Original Article

Complementarity between Microarray and Immunoblot for the Comparative Evaluation of IgE Repertoire of French and Italian Cypress Pollen Allergic Patients

(*Cupressus sempervirens* / pollen allergens / immunoblot / allergen microarray)

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Abstract. Cypress pollen represents the primary cause of respiratory allergies in Mediterranean areas. Patients allergic to *Cupressus sempervirens* pollen (*Cups*) (CPA) can be discriminated on the basis of the immunoglobulin E (IgE) binding to a basic 14 kDa protein (BP14) or to high-molecular-weight (HMW) glycoproteins only. Specific IgE repertoires of two differentially exposed CPA cohorts, French and Italian, were investigated using an IgE microarray system (some known major allergens from several allergenic sources) and individual IgE immunoblotting (IB) of whole *Cups* pollen extract separated by SDS-PAGE (all allergens from one allergenic source: cypress pollen). The prevalence of sensitization to BP14 was higher in French (37 %) than in Italian patients (17 %) and major differences were observed in IgE reactivities to lipid transfer proteins (LTPs). Thirty percent of the Italian CPA (4 % in the French group) had specific IgE against the *Parietaria* pollen LTP, independently of IB subgroups. Regarding peach LTP sensitization, all Pru p 3+ Italian CPA (10 %) were in the HMW+ subgroup, while Pru p 3+ French CPA (20 %) were all included in the BP14+ subgroup. BP14 sensitization is likely a marker of *Cups* exposure and is, in French CPA, significantly correlated to Pru p 3 sensitization. The IgE immunoblot and microarray are complementary tools that highlight differences in the subtle sensitization profile between groups of patients in comparative studies.

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Abbreviations: 1-DE SDS-PAGE – one-dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis, 2-DE – two-dimensional gel electrophoresis, ANOVA – analysis of variance, BCIP – 5-bromo-4-chloro-3-indolyl phosphate, BP14 – 14 kDa basic protein, CPA – cypress pollen allergic patients, *Cryj – Cryptomeria japonica*, *Cupa – Cupressus arizonica*, *Cups – Cupressus sempervirens*, FEIA – fluorescence enzyme immunoassay, HMW – high molecular weight, IB – immunoblotting, IgE – immunoglobulin E, ISAC® – Immuno Solid-phase Allergen Chip, IUIS – International Union of Immunological Societies, LTP – lipid transfer protein, Mr – relative molecular mass, NBT – nitro blue tetrazolium, NCa – cyanogen bromide-activated nitrocellulose sheet, PBS – phosphate-buffered saline, Tw – Tween 20.

Introduction

Cypress pollen is considered the primary cause of rhino-conjunctivitis and allergic asthma in areas surrounding the Mediterranean basin (Boutin-Forzano et al., 2005). The cypress, *largo sensu*, belongs to the Cupressaceae family in which four species have been studied in detail concerning their high allergenicity: *Cupressus arizonica* (*Cupa*, Arizona cypress), *Cupressus sempervirens* (*Cups*, Italian cypress), *Juniperus ashei* (mountain cedar) and *Cryptomeria japonica* (*Cryj*, Ja-

panese cedar) responsible for well-studied allergic disorders in Japan (Okamoto et al., 2009). A study performed in 23,077 Italian allergic patients using the Immuno Solid-phase Allergen Chip (ISAC® Uppsala, Sweden) microarray technique showed that among 75 allergens, Cup a 1 (the major allergen of *Cupa*) exhibited the highest prevalence of immunoglobulin E (IgE) sensitization (about 43 %) (Scala et al., 2010).

Proteins belonging to pectate lyase, polygalacturonase, thaumatin-like protein and Ca-binding protein families currently constitute groups 1 to 4 of cypress cedar allergens, respectively (www.allergen.org or www.allergome.org). In *Cups* pollen, only two allergens are officially classified in the data bank of the International Union of Immunological Societies (IUIS). The pectate lyase Cup s 1 represents the major allergen and is highly cross-reactive with Cup a 1 and Cry j 1 (Arilla et al., 2004), whereas Cup s 3, identified using cDNA cloning and homology sequence analysis, seems to be absent or poorly expressed in *Cups* pollen grains (Togawa et al., 2006). Based on inhibition assays and specific IgE cross-reactivity, *Cups* pollen extracts were also suspected to contain other allergenic components belonging to the profilin and β-galactosidase protein families (Barderas et al., 2004; Bistoni et al., 2005).

We have recently reported that the use of detergent, chaotropic agents or saline conditions can solubilize additional allergens from the *Cups* pollen (Shahali et al., 2010). Furthermore, proteomic analysis using combinatorial peptide ligand libraries as selective purification techniques of extracted pollen proteins led to the description of 10 unreported allergens (Shahali et al., 2012a). In particular, we identified, with > 65 % coverage in the protein sequence, the polygalacturonase of *Cupressus sempervirens* (putative Cup s 2) (data not shown, Shahali et al., 2012b) and we showed that the IgE reactivity to a 14 kDa basic allergen (BP14) in *Cups* pollen extracts could discriminate between two types of cypress pollen allergic patients (CPA). A first set of patients displayed heterogeneous IgE reactivity to high-molecular-weight (HMW, > 30kDa) allergenic glycoproteins, while specific IgE of a second set of patients mainly bound to BP14 (Shahali et al., 2012c).

The diagnosis of type 1 hypersensitivity is mainly based upon an evocative anamnesis and clinical history and *in vivo* skin prick tests. Then, *in vitro* detection of specific IgE against sensitizing molecular allergens can be performed using either commercial procedures in single or multi-array (Mari et al., 2010) or immunoprint after separation of proteins from the raw extract by 1- or 2-dimensional electrophoresis (Le Mao et al., 1998; Poncet et al., 2010).

The aim of this study was to compare the IgE repertoire of CPA from France and Italy using, on one side, immunoblots with a *Cups* pollen extract and, on the other side, IgE microarray assay. Total IgE content as well as specific reactivity towards a commercial *Cups* extract in classical immunoCAP (t23) assay were also evaluated. The results showed that the two analytical methods are complementary and suggest that co-sensitization and/or cross-reactivity and exposure play a role in shaping the subtle IgE repertoire in cypress pollen allergic patients.

Material and Methods

Patient sera

This study was approved by the institutional ethical committees (approval numbers: 2011-A00211-40 and 106-CE-2005) and written informed consent was obtained from the patients. Fifty-one CPA from Marseille, France, and 30 CPA from Rome, Italy, were selected according to their clinical symptoms (rhinitis, conjunctivitis and asthma) during the cypress pollinating season. The mean age of the French cohort was 40.3 years (range 10–74) in a group of 28 females and 23 males, and the mean age of the Italian cohort was 32.7 years (range 11–62) in a group of 16 females and 14 males. For each immunoblot analysis, the serum from a healthy individual was used as a negative control.

Cypress pollen protein extractions

Cups pollen was supplied by Allergon AB (Angelholm, Sweden). One hundred mg of pollen was incubated for 18 h in a buffer containing 4% SDS. The pollen suspension was then centrifuged at 18,000 *g* for 20 min at 4 °C, as previously described (Shahali et al., 2010). The supernatant was collected and stored in aliquots at -20 °C until use. The protein concentration in supernatants was measured with Bradford protein assay (Pierce, Thermo Fischer Scientific, Rockford, IL) in samples diluted to reduce the concentration of SDS < 0.1% according to the manufacturers' recommendations in order to prevent interferences. Bovine serum albumin, used for the protein calibration curve, was diluted in 0.1% SDS solution.

One-dimensional gel electrophoresis

For 1-DE SDS-PAGE separation, extracted proteins in 38 mmol.l^{-1} Tris buffer pH 6.8 containing 4% (w/v) SDS were applied to a thin 8–18% gradient polyacrylamide gel (ExcelGel, GE Healthcare, Uppsala, Sweden) and run in a flat-bed electrophoretic chamber (Multiphor II, GE Healthcare) at 12 °C. The gel was then either transferred onto a cyanogen bromide-activated nitrocellulose (NCa, Demeulemester et al., 1987) sheet (Optitran®BA-S 83, Schleicher and Schuell, Dassel, Germany) for Western blotting assays or stained for detection of the separated proteins. Molecular mass (Mr) markers (GE Healthcare) ranging from 14.4 to 94 kDa were used as comparative references.

Immunoblotting

Electroblotting of separated proteins was performed onto NCa sheets with a semidry Novablot apparatus (LKB, Uppsala, Sweden) following the manufacturer's instructions (1 h, 1 mA/cm^2). The membranes were then dried and blocked with PBS containing 0.3% (v/v)

Tween 20 (Sigma-Aldrich St Louis, MO) (PBS-Tw) for 1 h at 20 °C. For 1-DE screening, each NCa was then cut in 2.5 mm wide strips that were individually incubated with 1 : 10 diluted patient or control sera in PBS-Tw 0.1% (overnight at 20 °C). Each membrane was washed three times for 10 min in PBS-Tw 0.1 % (v/v) and incubated during 2 h at 20 °C with 1 : 700 dilution of alkaline phosphatase (AP)-conjugated goat anti-human IgE (Sigma-Aldrich). The AP activity was detected using 5-bromo-4-chloro-3-indolyl phosphate (BCIP, Sigma-Aldrich) and nitro blue tetrazolium (NBT, Sigma-Aldrich) in 0.1 mol.l $^{-1}$ Tris acetate buffer pH 9.5.

Total and specific IgE evaluation

Total IgE levels were assayed by nephelemetry (BNII, Siemens, Marburg, Germany) and expressed in IU.ml^{-1}. Specific IgE to cypress allergen (*Cupressus sempervirens*, reference code t23) were quantified with the widely used fluorescence enzyme immunoassay (FEIA) ImmunoCAP® (Thermo Fischer Scientific) in the ImmunoCAP 250 apparatus, as recommended by the manufacturer. The detection limit was 0.10 kU.l^{-1}. Specific IgE evaluation was performed in 46 French and 30 Italian CPA using the commercial allergen microarray ImmunoCAP ISAC® 103 according to the manufacturer's instructions. The raw data were expressed as ISAC standardized units (ISU) and levels above 0.1 ISU were considered positive.

Statistical analysis

The differences in variables were evaluated using the analysis of variance (ANOVA) and Fisher's exact test.

Results

Immunoblot: IgE sensitization to BP14 is lower in prevalence in Italian patients

Sera from French (N = 51) and Italian (N = 30) patients were screened by 1-D immunoblots using SDS *Cups* pollen extract. In agreement with our previous results, three IgE reactivity patterns were observed. IgE from some patient sera bound BP14 with or without reactivity to HMW allergens (19 in French patients, i.e. 37 %, and five in Italian patients, i.e. 17 %, see lines "Immunoblot" in Table 1). IgE from other patient sera only bound to HMW and not to BP14 (41 % and 60 % of French and Italian CPA, respectively), and the third category was negative for all proteins of the extract (22 % of the French patients and 23 % of the Italian cohort). The results of three representative sera for BP14 and HMW subgroups and one for the negative subgroup are depicted in Fig. 1 for French and Italian patients (Fig. 1A and 1B, respectively). These three groups will be referred to as immunoblot (IB) subgroups, BP14+, HMW+ and Neg in the rest of the study.

Table 1. IgE reactivity of French and Italian cohorts to allergens or specific extract from Cupressus sempervirens using different techniques

Technique	Cypress allergen or extract	French$_a$	Italian$_a$
Microarray	Cup a 1	91	100
Microarray	Cry j 1	85	100
FEIA$_b$	Cup s (t23)	89	100
Immunoblot	Cup s HMW	41	60
Immunoblot	Cup s BP14	37	17

$_a$ Results are expressed in % positive patient serum
$_b$ Fluorescence enzyme immunoassay

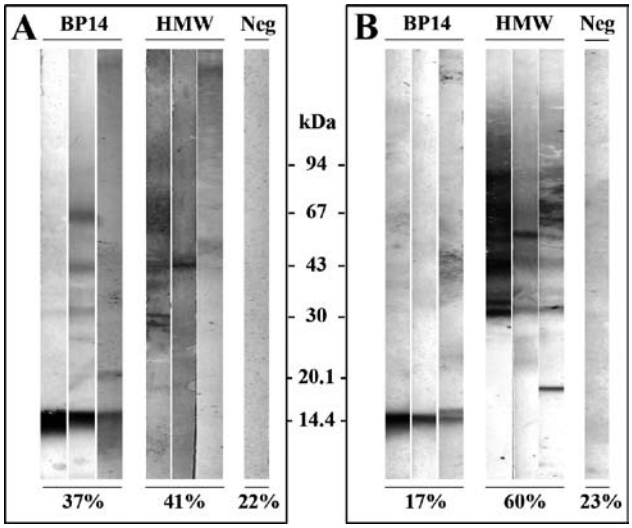

Fig. 1. Three immunoblot patterns obtained with representative patients' sera. An SDS protein extract of *Cupressus sempervirens* pollen separated in SDS-PAGE was blotted onto an NCa membrane. Each strip was individually incubated with sera from French (N = 51) and Italian (N = 30) CPA. Three representative patterns for BP14 and HMW and one for negative (Neg) sera are shown for French (**A**) and Italian (**B**) CPA. Percentages in each subgroup and molecular mass (kDa) are indicated.

Microarray: a minority of cypress allergic patients are monosensitized

IgE immunoreactivity results are separately represented in Fig. 2 for French patients and in Fig. 3 for Italian patients. Demographic and clinical characteristics are given on the left sides of Figs. 2 and 3 (the corresponding letter codes for molecular allergens and extracts are presented in Table 2) and specific global percent reactivity to cypress allergens is shown in Table 1. Most of the patients exhibited rhinoconjunctivitis in both cohorts and, independently of the IB subgroups, asthma was also observed in 35 % of French and 10 % of Italian CPA. With regard to microarray results (right side of both Figs. 2 and 3), the proportion of monosensitized versus polysensitized CPA was quite low both in French (13 %) and Italian (10 %) patients. The proportion of pollen-polysensitized patients was similar in French and Italian cohorts, 17 % versus 20 %, respec-

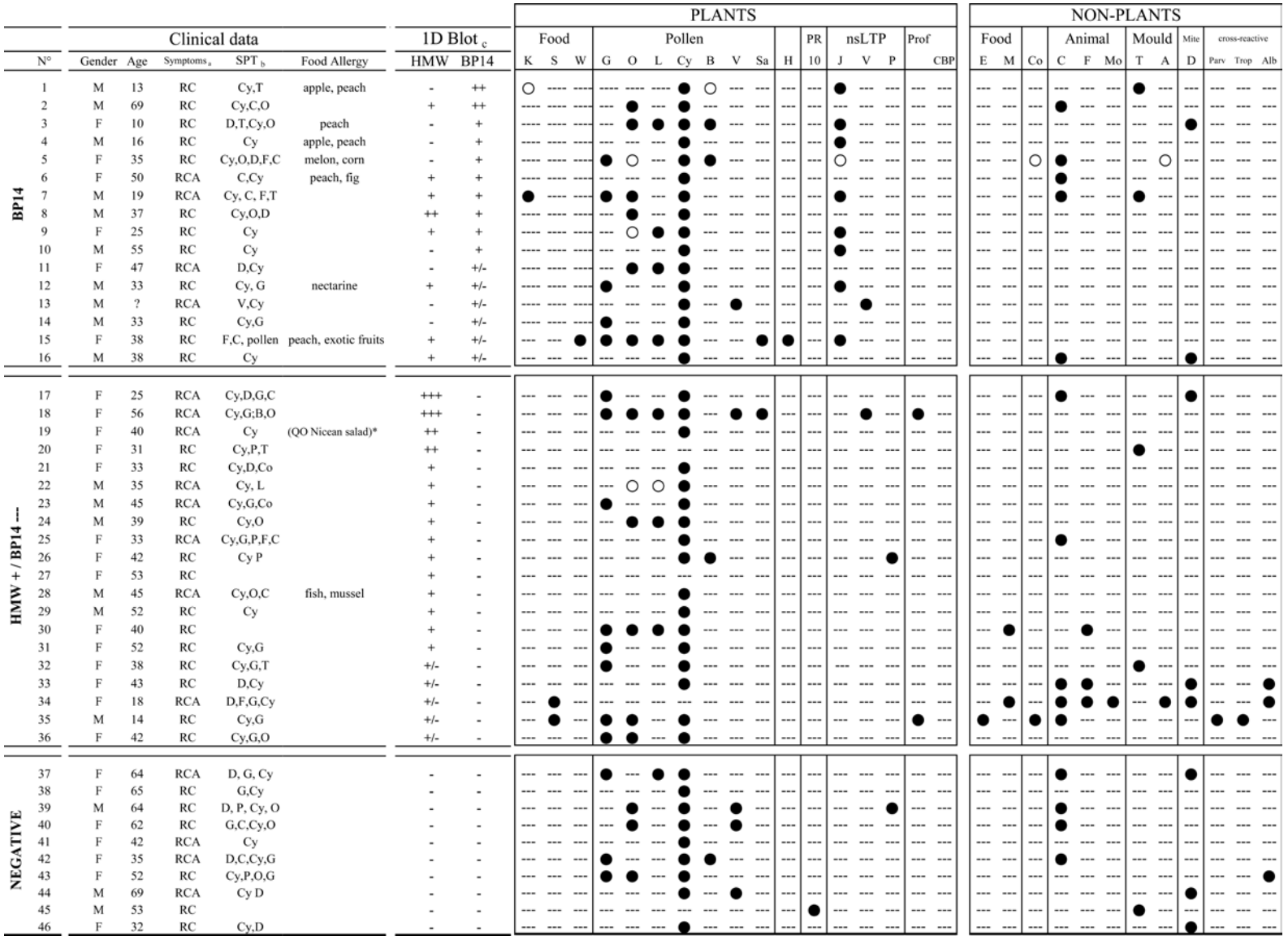

Fig. 2. Clinical and biological data of 46 French CPA

a – symptoms included: R: rhinitis; C: conjunctivitis; A: asthma. b – SPT: Skin Prick Test with standardized extracts. * – QO: Quincke oedema. c – results of IgE immunoblot are semi-quantitatively expressed according to the intensity of the reaction: - : negative; +/- : low; + : medium; ++ : high; +++ : very high. Close circle: positivity (ISU > 0.3). Open circle: low positivity (0.1 < ISU < 0 .3). --- : negative (ISU < 0.1). See Table 2 for letter code correspondence.

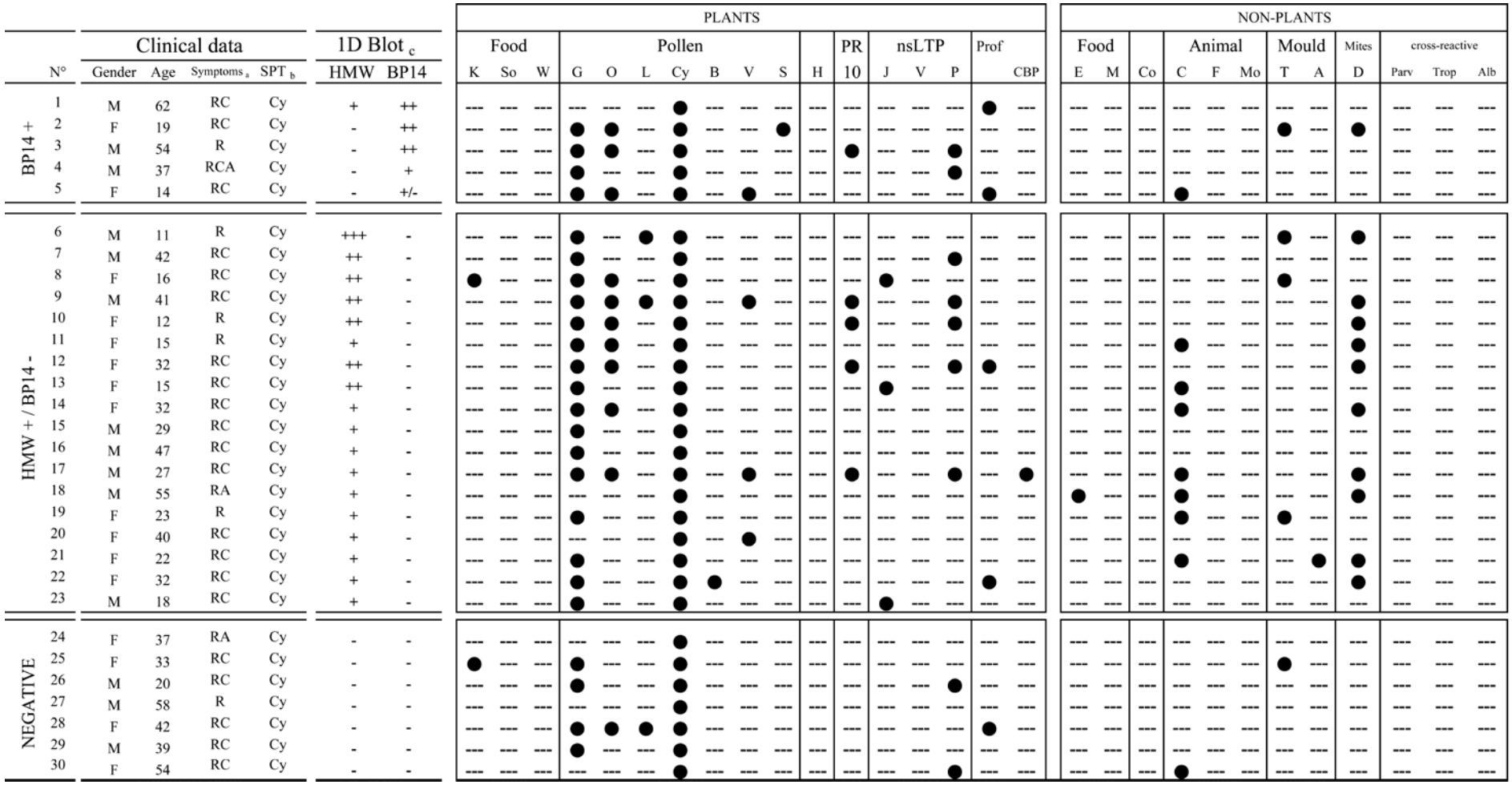

Fig. 3. Clinical and biological data of 30 Italian CPA

a – symptoms included: R: rhinitis; C: conjunctivitis; A: asthma. b – SPT: Skin Prick Test with standardized extracts. c – results of IgE immunoblot are semi-quantitatively expressed according to the intensity of the reaction. - : negative; +/- : low; + : medium; ++ : high; +++ : very high. Close circle: positivity (ISU > 0.3). --- : negative (ISU < 0.1). See Table 2 for letter code correspondence.

Table 2. Correspondence letter code for molecular allergens and extracts used for Figs. 2 and 3. For SPT reported in Fig.2, the letter code represents the total extract source.

	Code	Source	Species	Molecular Allergen
Plant food	**K** **S** **W**	Kiwi Soybean Wheat		nAct d 2 nGly m 5, 6 nTri a 18 nTri a gliadin
Pollen	**G** **O** **L** **Cy** **B** **V** **S**	Grass pollen Olive pollen Plane pollen Cypress pollen Ragweed pollen Mugwort pollen Saltwort pollen	Bermuda Timothy Arizona cypress Japanese cedar	nCyn d 1 rPhl p 1, 2, 5 , 6, 11 nPhl p 4 nOle e 1 rPla a 1 nPla a 2 Cup a 1 Cry j 1 nAmb a 1 nArt v 1 nSal k 1
	H	Latex		rHev b 5, 6
	PR10	PR10 family	Birch pollen Alder pollen Hazel pollen Hazelnut Apple Peach Soybean Peanut	rBet v 1 rAln g 1 rCor a 1.11 rCor a 1.41 rMal d 1 rPru p 1 rGly m 4 rAra h 8
LTP family	**J** **V** **P**	Peach Mugwort Wall pellitory	 *(Parietaria)*	nPru p 3 nArt v 3 rPar j 2
Profilin family	**Prof**	Profilins	Birch Olive Latex Mercury Timothy	rBet v 2 nOle e 2 rHev b 8 rMer a 1 rPhl p 12
	CBP	Ca-binding protein	Birch Timothy	rBet v 4 rPhl p 7
	E **M** **Co**	Egg Milk Cockroach		nGal d 3 ovotransferrin nBos d lactoferrin rBla g 5
	C **F** **Mo**	Cat Dog Mouse		rFel d 1, 4 rCan f 1, 2 nMus m 1
Mould	**T** **A**	Alternaria Aspergillus		rAlt a 1, 6 rAsp f 4
	D	House dust mite	*D. pteronyssinus* *D. farinae*	nDer p 1, 2 nDer f 1, 2
	Parva	Parvalbumin	Carp Cod	rCyp c 1 rGad c 1
	Tropo	Tropomyosin	Shrimp Mite Cockroach Anisakis	rPen a 1 nPen i 1 nPen m 1 rDer p 1 Bla g 7 rAni s 3
	Alb	Animal serum albumin	Cow Cat Dog Horse	nBos d 6 nFel d 2 nCan f 3 nEqu c 3

tively. In contrast, the proportion of Italian patients allergic to multi-categories of allergens (i.e. all pollen plus other allergens) was high (63 %), while it only reached 46 % in French patients. Patients with the pollen sensitization limited to cypress along with sensitization to non-pollen allergens represented 15 % for French and 7 % for Italian CPA. These mono-, pauci- and poly-sensitizations seemed to be independent of the three IB subgroups.

Individual levels of specific IgE reactivity to Cup a 1, Cry j 1 and *Cups* as well as total IgE are graphically shown in Fig. 4 for each IB subgroup. Specific IgE to *Cups* (t23), Cup a 1 and Cry j 1 values were significantly higher in the Italian patient group than in the French one independently of IB subgroups ($P < 0.05$)

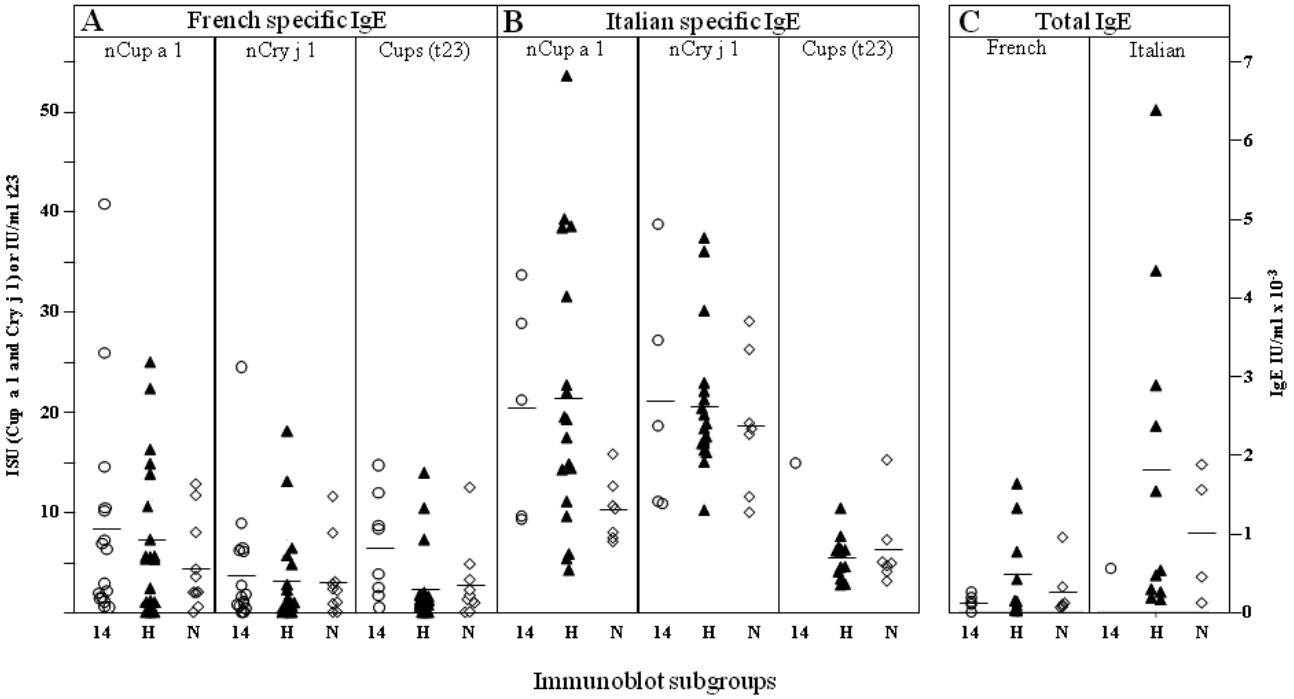

Fig. 4. Individual distribution plots of specific (**A** and **B**) and total IgE (**C**) levels in French and Italian CPA and for each IB subgroup

14: open circles: BP14 subgroup; **H**: close triangles: HMW subgroup; **N**: open diamond: Neg subgroup. Horizontal bars in each subgroup represent the mean value. Total IgE was quantified by nephelemetry and expressed in $IU.ml^{-1}$. ImmunoCAP-specific IgE levels to the whole *Cups* pollen extract (t23) were indicated in $IU.ml^{-1}$. The level of specific IgE to nCup a 1 and nCry j 1 was determined with ImmunoCAP ISAC and expressed in ISU.

and total IgE levels showed a tendency to be lower in French than in Italian CPA patients (P = 0.1019) (Fig. 4). While similar values of Cup a 1 and Cry j 1 specific IgE were observed in the Italian cohort (mean 18.6 and 20.4 ISU, respectively), they were significantly lower for Cry j 1 than for Cup a 1 in the French patients independently of IB subgroups (mean 3.4 and 7.1 ISU, respectively, P = 0.0129). Interestingly, *Cups* (t23) specific IgE were significantly higher (P < 0.05) in the BP14 subgroup as compared with HMW and Neg subgroups in the French patients.

LTPs are the discriminating allergens

Data of Fig. 2 and 3 were also globally interpreted as histograms in Fig. 5, pointing out the percentages of positive sera for allergens or groups of allergens in order to compare French and Italian repertoires. The overall qualitative diversity was similar and four allergen sources showed quantitative differences. Grass pollen (35 % vs. 80 %), PR10 (2 % vs. 17 %), profilin (4 % vs. 17 %) and house dust mite (17 % vs. 37 %) sensitizations were higher in Italian than in French CPA. However, the most striking qualitative and quantitative difference was found for LTP sensitization. Italian CPA were significantly more sensitized to *Parietaria* pollen LTP as compared to French CPA (30 % versus 4 %, respectively, P = 0.004), whereas sensitization to peach LTP reached 20 % in the French and 10 % in the Italian CPA. This outcome prompted us to assess how these reactivities were distributed among each IB subgroup. While the reactivity of CPA to the main type of allergens – pollen, animals and house dust mites – was equally distributed among the three IB subgroups, both in French and Italian patients (data not shown), the reactivity to peach LTP was strictly restricted to BP14 IB subgroup in French CPA. The nine Pru p 3+ French patients were all included in the BP14+ patient subgroup, while the three Pru p 3+ Italian patients were in the HMW+ subgroup (Fig. 6). This correlation is statistically significant (P = 0.01) and is consistent with the anamnetic investigation revealing a higher proportion of fruit allergy in the BP14+ French patient subgroup than in the HMW+ subgroup (left side of Fig. 2). Interestingly, no preferential association was found for the reactivity to *Parietaria* pollen LTP Par j 2 in Italian patients since it was found in the three IB subgroups (Fig. 6).

Discussion

Aside from the clinical symptoms collected by a physician in a traditional anamnesis, diagnosis is usually established with skin prick test and biological test evaluating the presence of specific IgE against a panel of allergens.

An optimized total extract has the advantage of containing the whole collection of components to which the individual has been exposed and is thus crucial to decipher the complete repertoire of a patient's IgE against a

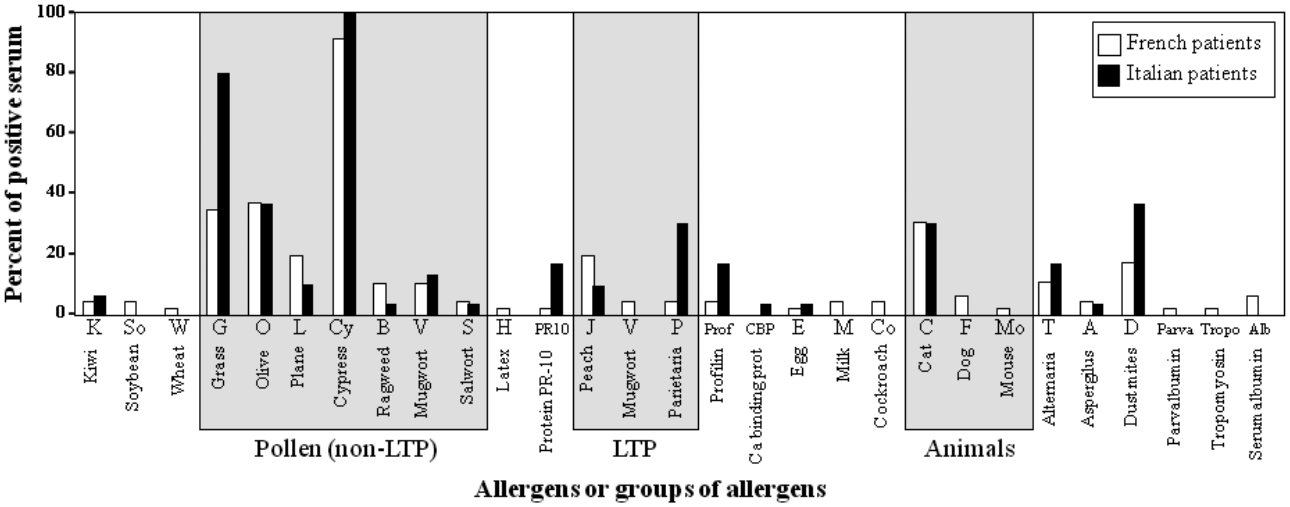

Fig. 5. Comparative distribution of IgE reactivities in French and Italian CPA patients. Data from the commercially available microchip array ISAC® (103 allergens) are presented as percentages of positive sera for each allergen or group of allergens. White histograms: French CPA, black histograms: Italian CPA. Pollen allergenic sources, LTP and animal-derived allergens are grouped in boxes to facilitate the comparisons. See Table 2 for letter code correspondence.

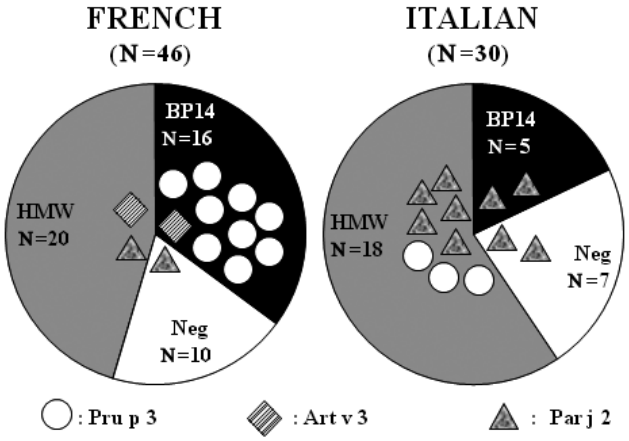

Fig. 6. Distribution of LTP sensitization obtained by microarray ISAC® (103 allergens) within IB subgroups in French and Italian patients. For each subgroup, HMW, BP14 and Neg, the number of patients is indicated. ○ : patient sensitized to peach LTP (Pru p 3); ◈ : patient sensitized to mugwort LTP (Art v 3); ▲ : patient sensitized to *Parietaria* LTP (Par j 2).

given source. Electrophoresis constitutes a useful tool to separate proteins from an allergenic extract and double dimension gel electrophoresis offers the best resolution (Le Mao et al., 1998; Shahali et al., 2010; Poncet et al., 2010). Coupled to immunoblotting with the patient's IgE and mass spectrometry of recognized allergens, this immunoproteomic approach evaluates the diversity of sensitizing allergens as well as the diversity of the individual IgE immune response. However, since the technique has not yet been miniaturized, it is tedious, time- and reagent-consuming when several allergenic sources have to be studied.

In contrast, a multiplexed microarray device, used for *in vitro* diagnosis, consumes low amounts of serum, can be automated and delivers the results of IgE binding on more than 100 molecular native or recombinant allergens covering representative group markers of plant and non-plant allergenic sources (Mari et al., 2010). A large repertoire of allergen reactivities can thus be explored in selected physio-pathological models on already known and well characterized molecular allergens. One obvious drawback is, of course, the inability to evaluate reactivity to a molecular allergen that has not been previously reported. Non-hydrosoluble allergens, for example, represent one such class that has remained poorly studied until now (Godfrin et al., 2007).

Consequently, for the purposes of research, these two techniques can be considered as complementary since immunoblots yield the complete allergen repertoire from a given allergenic source and the microarray gives a global reactivity overview against several allergenic sources.

We combined these two technologies to study the cypress pollen allergy, a pervasive pollinosis in areas surrounding the Mediterranean basin. Very few studies are available on the IgE repertoire of cypress-allergic French patients (Boutin-Forzano et al., 2005; Caimmi et al., 2013), whereas this pollinosis has been better documented in Italy and Spain (Corsico et al., 2000; Papa et al., 2001; Tordesillas et al., 2011). We previously reported that CPA can be classified into two groups according to IgE immunoblot patterns after migration of a cypress extract in 1-D and 2-D gel electrophoresis: CPA with anti-HMW allergen reactivities and those expressing IgE against BP14, a basic protein of 14 kDa (Shahali et al., 2010; 2012b, c). Proteomic analysis of cypress pollen extract in two-dimensional gel electrophoresis (2-DE) showed that the only protein found at 14 kDa in a PBS cypress extract is the cationic BP14 (Shahali et al., 2012b, c), making the immunoblot after 1-DE a valuable and reliable method to assess the CPA profile.

Comparison of the IgE immunoblot patterns of two CPA cohorts, one from Rome, Italy and the other from Marseille, France showed that the anti-BP14 specificity was less represented in Italian patients. Together with microarray evaluation of sensitization patterns and determination of Cup a 1, Cry j 1 and *Cups* specific IgE levels, our results point out some differences that might be attributed to variable exposure to local trees and/or other allergenic environments. Indeed, as compared to the Arizona cypress (*Cupressus arizonica*) and Japanese cedar (*Cryptomeria japonica*), the Italian cypress (*Cupressus sempervirens*) pollen is richer in BP14 (Shahali et al., 2012c) (with or without SDS in extraction buffer – unpublished data) and is the most abundant species in the south of France, where it represents more than 80 % of Cupressaceae. Conversely, the Arizona cypress is predominant in urban and peri-urban areas in Italy (Christian Pichot, INRA, France, personal communication). This might be one of the main reasons explaining why patients allergic to cypress pollen and showing anti-BP14 IgE are more frequent in the south of France.

Microarray experiments also revealed some differences in sensitization to LTPs. Thirty percent of Italian CPA reacted to *Parietaria* pollen LTP (Par j 2), independently of IB subgroups, and 20 % of French patients reacted to peach LTP (Pru p 3) with a strong correlation with the BP14 subgroup. This correlation was not observed for the three Italian patients sensitized to Pru p 3 that fall into the HMW subgroup. LTPs constitute a group of heterogeneous allergens where no IgE cross-reactivities are found between *Parietaria* pollen and food LTP, including peach (Egger et al., 2010; Tordesillas et al., 2011). We confirmed these results since no Pru p 3/Par j 2 co-sensitization was observed, either in French or in Italian CPA. This result is also in agreement with the absence of detected cross-reactivity between cypress pollen allergens and plant-derived food in cypress pollen-monosensitized Italian patients (Panzani et al., 2010).

A relationship between cypress pollen and peach allergy has already been reported (Hugues et al., 2006; Delimi et al., 2007), and the authors suggested, upon immunoblot inhibition experiments, that proteins around 45 kDa might be the cross-reactive allergens (Hugues et al., 2006). The fact that Pru p 3-reactive CPA were found in the BP14 subgroup (in French patients only) was unexpected and raised the question of the nature of BP14. LTPs are small in molecular masses (6–14 kDa) and basic in terms of isoelectric point values. These characteristics are shared with BP14 and several experiments were performed to unravel the physico-chemical nature of BP14. These experiments included chromatographic methods specifically adapted to purify LTPs, immunoblots using specific anti-Pru p 3 rabbit antibodies and more than 25 trials of characterization by mass spectrometry analysis. Analysis of the accumulated data led to the conclusion that there is no obvious direct immunochemical and structural relationships between known LTPs and BP14. Homology searches using a sequence of nine N-terminal amino-acids of BP14 (unpublished data) confirmed that this protein is currently absent from data banks and displays no identical characteristics with the Cupressaceae pollen LTP described as an allergen in Japanese cedar (Ibrahim et al., 2010). An LTP has also been immunochemically described in *Cupressus arizonica* pollen (Sanchez-Lopez et al., 2011). Efforts are still ongoing in our group to structurally characterize BP14 whose IgE response seems to be a specific marker of sensitization to *Cups* pollen (Shahali et al., 2012b) and is associated to Pru p 3 sensitization in French CPA only.

In conclusion, by combining two component-resolved explorative techniques, we highlighted some of the differences shaping the IgE repertoires of French and Italian CPA. These differences are related to the allergenic biodiversity of the local environmental exposure, either the species of cypress, *arizonica* versus *sempervirens,* or the co-sensitization source, peach or *Parietaria* LTP. Using immunoblot, the IgE reactivity against numerous allergens in one allergenic source was revealed, and using microarray, some representative allergens of several allergenic sources were studied. These approaches have proved to be complementary and were used here to efficiently decipher and refine sensitization profiles of French and Italian patients suffering from a common cypress allergy widely distributed around the Mediterranean basin.

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