

Original Article

Prevalence of Pol d 1 Sensitization in *Polistes dominula* Allergy and Its Diagnostic Role in Vespid Double-Positivity

Maria Beatrice Bilò, MD^{a,b,*}, Matteo Martini, MD^{c,*}, Patrizia Bonadonna, MD^d, Barbara Cinti, BS^e, Mirella Da Re, PhD^f, Oretta Gabrielli, MD^d, Francesco Olivieri, BS^d, Valeria Salgarolo, PhD^f, Giovanna Zanoni, MD^d, and Danilo Villalta, MD^f
Ancona, Parma, Pordenone, and Verona, Italy

What is already known about this topic? Although *Polistes* venom allergy is common, diagnosis of primary sensitization versus vespid cross-reactivity is challenged by the availability of only Pol d 5 as a *Polistes* molecule in diagnostics.

What does this article add to our knowledge? Pol d 1 is the most frequent allergen in *Polistes*-sensitized patients (97% to 100%). The diagnostic accuracy of Pol d 1 is good for detecting primary sensitizations in double-sensitized patients (area under the curve: 87% to 99%).

How does this study impact current management guidelines? Pol d 1 can increase the diagnostic accuracy of *Polistes* primary sensitizations if implemented in clinical practice, reducing the use of cumbersome and expensive in vitro testing (ie, CAP-inhibition and basophil activation tests) and unnecessary double-venom immunotherapies.

BACKGROUND: Stings by *Polistes* species frequently cause allergic reactions. However, standard allergy diagnostics are often unable to differentiate between primary sensitization and cross-reactivity in case of *Vespula/Polistes* double-sensitization because antigen 5 is the only *Polistes* venom molecule currently available in diagnostics (Pol d 5).

OBJECTIVE: To evaluate the frequency of phospholipase A1 in *Polistes* venom allergy (Pol d 1) and its diagnostic role in vespid allergy.

METHODS: We performed component-resolved diagnostics in patients with vespid allergic reactions who were positive to *Polistes* venom. A prevalence analysis was performed and the diagnostic accuracy of Pol d 1 was evaluated to detect primary *Polistes* sensitization in double-sensitized patients.

RESULTS: Blood samples were collected from 132 patients. Pol d 1 was present in 97% to 100% of 128 *Polistes*-positive patients. It was frequently involved in case of positivity to a single *Polistes* molecule (48% in double- and 80% in mono-sensitized patients). Furthermore, Pol d 1 was positive in 95% of Pol d 5-negative subjects. The diagnostic accuracy of Pol d 1 was good (folded type: area under the curve = 87%; 82% sensitivity and 77% specificity at the best cutoff of 5.773), and even better when used combined with the whole extract ratio (area under the curve = 99%; 91% sensitivity and 100% specificity).

CONCLUSIONS: The study shows that Pol d 1 is the most frequent *Polistes* allergen in Italian patients. It can distinguish *Polistes* primary sensitizations with good diagnostic accuracy, which supports its use in clinical practice. © 2021 Published by Elsevier Inc. on behalf of the American Academy of Allergy, Asthma & Immunology (J Allergy Clin Immunol Pract 2021;■:■-■)

Key words: *Polistes*; *Vespid*; Cross-reactivity; Double-sensitization; Pol d 1; Phospholipase A1; Pol d 5; Venom immunotherapy; CAP-inhibition; Hymenoptera allergy

Author has made corrections in ce:affiliation. Carry out the corrections in sa:affiliation also*Department of Clinical and Molecular Sciences, Università Politecnica delle Marche, Ancona, Italy

^bAllergy Unit, Department of Internal Medicine, Azienda Ospedaliero-Universitaria Ospedali Riuniti, Ancona, Italy

^cGlobal Clinical Development, Chiesi Farmaceutici S.p.A., Parma, Italy

^dAllergy Unit, Azienda Ospedaliera Universitaria Integrata, Verona, Italy

^eLaboratory Medicine, Ospedaliero-Universitaria Ospedali Riuniti, Ancona, Italy

^fImmunology and Allergy Unit, Santa Maria degli Angeli Hospital, Pordenone, Italy

*These authors contributed equally to this work.

Conflicts of interest: The authors declare that they have no relevant conflicts of interest.

Received for publication April 3, 2021; revised May 22, 2021; accepted for publication May 25, 2021.

Available online ■■

Corresponding author: Maria Beatrice Bilò, MD, Allergy Unit, Department of Internal Medicine, University Hospital Ospedali Riuniti, Via Conca 71, 60127 Ancona, Italy. E-mail: m.b.bilo@univpm.it.

2213-2198

© 2021 Published by Elsevier Inc. on behalf of the American Academy of Allergy, Asthma & Immunology

<https://doi.org/10.1016/j.jaip.2021.05.033>

INTRODUCTION

Stinging insects that most frequently cause Hymenoptera venom allergy in developed countries are bees of the Apidae family and wasps of the Vespidae family (composed of the Vespinae and Polistinae subfamilies). In southern Europe, in addition to *Vespula* (V) and hornets, some species of *Polistes*, such as *Polistes dominula* (P) are a frequent cause of allergic reactions.^{1,2} Moreover, *P dominula* has invaded the United States,³ South Africa,⁴ and central Europe.⁵ It will probably become more important in the future in different parts of the world, in addition to other well-known *Polistes* species that are currently present (eg, *Polistes annularis*, *Polistes apachus*, *Polistes exclamans*,

Abbreviations used

AUC- Area under the curve

CRD- Component-resolved diagnostic

P- *Polistes dominula*

ROC- Receiver operating curve

sIgE- Specific IgE

SR- Systemic reaction

V- *Vespula*

Polistes fuscatus, and *Polistes metricus*). In southern Europe, double-sensitization to V and P is much more frequent than to V/P and honeybee venom,⁶⁻⁸ and standard allergy diagnostics (skin test and serum IgE to whole extract) are mostly unable to differentiate between primary sensitization and cross-reactivity.⁹

Radioallergosorbent and CAP-inhibition techniques appear to be useful in cases of double V-P cosensitization^{6,7,10,11}; however, they may be relatively expensive and results could prove difficult to interpret.¹²

More recently, component-resolved diagnostics (CRD) identify molecule-specific IgEs using recombinant or natural allergenic epitopes, with important consequences for both diagnosis and therapeutic management. However, although CRD is able to distinguish allergies adequately to honeybee venom and vespid venom (particularly V), this is not the case when differentiation between allergies to various vespid venoms is required. Although P venom is devoid of carbohydrate determinants,¹³ definite discrimination is rarely possible owing to the high degree of cross-reactivity among the major allergens of these venoms.⁸ Because the only P venom allergen currently available for CRD on the most common sIgE assay platform is Pol d 5, the additional availability of other allergens from vespid venoms for CRD would represent an added value for advanced precision diagnostics in Hymenoptera venom allergy.⁶

Therefore, the objectives of this study were to evaluate, in a real-world setting (1) the frequency of P phospholipase A1 (Pol d 1) sensitization in vespid venom allergy, with a prevalence analysis; (2) and its diagnostic role in vespid allergy, particularly in the case of double-sensitization, when other available allergenic molecules (antigen 5s) are unable to characterize the P sensitization pattern.

METHODS

We collected the medical history and clinical examination of all patients with allergic reactions by vespid stings and then consecutively collected their blood samples at three allergy centers in northern and central Italy (S. Maria degli Angeli Hospital in Pordenone, Azienda Ospedaliero-Universitaria in Ancona, and Azienda Ospedaliera Universitaria Integrata in Verona), from 2016 to 2020. Patients were tested for sensitization to both P and V by EUROLINE DPA-Dx insect venom profile (Euroimmun, Lübeck, Germany). EUROLINE is a semiquantitative immunoblot assay for determining allergen-specific IgE.¹⁴ The profile applied in this study was composed of the following allergen extracts and molecules: *V. vulgaris* venom, honeybee venom, *Polistes* ssp. venom, *Vespa crabro* venom, Api m 1, Api m 2, Api m 4, Api m 10, Ves v 1, Ves v 5, Pol d 1, and Pol d 5, as well as a carbohydrate determinant marker. Two different types of recombinant phospholipase A1 were used for testing: Pol d 1a (native-like folded protein) and Pol d 1l (an artificial, unfolded variant, was produced under denaturing conditions,

yielding a protein with primarily linear epitopes). All patients positive for P were selected for analysis. Serum IgE levels specific to the extracts and molecules (Pol d 5 and Pol d 1) were measured. Incubations were performed according to the manufacturer's instructions on the EUROBlotOne instrument (Euroimmun, Lübeck, Germany). In brief, 210 µL of patients' sera diluted 1:2.5 was incubated at room temperature. After the first three washing steps, strips were incubated with alkaline phosphatase-conjugated anti-human IgE, and after the second three washing steps with chromogen/substrate solution. The reaction was stopped by washing with distilled water. The strips were placed on the evaluation protocol, dried, and evaluated with the EUROLIneScan program (Euroimmun).

Prevalence analysis of sensitization to the P molecules was performed and their frequencies were reported for the overall P-sensitized subjects, doubly-sensitized ones, and P mono-sensitized ones. According to the manufacturer's instructions, the threshold of negative results was less than 0.35 kU/L. Chi-square test was used for comparisons between double and mono-sensitized groups. For subjects with levels of 0.35 kU/L or greater, median serum IgE values with interquartile ranges were also reported. Specific IgE values less than 0.35 and greater than 100 kU/L were considered to be 0.34 and 101 kU/L, respectively, for the quantitative analysis. Mann-Whitney test was used to compare continuous variables.

A subgroup analysis was performed in patients sensitized to the whole P venom extract but negative to Pol d 5 (Pol d 5 negative subgroup), to characterize the role and magnitude of Pol d 1 in these subjects.

A CAP-inhibition test was performed in a subgroup of patients with similar values of specific IgE (sIgE) to P and V whole extract and antigen 5 (CAP subgroup), to assess sensitization to P or V.¹⁰ Results were used as the standard reference to build receiver operating curves (ROCs) to investigate the diagnostic accuracy of Pol d 1 in detecting P sensitization (AUC, sensitivity, and specificity), comparing different IgE ratios. The P/V ratios of the sIgE to these extracts and antigens were chosen for evaluation: whole extracts, antigen 5 (ie, Pol d 5/Ves v 5), phospholipase A1 (ie, Pol d 1/Ves v 1), and all antigens (ie, [Pol d 1 + Pol d 5]/[Ves v 1 + Ves v 5]). Cutoff values for each sIgE ratio, together with sensitivity and specificity, were calculated from the ROC analysis: the Youden index (ie, cutoff at the highest sum of sensitivity and specificity) and the 100% specific cutoff (ie, cutoff with a specificity of 100%). The obtained cutoff values were then applied to the all CAP subgroup, to compare their diagnostic ability with CAP-inhibition. A logistic model was used to evaluate the diagnostic accuracy of the P/V ratio of whole extracts combined with the P/V ratio of all available antigens.

We used STATA software (version 13, StataCorp, College Station, Tex) for analyses. Participants were informed about the study and signed informed consent spontaneously, according to Good Clinical Practice. Because all interventions were part of routine clinical practice, formal approval by the Ethics Committee was not needed and was not requested.

RESULTS

A total of 132 blood samples from White adult patients were consecutively collected. Four were excluded because V mono-sensitized; the remaining 128 were included in the analysis. Seventy percent of patients were males and 30% were females, median age of 55 years (interquartile range, 38-65 years). All reported allergic reactions to vespid stings, 64.5% had systemic

TABLE I. Prevalence of molecular allergens in *Polistes*-sensitized patients (positive to whole *Polistes* extract and/or *Polistes* molecules)

Molecule	Total (n = 128)	Double-sensitized (n = 116)	Mono-sensitized (n = 10)	P*
Whole <i>Polistes</i> extract	126 (98%)	116 (100%)	10 (100%)	0
Pol d 1(l)	104 (81%)	98 (84%)	6 (60%)	.004
Pol d 1(a)	100 (78%)	93 (80%)	7 (70%)	.672
Pol d 5	64 (50%)	62 (53%)	2 (20%)	.015
Pol d 1 (l and/or a)	123 (96%)	113 (97%)	10 (100%)	.573
Negative to all <i>Polistes</i> molecules†	3 (2%)	3 (3%)	0	n.a.
Negative to whole <i>Polistes</i> extract‡	2 (2%)	0	0	n.a.

a, folded type; l, unfolded type; n.a., not applicable.

Bold values show statistical significance at $P < .05$.

*Double versus mono-sensitized patients (chi-square test).

†Positive to whole *Polistes* extract.

‡Positive to *Vespula* and to Pol d 1a only.

TABLE II. Specific IgE values

Molecule	Total (n = 128)	Double-sensitized (n = 116)	Mono-sensitized (n = 10)	P*
Whole <i>Polistes</i> extract	34.1 (12.8-62.9)	38.9 (14.4-68.1)	13 (0.9-26.2)	.002
Pol d 1(l)	21.9 (5.1-57)	22.3 (4.8-58)	14.1 (8.1-30.6)	.019
Pol d 1(a)	15.4 (2.5-41.1)	21 (2.5-44.6)	2.9 (1.1-15.4)	.049
Pol d 5	1.4 (0.5-7.8)	1.4 (0.5-8.8)	1.9 (0.6-3.2)	.025

a, folded type; l, unfolded type.

Results are expressed in kU/L median values (interquartile range). Bold values show statistical significance at $P < .05$.

*Double versus mono-sensitized patients (Mann-Whitney test).

TABLE III. Frequency of *Polistes* molecular allergens (as mono-allergens)

Molecule	Total (n = 128)	Double-sensitized (n = 116)	Mono-sensitized (n = 10)
Pol d 1(l)	9 (7%)	8 (7%)	1 (10%)
Pol d 1(a)	17 (13%)	13 (11%)	4 (40%)
Pol d 5	0	0	0
Pol d 1 (l and/or a)	61 (48%)	53 (46%)	8 (80%)

a, folded type; l, unfolded type.

reactions (SRs), 21.8% had large local reactions, and 13.7% had both reactions.

Double-sensitization to whole-venom extracts was observed in most of the study population ($n = 116$; 90.6%); the others were either P mono-sensitized ($n = 10$; 7.8%) or negative to whole P extract but positive to Pol d 1a ($n = 2$; 1.6%) (Table I). Median sIgE values to each extract or molecule are reported in Table II. No significant correlations were found between type of reaction (ie, SR or large local reactions) and sensitization patterns or sIgE level.

Prevalence analysis of Pol d 1 and Pol d 5 sensitization

Overall, Pol d 1 was the most frequent P allergen (regardless of the folded or unfolded structure) in both double-sensitized patients (97%) and mono-sensitized patients (100%) compared with Pol d 5 (53% and 20% in the double- and mono-sensitized group, respectively) (Table I). Pol d 1a frequency was more frequent in double (80%) than in mono-sensitized patients (70%), but the difference was not statistically significant ($P = .672$). On the other hand, Pol d 1l was significantly more frequent in the double-sensitized group (84%) than in the mono-sensitized group (60%) ($P = .004$).

Pol d 1 was involved in 48% of patients who were positive to a single P molecule; it was even more frequent in P mono-sensitized

patients (80%) (Table III). Pol d 1a as the only P positive molecule was present in 13% of P-sensitized patients, compared with 7% of Pol d 1l. This difference between phospholipase A1 types was reflected both in double- and mono-sensitized patients (Table III).

Conversely, antigen 5 was present in only 53% of double-sensitized patients, with a significant lower frequency in P mono-sensitized patients (20%; $P = .015$) (Table I). Furthermore, antigen 5 was not involved in cases of positivity to a single P molecule (Table III).

Three patients (2%) were positive to the whole P venom extract but negative to all P molecules (Table I).

Sixty-two patients (48% of 128 subjects) were positive to the whole P venom extract but negative to Pol d 5; therefore, they were included in the Pol d 5 negative subgroup (Table IV). Almost all subjects had anti-Pol d 1 sIgE ($n = 59$; 95% of this subgroup). The remaining three patients (5%) were negative to all P molecules. Pol d 1a was more frequent than Pol d 1l (81% and 71%, respectively). This difference was even bigger in the mono-sensitized group (88% and 50%), but not statistically significant. Concerning the median values of sIgE, in this Pol d 5 negative subgroup, the only significant difference was observed for values of whole P venom extract between double- and mono-sensitized patients (20.6 and 3.2 kU/L, respectively; $P = .032$).

TABLE IV. Frequency of molecular allergens (with specific IgE values) in *Polistes*-sensitized patients who were negative to Pol d 5 (n = 62)

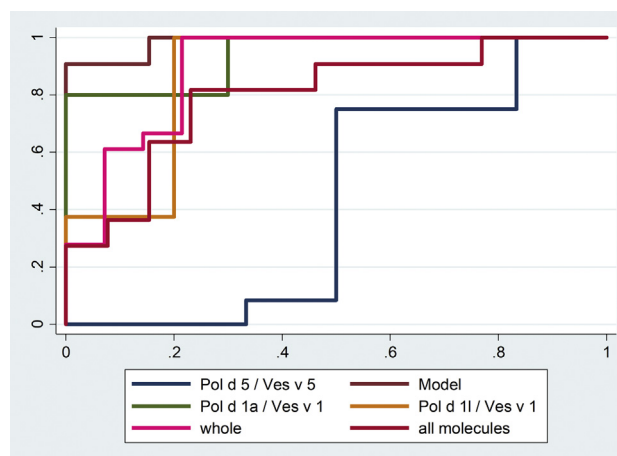
Molecule	Total (n = 62)			Double-sensitized (n = 54)			Mono-sensitized (n = 8)			P	
	N	sIgE	IQR	N	sIgE	IQR	N	sIgE	IQR	*	†
Whole <i>Polistes</i> extract	62 (100%)	19.2	3.2-44.6	54 (100%)	20.6	3.4-45.5	8 (100%)	3.2	0.7-22.3	n.a.	.032
Pol d 1(l)	44 (71%)	12.8	3.1-23.6	40 (74%)	13.8	3.1-26.7	4 (50%)	9.4	4.3-14.1	.162	.108
Pol d 1(a)	50 (81%)	6.5	2.3-27.1	43 (80%)	8.1	2.3-28.9	7 (88%)	2.9	1.1-15.4	.599	.650
Pol d 5	0	n.a.		0	n.a.		0	n.a.		n.a.	n.a.
Pol d 1 (l or a)	59 (95%)	n.a.		51 (94%)	n.a.		8 (100%)	n.a.		.494	n.a.
Negative to all molecules	3 (5%)	n.a.		3 (6%)	n.a.		0	n.a.		n.a.	n.a.

a, folded type; *IQR*, interquartile range; l, unfolded type; n.a., not applicable.

Median-specific IgE values are expressed in kU/L. Bold values show statistical significance at $P < .05$.

*Double versus mono-sensitized patients (frequency of positive subjects, chi-squared test).

†Double versus mono-sensitized patients (specific IgE values, Mann-Whitney test).

**FIGURE 1.** Diagnostic accuracy of specific IgE ratios in detecting *Polistes* sensitization (receiver operating characteristics analysis): All molecules : $\frac{(\text{Pol d 1} + \text{Pol d 5})}{(\text{Ves v 1} + \text{Ves v 5})}$; Whole : $\frac{\text{Polistes whole extract}}{\text{Vesula whole extract}}$; Model : All molecules + Whole.

Diagnostic accuracy of Pol d 1 for *Polistes* sensitization in double-sensitization

Similar values of P and V sIgE for the whole extract (35.6 ± 30.5 and 21.9 ± 20.0 kU/L, respectively) and antigen 5 (Pol d 5 = 9.7 ± 21.9 kU/L; Ves v 5 = 10 ± 18.4 kU/L, respectively) were identified in 52 patients (CAP subgroup, 41% of the total 128 patients); their samples were tested with the CAP-inhibition test. The results showed that 21 subjects were sensitized to P (40%) and 14 subjects to V (27%). In the remaining 17 subjects (33%), the test showed either undetermined results (ie, it was unable to discriminate between P or V owing to technical limitations) or genuine double-sensitizations in 13 and 4 patients, respectively.

The P/V ratio of the whole venom extract showed good diagnostic accuracy in detecting P sensitization compared with the standard reference obtained from CAP-inhibition (area under the curve [AUC] = 0.89; standard error [SE] = 0.06; 95% confidence interval [CI], 0.77-1.00) (Figure 1). The Youden cutoff was 1.759 (sensitivity = 94%, specificity = 79%). It was able to classify 89% of subjects correctly. The 100% specific cutoff was 7.195 (sensitivity = 28%, percentage of correctly classified subjects = 63%).

The diagnostic accuracy of the phospholipase A1 ratio was good for Pol d 1a (Pol d 1a/Ves v 1) as well (AUC = 0.87; SE = 0.13;

95% CI, 0.61-1.00) (Figure 1). The value of 11.10 was identified as both the Youden and the 100% specific cutoff (sensitivity = 80%; specificity = 100%), and it was able to classify 89% of subjects correctly. On the other hand, the AUC of the Pol d 1l/Ves v 1 ratio was lower (AUC = 0.75; SE = 0.14; 95% CI, 0.48-1.00; 100% specific cutoff: 8.226; Youden cutoff: 1 with 40% specificity and 100% sensitivity) (Figure 1), and the Pol d 5/Ves v 5 ratio showed poor accuracy for the diagnosis of P sensitization (AUC = 0.63; SE = 0.16; 95% CI, 0.33-0.94; 100% specific cutoff: 166.7; Youden cutoff: 1 with 50% specificity and 92% sensitivity) (Figure 1).

When all of these molecules were summed to obtain the P/V ratio of all antigens (Pol d 1 + Pol d 5)/(Ves v 1 + Ves v 5), the diagnostic accuracy was fair (AUC = 0.80; SE = 0.09; 95% CI, 0.61-0.98) (Figure 1). The Youden cutoff was 5.773 (sensitivity = 82%; specificity = 77%) and it was able to classify 83% of subjects correctly. The 100% specific cutoff was 78.38 (sensitivity = 27%; percentage of correctly classified subjects = 64%).

After the P/V ratio of the whole venom extract was combined with the P/V ratio of all allergens in a logistic model for the diagnosis of P sensitization, using CAP-inhibition results as the reference standard, the AUC reached 99% (Figure 1) with 100% of the P group correctly classified (specificity) and 91% of the V

TABLE V. Venom immunotherapy prescriptions according to CAP-inhibition testing and specific IgE ratios with area under the curve (AUC) of 80% or greater (n = 52)

Test	CAP inhibition	Polistes whole extract Vespula whole extract	Pol d 1a Ves v 1	(Pol d 1 + Pol d 5) (Ves v 1 + Ves v 5)
AUC	n.a.	89%	87%	80%
Cutoff*	n.a.	1.759	11.1	5.773
Sensitivity/specificity	n.a.	94%/79%	80%/100%	82%/77%
<i>Polistes</i>	21 (40%)	33 (63%)	36 (69%)	30 (58%)
<i>Vespula</i>	14 (27%)	18 (35%)	4 (8%)	21 (40%)
Both†	17 (33%)	1 (2%)	12 (23%)	1 (2%)

n.a., not applicable.

*Best cutoff (Youden).

†Both *Polistes* and *Vespula* immunotherapies are prescribed in case of genuine double-allergy or undetermined diagnosis.

group correctly classified (sensitivity) at the best cutoffs (Youden and 100% specific cutoff) identified in this model for P/V whole extract (2.773) and P/V of all allergens (3.551).

Table V lists the percentages of P, V, and undefined diagnoses after these ratios with an AUC of 80% or greater were applied; they may reflect the prescription of venom immunotherapies. On the other hand, with the 100% specific cutoff values, the percentages obtained were P = 67%, V = 31%, and not defined = 2%.

DISCUSSION

Prevalence analysis

We studied the prevalence of Pol d 1 in a population of vespid-sensitized patients consecutively enrolled in northern and center Italy. Most were double-sensitized to P and V (90.6%).

Our results showed that the major allergen in P sensitization was Pol d 1, because it was by far the most frequent positive antigen in the overall study population (96%), ranging from 97% to 100% in the double- and mono-sensitized populations, respectively. Furthermore, it was the only P allergen involved in sensitization to a single P antigen. On the other hand, Pol d 5 was positive in only 50% of the overall population (53% in double-sensitized patients and 20% in the mono-sensitized group), and it was not present in sensitization to a single P antigen.

These figures are consistent with the only two previous studies on Pol d 1 in V/P double-sensitized patients, reporting a Pol d 1 frequency of 87%–62%, compared to 70%–15% of Pol d 5, despite the different methods and small sample sizes (45 and 13 subjects analyzed).^{6,15}

Concerning the type of Pol d 1, Pol d 1a would resemble the natural-like variant contained in the venom, which has natural-like folding, and Pol d 1l is an artificial, unfolded variant with primarily linear epitopes. This unfolded type (Pol d 1l) was more frequent than the folded one (Pol d 1a) in the overall population (81% vs 78%) and in double-sensitized patients (84% vs 80%), but it was less frequent in the P mono-sensitized group (60% vs 70%).

The prevalence was different in patients sensitized to a single P molecule, because Pol d 1a was always more represented than Pol d 1l. Results from previous studies support the hypothesis that patterns of sensitization may be different between double- and mono-sensitized subjects^{16,17}; this might be consistent with our results showing a different distribution of Pol d 1a and Pol d 1l in these two groups. However, further studies on the characterization of folded and unfolded types of sensitization are needed to

confirm the differential diagnosis of vespid allergy based on differences in recognition patterns.

Currently, the presence of a single P allergen (Pol d 5) in a monoplex platform hampers an accurate diagnosis of P sensitization, because a negative Pol d 5 does not rule out possible significant and neglected sensitization to Pol d 1. Among other allergens, Pol d 1 may have an important role. In our study, Pol d 1 sensitization was present in almost all the patients (95%) of the Pol d 5-negative subgroup (ie, positive to the whole P venom extract but negative to Pol d 5), confirming its role of major allergen in P allergy. Only three patients (5%) had no Pol d 1 sensitization in this subgroup, and they were negative to Pol d 5, too. This suggests that additional P antigens not yet available might be relevant at least in a small percentage of P-sensitized patients.⁹ Although all three patients reported SR to stings, the clinical relevance of these unknown antigens is hard to predict, because the subjects were P/V double-sensitized.

Concerning sIgE median values, anti-Pol d 1 IgE levels were higher than anti-Pol d 5 levels in the overall study population. In particular, anti-Pol d 1 IgE levels were higher than those of anti-Pol d 1a, especially in the P mono-sensitized patients (14.1 vs 2.9 kU/L).

Significant differences were observed even between double- and mono-sensitized patients, because both the whole extract and the molecules, except for Pol d 5, had higher values in the double-sensitized group, compared with the mono-sensitized one (Tables II and III). Similar results were observed in double-sensitized population of bee venom–allergic patients.¹⁶

CAP-inhibition analysis

The usefulness of CRD in resolving challenging cases of P/V double-sensitization is a long-standing issue. Several studies have tried to discover possible ways to combine available P and V molecules to distinguish between genuine double-sensitization and clinically insignificant cross-reactivity. A Pol d 5 to Ves V 5 double ratio was previously proposed, but unfortunately, the diagnostic accuracy has not met minimum requirements for implementation in clinical practice.^{7,10,18} It is well-acknowledged that the lack of additional P antigens, in addition to the only available Pol d 5, is probably the biggest limitation in these studies, together with the choice of an arbitrary cutoff not supported by ROC analyses (double ratio [ie, cutoff = 2]).

According to literature, measuring the levels of sIgE against the four major allergens phospholipases A1 (Pol d 1 and Ves v 1) and antigens 5 (Pol d 5 and Ves v 5) of P and V allows reliable

identification of the allergy-eliciting venom in 69% of allergic patients with double sensitization.⁶

Therefore, considering the magnitude of phospholipase A1 in double-sensitizations, as also suggested by our analysis of prevalence, in this study we implemented Pol d 1 in the diagnostic algorithm and tested different ratios in the CAP subgroup at the best cutoffs obtained from the ROC analysis.

The P/V ratio with the whole extract had good diagnostic accuracy (AUC = 89%) with good sensitivity (94%) but low specificity (79%) at the best cutoff (1.759). The accuracy of the Pol d 1a to Ves v 1 ratio had similar accuracy (AUC = 87%) with higher sensitivity (80%) and specificity (100%), but the best cutoff was high (11.10) for use in clinical practice. Conversely, the diagnostic accuracy was low for Pol d 1l/Ves v 1 (AUC = 75%) and Pol d 5/Ves v 5 (AUC = 63%). Nevertheless the biological rationale behind using either phospholipase A1 or antigen 5 is probably poor, because the contribution of both should be considered for P sensitization, as was supported by our prevalence analysis. Therefore, we tested the ratio of all available antigens (ie, [Pol d 1 + Pol d 5]/[Ves v 1 + Ves v 5]), which showed just fair diagnostic accuracy (AUC = 80%), not as good as the whole extract ratio, but with the best cutoff (5.773), closer to that observed with the whole P/V ratio, and more convenient in clinical practice than the high Pol d 1a/Ves v 1 cutoff.

The best result was obtained by combining, in a logistic model, the two ratios with the strongest rationale: the whole extract and all antigen ratios. With this new ratio, we reached excellent accuracy (AUC = 99%; and 100% specificity and 91% sensitivity) with cutoff values of 2.773 for the P/V whole extract and 3.551 for the all-antigens ratio.

For venom immunotherapy prescription, a comprehensive assessment of clinical data, history (eg, identification of the culprit Hymenoptera species), and in vivo and in vitro tests must be performed, with CAP-inhibition or basophil activation testing to be used in case of undefined diagnosis even after sIgE and CRD evaluation. After applying these ratios, the percentage of undefined diagnosis decreased markedly, even compared with CAP-inhibition testing, avoiding many unnecessary double-venom immunotherapies, with consequent benefits in terms of cost, time, and burden for patients, health care professionals, health care systems, and payers.

Some points highlight limitations related to our study of prevalence. First, many factors hinder comparability with other, similar studies. It is well-known that different tests and calibration approaches may show different results. Therefore, companies are encouraged to add Pol d 1 to their pipelines and develop accurate quantitative immunoassays to enhance the standardization and validation process of these new molecules. Different baseline characteristics of patients, such as geographical origin or sensitization pattern, may also have a confounding role in the comparisons. Furthermore, a P mono-sensitized population should be analyzed to investigate the actual role of the P antigens, to avoid possible confounders related to cross-reactivity. We performed a prevalence analysis on P mono-sensitized patients, but the sample size of this group was low compared with the overall study population ($n = 10$; 8%). On the other hand, some strengths can be highlighted in this study. First, the sample size was about double those of previous studies on Pol d 1.^{6,15} Second, for the first time, we implemented CAP-inhibition to investigate Pol d 1, which allowed both a formal analysis of Pol d 1 diagnostic accuracy and an estimate of its

actual impact in clinical practice (eg, venom immunotherapy prescription). Third, we investigated for the first time two Pol d 1 variants, reflecting different sensitization patterns. Nonetheless, further multicenter studies need to be performed in a broader population and in different geographic areas with V/P dual-sensitization problems, aiming to confirm our findings and investigating the role of other P antigens, in addition to Pol d 1 and Pol d 5, which are still overlooked but might have a role, as suggested by results from the three patients who were positive to the whole P extract but negative to all tested P molecules.

Our prevalence analysis, which was conducted in a real-world setting of Italian P-sensitized patients, showed that Pol d 1 seems to be the most frequent P allergen, supporting its crucial role in diagnostics. The increase in diagnostic accuracy, after implementation of Pol d 1, is particularly important in distinguishing real V/P co-sensitizations from not clinically significant cross-reactivities. A combination of a whole-extract ratio and an all-allergens ratio might be the best diagnostic tool to avoid or limit both complex tests (CAP-inhibition, basophil activation testing) and unnecessary double venom immunotherapy.

Acknowledgments

M. B. Bilò planned and designed the study, collected patients' data, and wrote the article. D. Villalta designed the study, collected patients' data, performed the tests, and wrote the article. M. Martini conducted data analyses and wrote the article. All other authors contributed to the collection, interpretation of data, and revision of the manuscript. All authors had access to the data and read and approved the final manuscript. The authors thank Euroimmun Diagnostics for kindly providing the reagents for the study free of cost, and Dr Henning Seismann for technical assistance. This was a non-industry sponsored study, planned and conducted by public institutions (S. Maria degli Angeli Hospital in Pordenone, Azienda Ospedaliero-Universitaria Ospedali Riuniti in Ancona, Azienda Ospedaliera Universitaria Integrata in Verona) without external funding.

REFERENCES

1. Blanca M, Garcia F, Miranda A, Carmona MJ, Garcia J, Fernandez J, et al. Determination of IgE antibodies to *Polistes dominulus*, *Vespula germanica* and *Vespa crabro* in sera of patients allergic to vespids. *Allergy* 1991;46:109-14.
2. Severino MG, Campi P, Macchia D, Manfredi M, Turillazzi S, Spadolini I, et al. European *Polistes* venom allergy. *Allergy* 2006;61:860-3.
3. Cervo R, Zacchi F, Turillazzi S. *Polistes dominulus* (Hymenoptera, Vespidae) invading North America: some hypotheses for its rapid spread. *Insectes Sociaux* 2000;47:155-7.
4. Eardley C, Koch F, Wood AR. *Polistes dominulus* (Christ, 1791) (Hymenoptera: Polistinae: Vespidae) Newly recorded from South Africa. *Afr Entomol* 2009;17:226-7.
5. Hoehrl N. Nesting behavior of the paper wasp *Polistes dominula* in Central Europe—a flexible system for expanding into new areas. *Ecosphere* 2015;6:182-92.
6. Monsalve RI, Vega A, Marqués L, Miranda A, Fernández J, Soriano V, et al. Component-resolved diagnosis of vespid venom-allergic individuals: phospholipases and antigen 5s are necessary to identify *Vespula* or *Polistes* sensitization. *Allergy* 2012;67:528-36.
7. Quercia O, Cova V, Martini M, Cortellini G, Murzilli F, Bignardi D, et al. CAP-inhibition, molecular diagnostics, and total IgE in the evaluation of *Polistes* and *Vespula* double sensitization. *Int Arch Allergy Immunol* 2018;177:365-9.
8. Schiener M, Eberlein B, Moreno-Aguilar C, Pietsch G, Serrano P, McIntyre M, et al. Application of recombinant antigen 5 allergens from seven allergy-relevant Hymenoptera species in diagnostics. *Allergy* 2016;72:98-108.
9. Bilò MB, Ollert M, Blank S. The role of component-resolved diagnosis in Hymenoptera venom allergy. *Curr Opin Allergy Clin Immunol* 2019;19:614-22.
10. Caruso B, Bonadonna P, Bovo C, Melloni N, Lombardo C, Senna G, et al. Wasp venom allergy screening with recombinant allergen testing. *Diagnostic*

- performance of rPol d 5 and rVes v 5 for differentiating sensitization to *Vespula* and *Polistes* subspecies. *Clin Chim Acta* 2016;453:170-3.
11. Hamilton RG, Wisenauer JA, Golden DB, Valentine MD, Adkinson NF. Selection of Hymenoptera venoms for immunotherapy on the basis of patient's IgE antibody cross-reactivity. *J Allergy Clin Immunol* 1993;92:651-9.
 12. Matricardi PM, Kleine-Tebbe J, Hoffmann HJ, Valenta R, Hilger C, Hofmaier S, et al. EAACI molecular allergology user's guide. *Pediatr Allergy Immunol* 2016;27:1-250.
 13. Blank S, Neu C, Hasche D, Bantleon FI, Jakob T, Spillner E. *Polistes* species venom is devoid of carbohydrate-based cross-reactivity and allows interference-free diagnostics. *J Allergy Clin Immunol* 2013;131:1239-42.
 14. Di Fraia M, Arasi S, Castelli S, Dramburg S, Potapova E, Villalta D, et al. A new molecular multiplex IgE assay for the diagnosis of pollen allergy in Mediterranean countries: a validation study. *Clin Exp Allergy* 2019;49:341-9.
 15. Galindo-Bonilla PA, Galán-Nieto A, Alfaya-Arias T, García-Rodríguez C, de la Roca-Pinzón F, Feo-Brito F. Component-resolved diagnosis in vespid venom-allergic individuals. *Allergol Immunopathol (Madr)* 2015;43:398-402.
 16. Kohler J, Blank S, Muller S, Bantleon F, Frick M, Huss-Marp J, et al. Component resolution reveals additional major allergens in patients with honeybee venom allergy. *J Allergy Clin Immunol* 2014;133:1383-9.
 17. Vachová M, Panzner P, Kopač P, Bidovec Stojkovič U, Korošec P. Routine clinical utility of honeybee venom allergen components. *J Allergy Clin Immunol Pract* 2018;6:2121-3.
 18. Savi E, Peveri S, Makri E, Pravettoni V, Incorvaia C. Comparing the ability of molecular diagnosis and CAP-inhibition in identifying the really causative venom in patients with positive tests to *Vespula* and *Polistes* species. *Clin Mol Allergy* 2016;14:3.