,2\pm0,15\%$, respectively). Interestingly also the defensine Amb a 4 reactivity was inhibited by *Ambrosia artemisiifolia* SPP in a reactive subject (Table 1, Experiment #3). On the other hand, *Ambrosia trifida* (giant ragweed) SPPs was able to induce only a slight inhibition of IgE reactivity to short ragweed by one patient' serum.

Cypress: cypress SPPs did not exert any significant inhibition of IgE reactivity to both whole pollen (18%) and the major allergen, Cup a 1 (22%) (Table 1).

Panallergen inhibition: interestingly, both *Olea europea* and *Phleum pratense* SPPs inhibited profilin IgE recognition, while no other pollen was able to do it. On the other hand, polcalcin IgE reactivity was inhibited only by the grass SPPs.

DISCUSSION

In this study, we analyzed the allergenicity of SPPs obtained from six different sources by osmotic shock, thus mimicking the conditions leading to SPPs release during thunderstorms. The SPPs were employed to absorb the IgE of sera from hypersensitive patients and absorbed and non-absorbed sera were tested on a commercially available multiplex platform containing both extracts and allergen components of a large number of allergen sources including all major allergens of the study pollens (23-27).

The allergenic potential is an essential characteristic of SPPs to be considered to understand which groups of hypersensitive patients are really at risk for severe asthma attacks during thunderstorms. However, also the percentage of SPPs-releasing pollen grains should be considered as it determines the atmosphere concentration of SPPs contributing to modulate the risk of thunderstorm-related asthma outbreaks. Our study demonstrated that both the percentage of SPPs-releasing pollen grains and the SPPs allergenicity are species-specific features and that the combination of these two factors describes the asthma outbreaks reported so far.

Our experiments clearly showed that most birch and cypress pollen grains released SPPs but they did not retain any allergenic activity and were unable to inhibit serum IgE reactivity to Bet v 1 and Cup a 1, respectively. This is in agreement with the observation by Buters et al. showing that in outdoor air samples collected with a high-volume cascade impactor, 93% of Bet v 1 was found in the PM > 10 μ m fraction and any in the particles smaller than 2.5 μ m (28). No data are available on Cup s 1 cypress allergen, although the highly cross-reactive *Cupressus arizonica* allergen Cup a 1 was measured in SPPs in one study (29). It is possible that upon hydration Cup s 1 is freely-diffused from pollen and not released associated with SPPs. Anyway, so far there are no reports about thunderstorm-related asthma outbreaks due to birch and cypress, which seems to agree with the lack of SPP allergenicity we found in our experiment.

On the contrary, olive and pellitory SPPs retained the total IgE binding capability. However, the very low percentage of SPPs-releasing pollen grains in these species suggested a risk of severe asthma episodes limited to those situations combining thunderstorm with very high olive or

pellitory pollen concentrations. This could explain the few reports of thunderstorm-related asthma attacks for these species. Similarly, for ragweed, a low percentage of pollen grains releasing SPPs containing allergens was observed. These SPPs showed a partial allergenic activity leading to only limited inhibition of serum IgE reactivity to ragweed pollen extract and Amb a 1. A relatively low release of SPPs into the atmosphere during thunderstorm along with a retained partial allergenicity of SPPs might explain the reason why during the ragweed pollen season, thunderstorm asthma episodes have never been reported. Indeed, recent studies showed that the major allergen Amb a 1 is present in SPPs in vitro (30) and is detectable in ambient air in the PM fraction between 10 and 2,5 μm , even though at a significantly lower concentration than in the PM > 10 μm fraction. (31). Taken together, present and previous findings, obtained with different methods, are all consistent with the presence of Amb a 1 in SPPs but the low concentration of SPPs in the atmosphere upon thunderstorm might be not high enough to elicit symptoms in allergic patients. Besides, the geographical distribution of ragweed might also make the combination of environmental factors leading to thunderstorm asthma less probable. Regarding giant ragweed SPPs, their low inhibiting activity on short ragweed IgE reactivity is fully in keeping with the finding of a previous study showing a different allergenicity of the major allergens of the two plants (32).

The only investigated species showing both a consistent percentage of SPPs-releasing pollen grains and a consistent SPPs allergenicity was *Phleum pratense*. Notably, grass pollens are the main group for whom thunderstorm-related asthma outbreaks have been reported so far. Thus, the sole association of IgE-binding allergens to SPPs after the release, although essential, seems not sufficient to provoke a severe asthma attack during a thunderstorm. A quite high concentration of SPPs seems also necessary and the pollen features related to its ability in SPP releasing seem to play a fundamental role.

Anyway, a detailed analysis of SPPs-associated allergens helps to better identify the grass pollen allergic subjects that are at higher risk of thunderstorm-related asthma. While the results with both olive pollen extract and Ole e 1 and with pellitory pollen extract and Par j 2, consistently with previous molecular aerobiology studies (33,34), were unequivocal (100% inhibition) suggesting that the respective SPPs retained a whole allergenicity, the results of inhibition

studies carried out with grass SPPs were particularly interesting. Grass pollen is the only source among those studied here that shows several allergenic proteins that may sensitize allergic patients to a different extent, producing a large number of allergic profiles (35). The inhibition exerted by the SPPs on IgE reactivity to the whole pollen extract was relatively limited (38% for *Lolium perenne*, and 65% for *Phleum pratense*). In contrast, the inhibition obtained for the single components studied showed large variability, ranging from 100% for Phl p 2 to 45% for Phl p 5, 25% for Phl p 6, and 38% for the major allergen Phl p 1. Thus, Phl p 1 was virtually missing or present in very small amounts on the grass SPPs. Unfortunately, we were not able to investigate other grass pollen allergenic components such as Phl p 4 or Phl p 11. Phl p 5 concentration in ambient air was extensively studied in the EU funded multi-centric project HIALINE, where this allergen was detected in the PM fraction between 10 and 2,5 μm and was found to be correlated with relative humidity (36). While data on Phl p 5 is consistent with the present study findings, less is known about the content of the other grass allergens in SPPs. A study on Lol p 1, a Group 1 grass allergen with a high cross-reactivity and the same biological function (Beta-expansin) as Phl p 1, showed an inverse correlation between the allergen and relative humidity, supporting the hypothesis that the release of group 1 allergen from pollen after hydration, if any, occurs with a different dynamic (36).

If these results had to be transferred into the clinical practice, this might theoretically suggest that grass pollen allergic subjects are at higher risk of thunderstorm-related asthma if they are sensitized to Phl p 2, whereas patients sensitized to Phl p 1 and Phl p 5 are less at risk. As far as we know the component-resolved diagnosis of patients who experienced thunderstorm-associated asthma has not been carried out so far. This study suggests that this might be the case. If such studies confirm our results, it could be possible to predict which grass pollen allergic patients are at risk of severe asthma during thunderstorms.

Thunderstorm asthma epidemics are quite infrequent and so far, limited to some geographical areas but they often have a dramatic impact on public health. Indeed, many patients with severe, sometimes fatal, asthma attacks during thunderstorms did not have a history of asthma but only of allergic rhinitis. Present findings support the hypothesis that osmotic shock plays a critical role in the increase of species-specific SPPs-associated allergens in the air during thunderstorms and

that this is a key mechanism that makes pollen-derived allergens able to penetrate the small airways. On the other hand, the study suggests that the amount of particles released after hydration does not provide any information about their allergenicity, which depends on the pollen type. In other words, more SPPs does not necessarily mean more allergens released.

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Figure 1 Percentage of SPPs-releasing pollen grains in the different allergenic plant species upon hydration. (A) Percentage trends after 0, 5, 10- and 20-minutes hydration. (B) Mean percentages after 10 minutes of hydration. Different letters mean statistically different ($P < 0.05$).

Fig.1

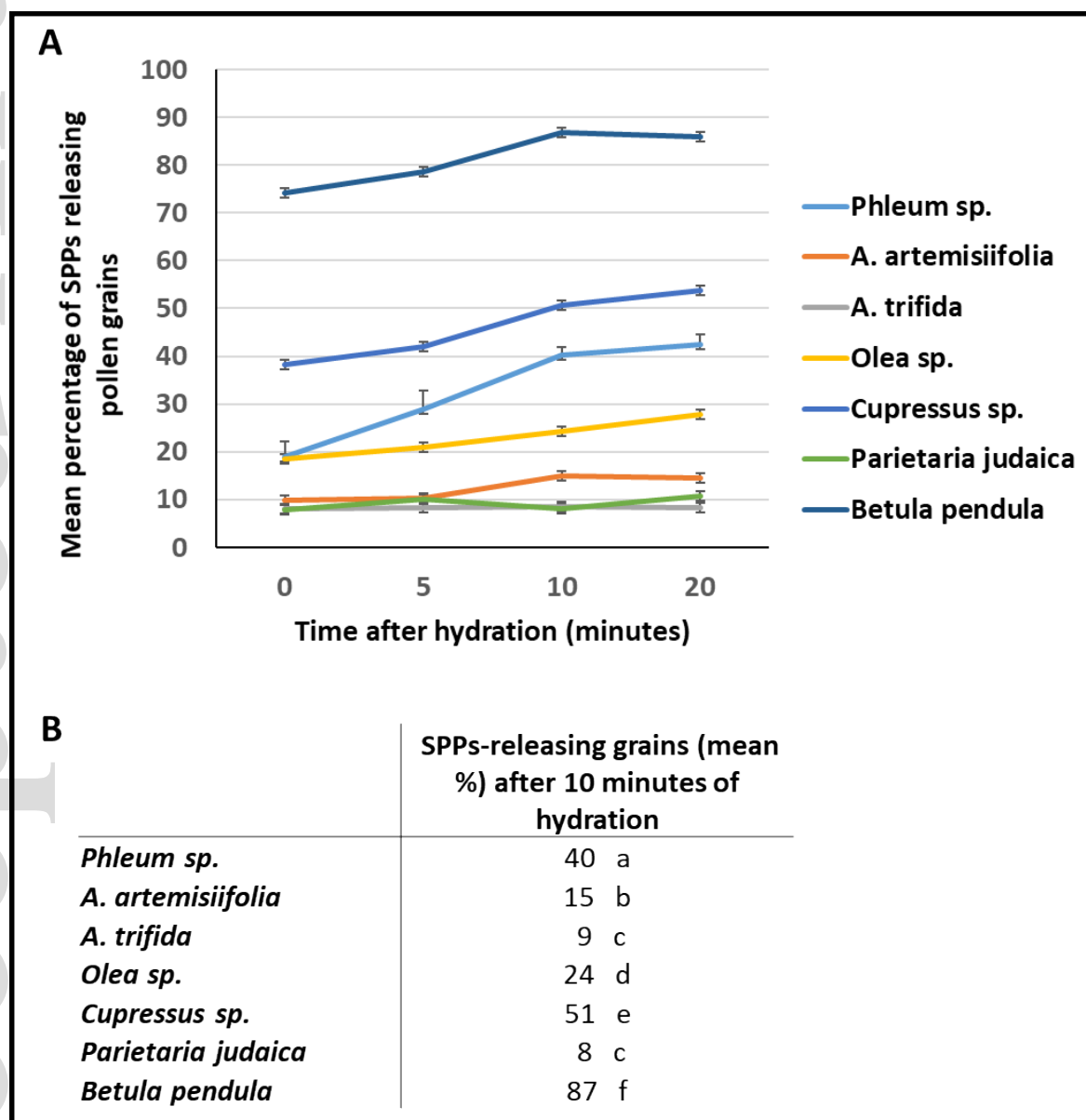


Table 1. Specific IgE reactivity before and after absorption with the sub-pollen particles of the respective allergen source.

SPP	Molecule	Biological Source (Biochemical designation)	Experiment #1			Experiment #2			Experiment #3		
			NI	I	% Inhibition	NI	I	% Inhibition	NI	I	% Inhibition
Olea sp	Fra e 1	<i>Fraxinus excelsior</i> (Ash)	11,04	<0,3	100,0%	0,83	<0,3	100,0%	0,32	<0,3	100,0%
		(Ole e 1-Family)	20,54	<0,3	100,0%	1,80	<0,3	100,0%	<0,3	<0,3	-
		<i>Ligustrum vulgare</i> (Common Privet)	4,23	<0,3	100,0%	nd	nd	nd	nd	nd	nd
	Ole e 1	<i>Olea europea</i> (Olive tree)	22,77	<0,3	100,0%	nd	nd	nd	nd	nd	nd
		(Ole e 1-Family)	21,04	0,3	98,6%	1,16	<0,3	100,0%	1,81	0,45	75,1%
	Ole e 2	(Profilin)	<0,3	<0,3	-	nd	nd	nd	nd	nd	nd
		<i>Syringa vulgaris</i> (Common lilac)	1,85	<0,3	100,0%	nd	nd	nd	nd	nd	nd
Phleum pratense		<i>Zea Mais</i>	6,58	0,62	90,6%	<0,3	<0,3	-	2,50	2,31	7,6%
		<i>Secale cereale</i> (Rye)	8,01	1,74	78,3%	0,47	<0,3	100,0%	10,34	4,09	60,4%
		<i>Paspalum Notatum</i>	1,89	<0,3	100,0%	0,34	0,35	0,0%	7,02	2,69	61,7%
		<i>Phragmites communis</i>	1,52	0,34	77,6%	0,74	0,42	43,2%	9,46	3,85	59,3%
		<i>Sorghum halepense</i>	0,99	<0,3	100,0%	nd	nd	nd	nd	nd	nd
	Lol p 1	<i>Lolium perenne</i> (Beta Expansin)	12,31	7,67	37,7%	4,57	3,40	25,6%	11,93	8,10	32,1%
		<i>Phleum pratense</i>	13,35	4,63	65,3%	nd	nd	nd	nd	nd	nd
	Phl p 1	(Beta-Expansin)	8,51	6,87	19,3%	6,28	3,77	40,0%	23,54	10,42	55,7%
	Phl p 2	(Expansin)	4,91	<0,3	100,0%	<0,3	<0,3	-	22,97	1,08	95,3%
	Phl p 5 0101	(grass Group 5/6)	20,13	8,09	59,8%	5,32	3,31	37,8%	24,10	14,88	38,3%
	Phl p 6	(grass Group 5/6)	5,13	3,49	32,0%	0,32	0,35	0,0%	4,93	2,86	42,0%
	Phl p 7	(Polcalcin)	0,37	<0,3	59,5%	<0,3	<0,3	-	<0,3	<0,3	-
	Phl p 12	(Profilin)	2,33	1,88	19,3%	1,05	<0,3	100,0%	<0,3	<0,3	-
Ambrosia artemisiifolia	Amb a 1	<i>Ambrosia artemisiifolia</i>	1,69	0,55	67,5%	5,06	0,57	88,7%	4,95	1,42	71,3%
		(Pectate Lyase)	4,55	1,35	70,3%	6,54	0,53	92,0%	<0,3	<0,3	-
	Amb a 4	(Defensin)	<0,3	<0,3	-	<0,3	<0,3	-	0,84	0,38	54,8%
Ambrosia trifida	Amb a 1	<i>Ambrosia artemisiifolia</i>	1,69	0,86	49,1%	5,06	2,45	51,6%	4,95	3,03	38,8%
		(Pectate Lyase)	4,55	3,55	22,0%	6,54	2,94	55,0%	<0,3	<0,3	-
	Amb a 4	(Defensin)	<0,3	<0,3	-	<0,3	0,00	-	0,84	0,83	0,0%
Betula pendula	Aln g 1	<i>Alnus glutinosa</i>	3,17	4,99	0,0%	nd	nd	nd	nd	nd	nd
		(PR-10)	7,8	7,03	9,9%	2,97	3,04	0,0%	4,29	4,50	0,0%
		(Polcalcin)	<0,3	<0,3	-	<0,3	<0,3	-	<0,3	<0,3	-
	Bet v 1	<i>Betula verrucosa</i>	11,35	9,18	19,1%	nd	nd	nd	nd	nd	nd
		(PR-10)	14,42	8,96	37,9%	9,29	9,97	0,0%	12,75	17,94	0,0%
		(Profilin)	6,93	7,51	0,0%	1,60	1,66	0,0%	<0,3	<0,3	-
	Bet v 6	(Isoflavon Reductase)	1,2	1,32	0,0%	<0,3	<0,3	-	<0,3	<0,3	-
		<i>Corylus avellana</i>	9,25	8,62	6,8%	5,96	6,12	0,0%	10,80	12,09	0,0%
	Cor a 1 0103	(PR-10)	9,29	8,09	12,9%	6,98	7,43	0,0%	19,25	22,18	0,0%
		<i>Fagus sylvatica</i>	1,43	1,39	2,8%	nd	nd	nd	nd	nd	nd
	Fag s 1	(PR-10)	nd	nd	nd	15,08	13,82	8,4%	6,70	9,00	0,0%
Cupressus sp	Cup a 1	<i>Quercus alba</i>	<0,3	<0,3	-	nd	nd	nd	nd	nd	nd
		<i>Juniperus ashei</i>	<0,3	<0,3	-	nd	nd	nd	nd	nd	nd
		<i>Cryptomeria japonica</i>	<0,3	<0,3	-	nd	nd	nd	nd	nd	nd
		<i>Cupressus arizonica</i>	<0,3	<0,3	-	nd	nd	nd	nd	nd	nd
Parietaria officinalis	Par j 2	(Pectate Lias)	11,14	9,11	18,2%	nd	nd	nd	nd	nd	nd
		<i>Parietaria judaica</i>	1,74	<0,3	100,0%	5,84	0,57	90,2%	31,85	2,59	92,0%
		nsLTP	6,76	<0,3	100,0%	7,64	1,09	85,7%	32,30	5,43	83,2%

NI: Not inhibited; I: After absorption with the sub-pollen particles of the respective allergen source. An inhibition <50% was considered as negative, 50-75% moderate (yellow), >75% complete (orange).. Exp#2 and 3 were carried out with the ALEX2 version

Table S1. Mean size of SPPs.

Sub-pollen particles (SPPs)	Mean size ± SD (diameter µm)
<i>Ambrosia artemisiifolia</i>	2.1 ± 1.2
<i>Ambrosia trifida</i>	2.8 ± 1.3
<i>Betula pendula</i>	3.5 ± 1.1
<i>Olea sp</i>	3.6 ± 1.7
<i>Cupressus sp</i>	2.9 ± 0.9
<i>Parietaria judaica</i>	1.9 ± 1.1
<i>Phleum pratense</i>	3.8 ± 1.1

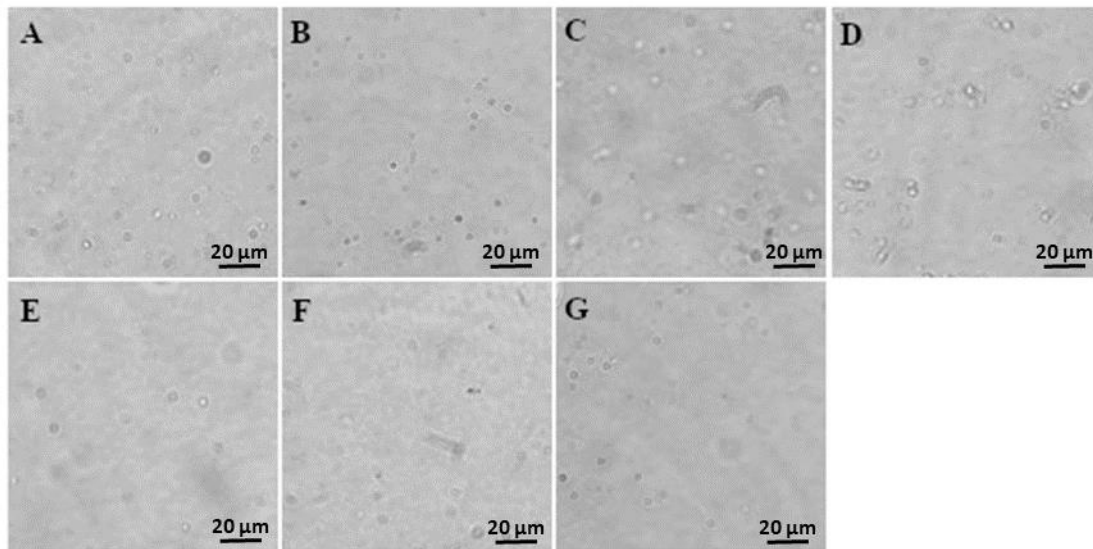


Figure S1

Representative pictures of SPPs - containing solutions obtained, with a Zeiss Axioplan light microscope connected to a Leica DC 300F camera (objective 40X), from:

- (A) common ragweed (*Ambrosia artemisiifolia* L),
- (B) cypress (*Cupressus sempervirens* L.),
- (C) timothy grass (*Phleum pratense* L.),
- (D) birch (*Betula pendula* L.),
- (E) giant ragweed (*Ambrosia trifida* L.),
- (F) olive tree (*Olea europaea* L.) and
- (G) pellitory (*Parietaria officinalis*, L.).