ORIGINAL ARTICLE

Assessing the Allergenic Relevance of *Vespula alascensis* Venom: Implications for Venom Immunotherapy

Slusarenko BO[1], Borrmann KF[2,3], Knappe C[1], Vogel J[4], Schnautz B[1], Kler S[3], Biedermann T[5], Brockow K[5], Darsow U[5], Carreno Velazquez T[6], Francescato E[7], Hewings SJ[6], Kramer MF[6,8], Heath MD[6], Dittmar G[2,3], Hilger C[3], Peters RS[4], Schmidt-Weber CB[1], Eberlein B[5], Blank S[1]

[1]Center of Allergy and Environment (ZAUM), Technical University of Munich, School of Medicine and Health and Helmholtz Munich, German Research Center for Environmental Health, Munich, Germany
[2]Department of Life Sciences and Medicine, University of Luxembourg, Belvaux, Luxembourg
[3]Department of Infection and Immunity, Luxembourg Institute of Health (LIH), Esch-Sur-Alzette, Luxembourg
[4]Leibniz Institute for the Analysis of Biodiversity Change (LIB), Museum Koenig Bonn, Arthropoda Department, Bonn, Germany
[5]Department of Dermatology and Allergy Biederstein, School of Medicine and Health, Technical University of Munich, Munich, Germany
[6]Allergy Therapeutics PLC, Worthing, United Kingdom
[7]Entomon SRL, Florence, Italy
[8]Bencard Allergie GmbH, Munich, Germany

J Investig Allergol Clin Immunol 2026; Vol. 36(2)
doi: 10.18176/jiaci.1108

Abstract

Background: Venom immunotherapy (VIT) is an effective treatment for yellow jacket venom (YJV) allergy. It is often based on mixtures of venom from different species. Recent taxonomic re-evaluation has revealed that widely used VIT preparations contain venom from *Vespula alascensis*, a North American species, rather than venom from the Palearctic *Vespula vulgaris*.
Objective: To assess the allergenic relevance of *V alascensis* venom for European patients and evaluate its suitability for inclusion in VIT preparations.
Methods: The ability of *V alascensis* and *V vulgaris* venom to activate effector cells from YJV-allergic patients from Germany was compared using the basophil activation test. Patients' sIgE reactivity was assessed using ELISA, inhibition ELISA, CAP inhibition, and Western blot. Proteomic analysis of the venoms was also conducted.
Results: Basophil activation tests showed highly similar activation profiles for *V alascensis* and *V vulgaris* venoms. Moreover, ELISA and inhibition analyses revealed comparable overall sIgE reactivity for both venoms. In contrast, Western blot analysis demonstrated similar sIgE profiles for antigen 5 allergens, although phospholipase A1 allergens were differentially detected. Proteomic analysis revealed no differences in the major allergens of the 2 venoms.
Conclusion: This study demonstrates the functional similarity between *V alascensis* and *V vulgaris* venoms in allergic responses. Moreover, it highlights the clinical relevance of *V alascensis* for YJV-sensitized European patients and supports its inclusion in YJV VIT preparations in Europe.

Key words: Allergen. Allergen-specific immunotherapy. Cross-reactivity. Hymenoptera venom allergy. Venom immunotherapy. *Vespula alascensis*. *Vespula vulgaris*. Yellow jacket venom.

Resumen

Antecedentes: La inmunoterapia con venenos (ITV) es un tratamiento causal y eficaz para los pacientes alérgicos al veneno de véspula (VV), a menudo se utilizan mezclas de veneno de diferentes especies de véspula. Una reciente reevaluación taxonómica ha revelado que algunos preparados de inmunoterapia con veneno ampliamente utilizados contienen veneno de *Vespula alascensis*, una especie norteamericana, en lugar de veneno de *Vespula vulgaris*, una especie paleártica (europa y asia).
Objetivo: Evaluar la relevancia alergénica del veneno de *V. alascensis* para los pacientes europeos y valorar su idoneidad para incluirlo en los preparados de inmunoterapia con venenos (ITV).
Métodos: Se comparó la capacidad del veneno de *V. alascensis* y *V. vulgaris* para activar células efectoras de pacientes alérgicos al VV de Alemania mediante pruebas de activación de basófilos. La reactividad sIgE de los pacientes se evaluó mediante ELISA, ELISA-inhibición, CAP- inhibición y western blot. Además, se realizó un análisis proteómico de los dos venenos.
Resultados: Las pruebas de activación de basófilos mostraron perfiles de activación muy similares para los venenos de *V. alascensis* y *V. vulgaris*. Además, los análisis ELISA y las pruebas de inhibición revelaron una reactividad sIgE global comparable para ambos venenos.

Por el contrario, el western blot demostró perfiles de sIgE similares para los antígenos 5, aunque la fosfolipasa A1 se detectó de forma diferencial en el perfil alergénico. El análisis proteómico no reveló diferencias en los principales alérgenos de los dos venenos.
Conclusiones: Este estudio demuestra la similitud funcional entre los venenos de *V. alascensis* y *V. vulgaris* en las respuestas alérgicas. Además, pone de relieve la importancia clínica de *V. alascensis* para los pacientes europeos sensibilizados a VV y respalda su inclusión en los preparados de ITV para los pacientes alérgicos a VV en Europa.

Palabras clave: Alérgeno. Inmunoterapia alérgeno-específica. Reactividad cruzada. Alergia al veneno de himenópteros. Inmunoterapia con veneno. *Vespula alascensis*. *Vespula vulgaris*. Veneno de véspula.

Summary box

- **What do we know about this topic?**
  Taxonomic re-evaluation revealed that widely used yellow jacket venom immunotherapy (VIT) preparations contain venom from *Vespula alascensis*, a North American species, rather than from *Vespula vulgaris*. Detailed venom characterization is essential, as the allergenic relevance of *V alascensis* venom in Europe remains largely unexplored.
- **How does this study impact our current understanding and/or clinical management of this topic?**
  This study demonstrates the functional similarity of *V alascensis* and *V vulgaris* venoms in allergic responses. It highlights the importance of sensitization to *V alascensis* for European patients, supporting its inclusion in yellow jacket VIT preparations for Europe.

Introduction

Yellow jacket (*Vespula* species) venom (YJV) allergy is a leading cause of severe anaphylaxis [1-3]. Venom immunotherapy (VIT) is an effective treatment, achieving cure in between 91% and 99% of cases [4-6]. Several commercially available VIT preparations are formulated as mixtures of venoms from different *Vespula* species to maximize epitope coverage across a broad spectrum of potential primary sensitizers within the genus.

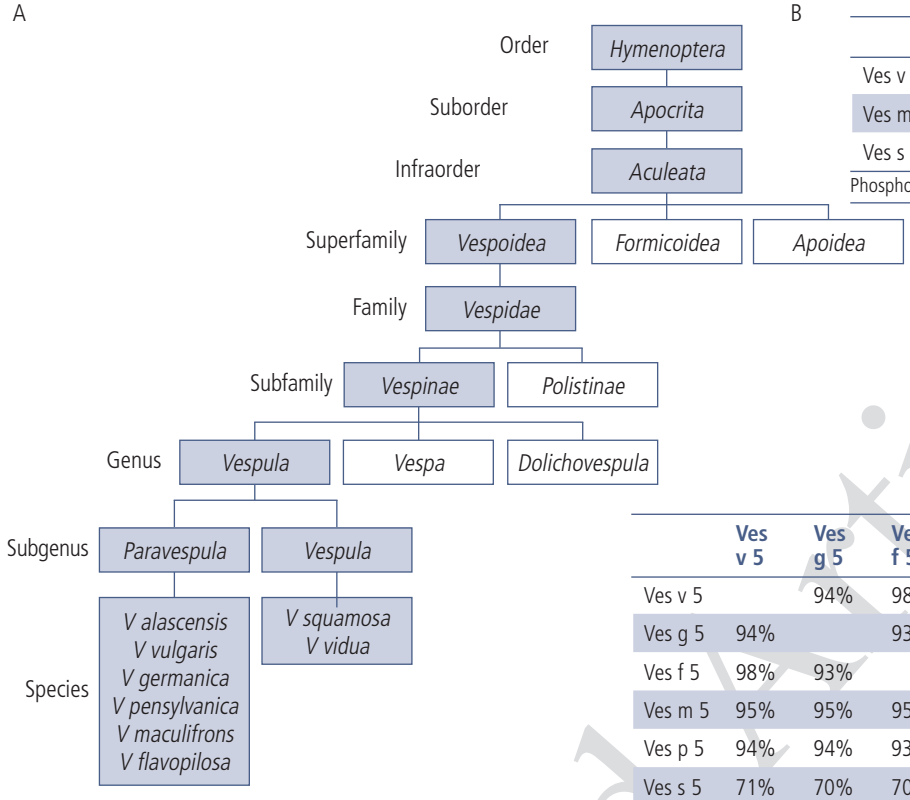
Nevertheless, these species are closely related, exhibiting a high degree of homology and, consequently, significant cross-reactivity between the relevant allergens [7,8]. The official WHO/IUIS Allergen Nomenclature list currently includes allergens from the yellow jacket species *Vespula flavopilosa*, *Vespula germanica*, *Vespula maculifrons*, *Vespula pensylvanica*, *Vespula squamosa*, *Vespula vidua*, and *Vespula vulgaris* [9]. Phylogenetic analyses show that, within the genus *Vespula*, the American species *V squamosa* and *V vidua* are distantly related to the other species, with *V squamosa* and *V vidua* belonging to the subgenus *Vespula*, and the remaining species belonging to the subgenus *Paravespula* (Figure 1A) [10,11]. As a result, the major venom allergens, phospholipase A1 (PLA1) and antigen 5 (Ag5), exhibit varying degrees of sequence identity between these 2 taxonomic groups. The Ag5 allergen sequences of all WHO/IUIS-listed species within the subgenus *Paravespula* show an intraspecific sequence identity of 93%-98%, whereas the interspecific sequence identity with the subgenus *Vespula* Ag5 amounts to 71%-74%. Similar levels of sequence identity are observed for the PLA1 allergens (Figure 1B).

V germanica and *V vulgaris* are originally Palearctic species but have also been introduced to many regions worldwide [12]. In contrast, the other species mentioned above are primarily restricted to North America, with limited distribution in parts of Central America [13].

Although *Vespula alascensis*, a species native to North America, was named in 1870, it was long treated as a taxonomic synonym of the related species *V vulgaris*. However, in 2010, it was recognized as a distinct species [14]. Therefore, contrary to previous assumptions, *V vulgaris* does not appear to be present in North America. As a result, commonly used VIT formulations with source materials originating from the US, such as Alutard SQ Wasp® (mixture of *V germanica*, *V alascensis*, *V maculifrons*, *V pensylvanica*, *V squamosa*, and *V flavopilosa* venoms) [15,16] and Venomil® Wasp (mixture of *V germanica*, *V alascensis*, *V maculifrons*, *V pensylvanica*, and *V squamosa* venoms) [17] contain *V alascensis* rather than *V vulgaris* venom.

V alascensis and *V vulgaris* are closely related species [11]. Nevertheless, the revised taxonomic classification creates a particular allergological gap, as virtually no studies exist on the allergological relevance of *V alascensis* venom. Currently, it remains unclear which existing data, particularly from studies conducted in the US, were derived using *V alascensis* or *V vulgaris* venom. However, given the presence of *V alascensis* venom in key VIT preparations, assessing its allergological relevance is essential. Therefore, this study aimed to provide a detailed comparative proteomic analysis and evaluation of the allergological significance of *V alascensis* and *V vulgaris* venoms.

A



B

	Ves v 1	Ves m 1	Ves s 1
Ves v 1		96%	71%
Ves m 1	96%		71%
Ves s 1	71%	71%	

Phospholipase A1 allergens.

	Ves v 5	Ves g 5	Ves f 5	Ves m 5	Ves p 5	Ves s 5	Ves vi 5
Ves v 5		94%	98%	95%	94%	71%	74%
Ves g 5	94%		93%	95%	94%	70%	74%
Ves f 5	98%	93%		95%	93%	70%	74%
Ves m 5	95%	95%	95%		93%	72%	73%
Ves p 5	94%	94%	93%	93%		72%	74%
Ves s 5	71%	70%	70%	72%	72%		80%
Ves vi 5	74%	74%	74%	73%	74%	80%	

Antigen 5 allergens.

Figure 1. Allergy-relevant *Vespula* species and their inter-relationships. A, Classification of *Vespula* species currently listed in the WHO/IUIS allergen nomenclature database and *Vespula alascensis*. From the full Hymenoptera tree, only a selection of allergy-relevant taxa are shown. B, Percentage of identity at protein sequence level of phospholipase A1 and antigen 5 allergens of *Vespula* species currently listed in the WHO/IUIS allergen nomenclature database.

Methods

Venoms

V alascensis venom extract was prepared by homogenizing 600 hand-dissected venom sacs in 30 mL of β-alanine-acetic acid buffer (25 mM β-alanine, 75 mM NaCl, 13 mM KCl, pH 4.6 [adjusted with acetic acid]) using a tissue grinder and then centrifuging at not less than 12 000 rcf for 30 minutes at 4°C. The supernatant was collected and sterile-filtered. Entomon Capillary Extracted Venom® *V vulgaris* sourced from New Zealand (NZ) or Italy (IT) was provided lyophilized by Entomon. Venomil® Wasp was acquired from Allergy Therapeutics. Proteins were determined using bicinchoninic acid assay (Thermo Fisher Scientific).

Patients

Blood and/or serum samples from 63 YJV-allergic patients (40 female and 23 male; mean age, 49.6 years) and 5 nonallergic controls from Southern Bavaria (Germany) were included in the study. YJV allergy was diagnosed based on a combination of clinical history and skin testing and/or measurement of sIgE to venoms and molecular allergens. Patients' clinical data are given in Table S1. Signed written consent was obtained from all participants before enrolment in the study. The study was conducted in accordance with the Declaration of Helsinki. The protocol was approved by the ethics committee of the Faculty of Medicine of the Technical University of Munich, Germany (approval numbers: 538/17S and 5478/12).

Sequence Analysis

The protein sequence analysis of allergens and the DNA barcode sequence analysis are described in Supplementary Methods.

Basophil Activation Test

Basophil activation tests (Flow CAST, Bühlmann Laboratories AG) with the different venoms were performed as previously described [18]. A brief description is given in the Supplementary Methods.

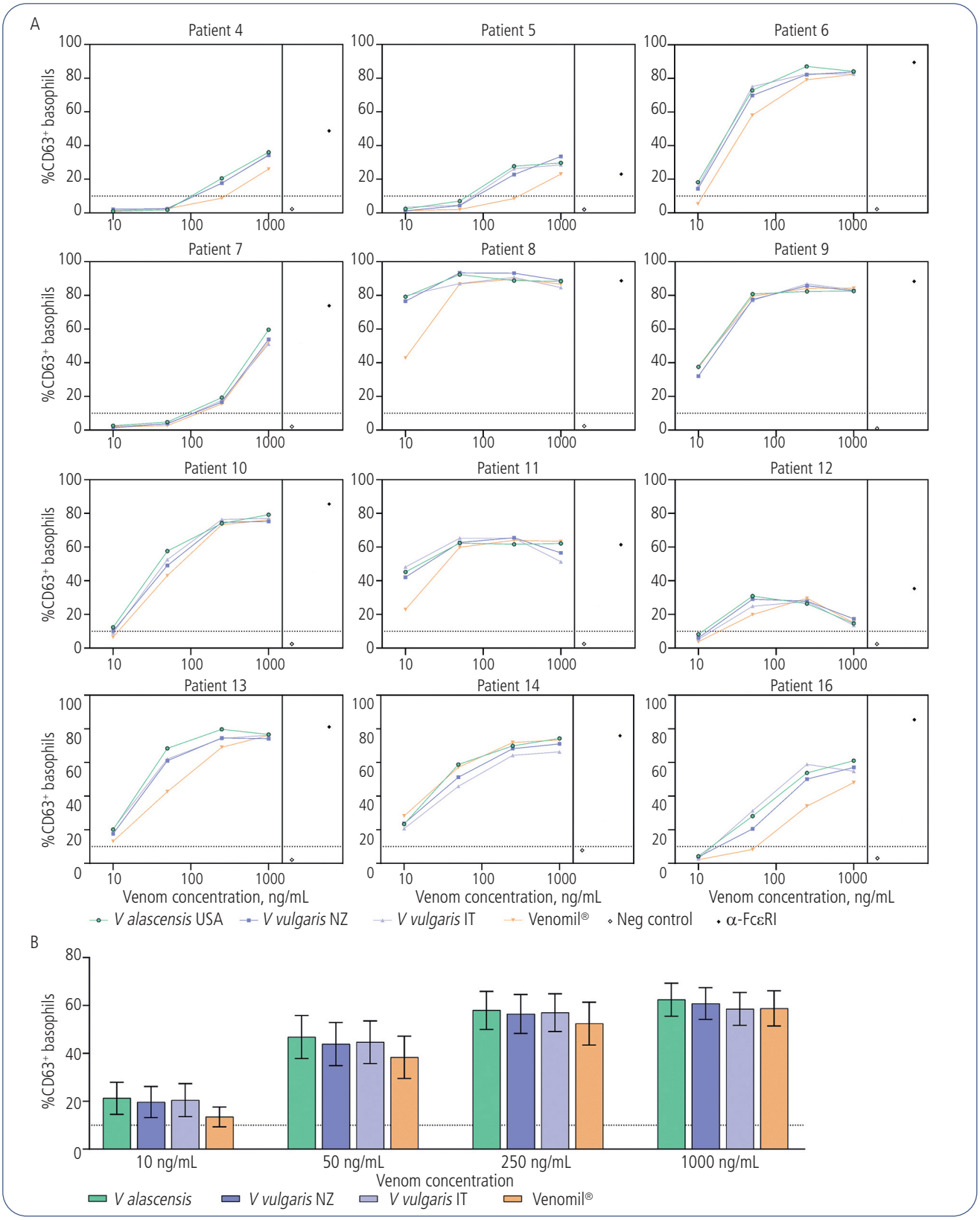


Figure 2. Basophil activation tests with *Vespula* species venoms. Basophils from yellow jacket venom–allergic patients from Germany were exposed to different concentrations of *Vespula alascensis* venom, *Vespula vulgaris* venom, and the therapeutic venom preparation Venomil®. Activation is shown as the percentage of CD63+ basophils out of total basophils. A dotted line indicates the cut-off of the assay. A, Dose-response curves of individual patients. Additionally, stimulation with anti-FcεRI (positive control) and plain stimulation buffer (negative control) is shown. B, Combined data from all patients. IT indicates Italy; NZ, New Zealand.

Measurement of sIgE Reactivity

A detailed description of the ELISA, inhibition ELISA, CAP-inhibition, SDS-PAGE, and Western blot analyses is given in Supplementary Methods.

Proteomic Analysis

A detailed description of the mass spectrometry analysis of gel bands and whole venoms is given in Supplementary Methods.

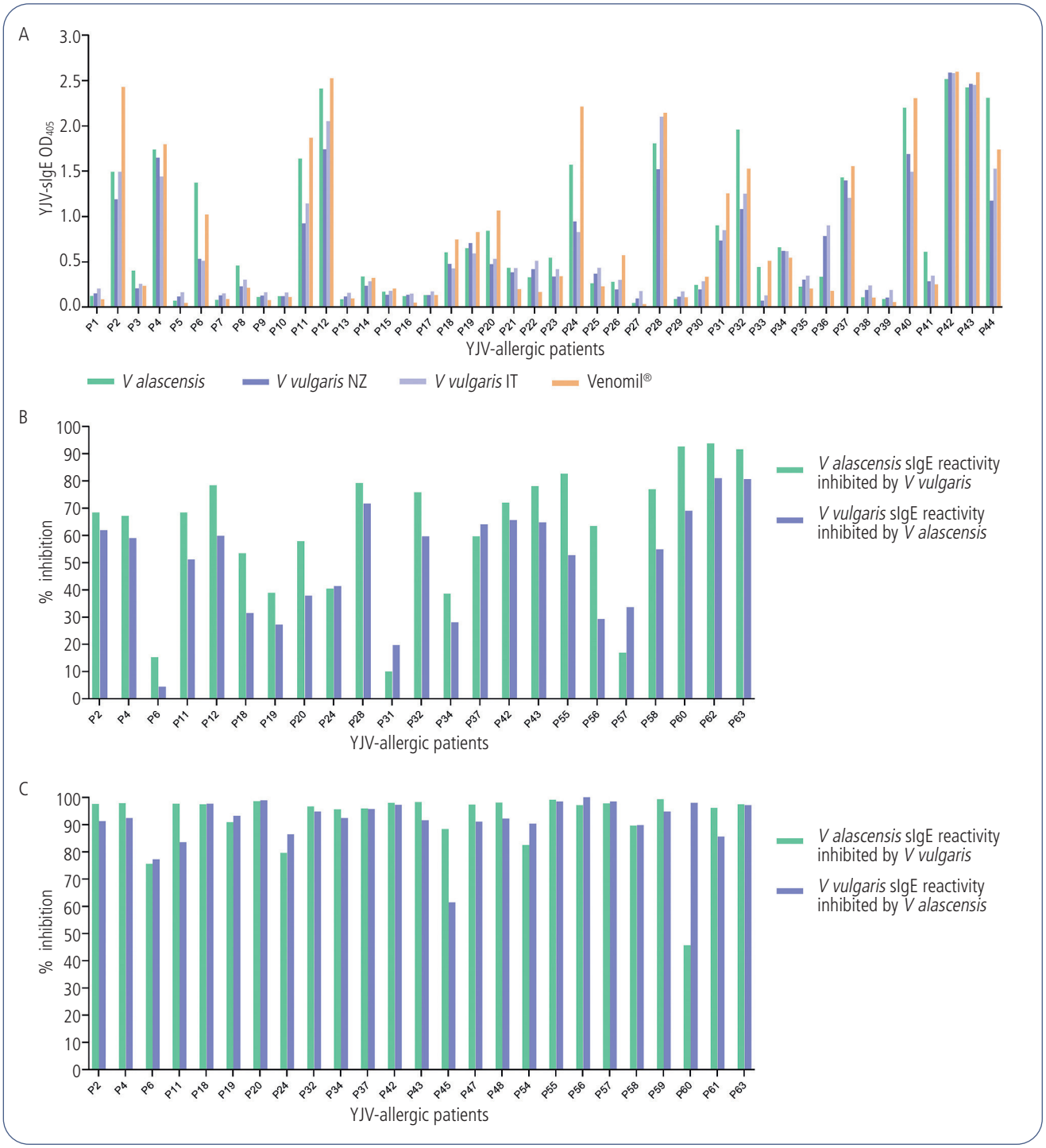


Figure 3. sIgE reactivity of yellow jacket venom–allergic patients from Germany with the different *Vespula* species venoms. A, sIgE immunoreactivity of patients in ELISA. B, Inhibitory capacity of *Vespula alascensis* and *Vespula vulgaris* (IT) venom on sIgE immunoreactivity against the respective other venom in ELISA. C, Inhibitory capacity of *V alascensis* and *V vulgaris* (IT) venom on sIgE immunoreactivity against the respective other venom in CAP-inhibition. IT indicates Italy; NZ, New Zealand; YJV, yellow jacket venom.

Results

Taxonomic Re-evaluation of V alascensis and V vulgaris

The results of the analyses of DNA sequence data in Carpenter and Glare [14] are clear, showing that the North American specimens differ from the Eurasian/New Zealand specimens (Figure 1 in Carpenter and Glare). However, specimen sampling was very limited (only 2 and 5 specimens, respectively), and the analyses lacked some standard methods and metrics.

The comparison of all 514 CO1 (cytochrome oxidase subunit 1) DNA barcode sequences (169 *V alascensis*, 345 *V vulgaris*, Supplementary Data S1) show that the 2 species can be molecularly differentiated from each other (Supplementary Results S1). Both species form an individual BIN in BOLD [19] (when corrected for misidentifications) and have a minimum interspecific difference of 6.2% and a maximum intraspecific difference of 3.1%.

Activation of Basophils in YJV-Allergic Patients

Basophil activation tests (BATs) were performed to assess the ability of the different *Vespula* venoms to activate effector cells (Figure 2 and S1). Dose-response curves were generated using blood samples from 13 YJV-allergic patients from Southern Bavaria, Germany prior to the initiation of VIT, as well as from 5 nonallergic controls.

Basophil activation (% $CD63^+$ basophils) in 12 responders with YJV allergy was characterized by very similar activation profiles across all tested venoms and venom concentrations (Figure 2A). No significant differences in basophil activation were observed between *V alascensis*, *V vulgaris*, and the venom mixture (Venomil®) (Figure 2B). One patient was identified as a nonresponder (Figure S1). The nonallergic controls exhibited no basophil activation in response to any of the venoms tested (Figure S1).

Specific IgE Reactivity of YJV-Allergic Patients

The sIgE reactivity to the different *Vespula* venoms was first assessed with ELISA using the sera of 44 YJV-allergic patients from Germany prior to the initiation of VIT (Figure 3A). Overall, the reactivity profiles of the sera across the different venoms were comparable. For nearly all patients with measurable YJV-sIgE reactivity in ELISA, pronounced reactivity was observed with *V alascensis* venom, *V vulgaris* venom (NZ and IT), and the therapeutic venom mixture Venomil®. Notably, 1 patient (P33) exhibited a positive signal almost exclusively with *V alascensis* venom and Venomil®. Interestingly, for several patients, including patients 6, 11, 24, and 40, the reactivity was slightly higher to these 2 venoms than to the 2 *V vulgaris* venom preparations.

To evaluate the cross-reactivity of patient sIgE antibodies between *V alascensis* and *V vulgaris* venom and to gain insights into the extent of shared epitopes, inhibition ELISA experiments were performed using 23 sera from YJV-allergic patients, which exhibited pronounced sIgE reactivity with the venoms in ELISA (Figure 3A). Both *V alascensis* and *V vulgaris* IT venom displayed similar inhibitory capacities (Figure 3B). While the inhibitory capacity of *V vulgaris* venom was slightly higher for most sera than that of *V alascensis* venom, the differences were not statistically significant. In addition, CAP inhibition was performed with sera from 25 patients using biotinylated *V alascensis* and *V vulgaris* (IT) venoms coupled to streptavidin ImmunoCAPs (Figure 3C). Similarly, in these experiments, the inhibitory capacity of both venoms was largely comparable for most patients. The percent inhibition values observed were even

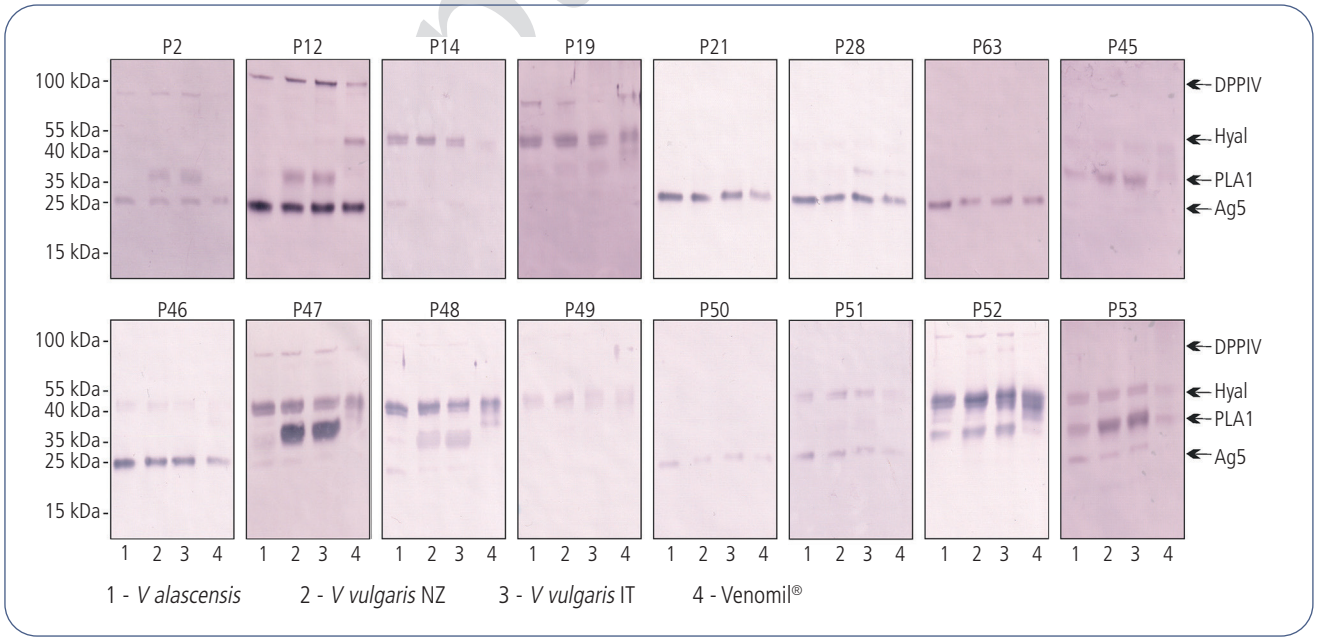


Figure 4. sIgE immunoreactivity of patients in Western blot. Ag5 indicates antigen 5; DPPIV, dipeptidyl peptidase IV; Hyal, hyaluronidase; IT, Italy; NZ, New Zealand; PLA1, phospholipase A1.

higher than those obtained in the ELISA analysis. Only in patients 45 and 50 was a higher inhibitory capacity observed for *V vulgaris* and *V alascensis* venom, respectively.

To gain more detailed insights into sIgE specificity at a component-resolved level, Western blot analyses were performed using 16 sera (Figure 4). For all sera clearly recognizing Ag5 allergens (patients 2, 12, 21, 28, 36, 46, 50, and 51), sIgE reactivity was comparable with Ag5 from *V alascensis* venom, *V vulgaris* venom, and the therapeutic venom mixture Venomil®, although the latter sometimes showed slightly weaker signal intensities (eg, patients 21, 28, and 46). Only patient 53, who exhibited weak Ag5 reactivity, showed distinctly weaker binding to Ag5 in Venomil®. It is important to note that Venomil® contains venom from 5 species, as opposed to a single species, to cover a broader epitope spectrum.

Hyaluronidase sIgE recognition was similar in patients 19, 45, 46, 47, 48, 49, 51, 52, and 53, with patients 45 and 46 showing only weak signal intensities. While patient 12 reacted exclusively to hyaluronidase from Venomil®, patient 14 showed higher reactivity to hyaluronidase from the other 3 venoms.

Dipeptidyl peptidase IV (DPPIV) sIgE reactivity (patients 2, 12, 19, 47, 48, and 52) was comparable between *V alascensis* and *V vulgaris* venoms but was weaker for Venomil®. This reduced reactivity corresponds to the lower DPPIV content observed in the SDS-PAGE analysis of Venomil® (Figure S2).

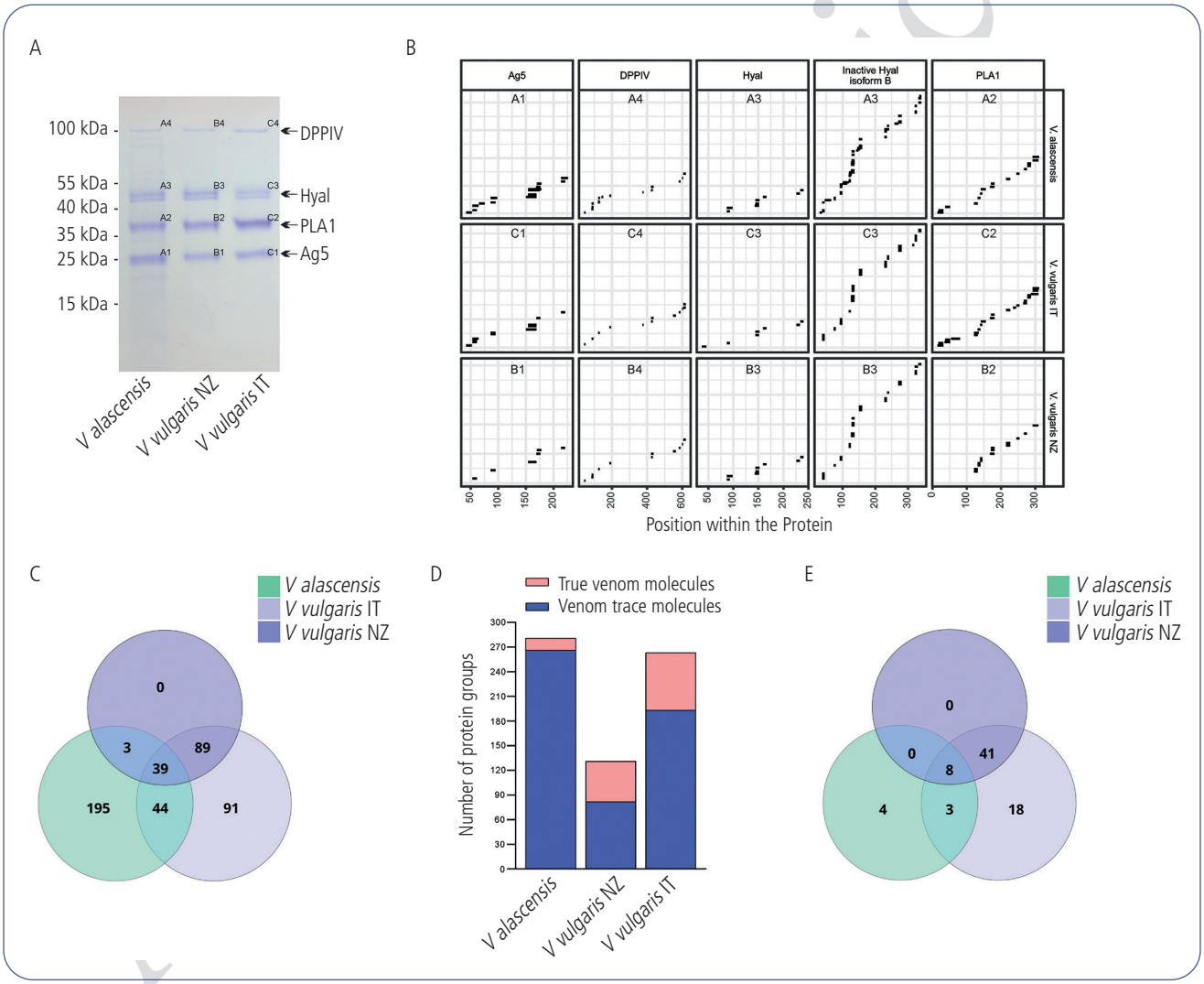


Figure 5. Proteomic analysis of *Vespula* species venoms. A, SDS-PAGE analysis. Alphanumeric labels within the gel indicate protein bands excised for mass spectrometry analysis. In samples A3, B3, and C3, the double bands were excised together, as further separation was not possible. B, Peptides from the proteins identified within the bands with the highest intensity with their positions within the protein sequences. The alphanumeric labels shown here correspond to those assigned to the gel bands in panel A. C, Number of protein groups identified in the whole venom extracts, with overlapping regions showing shared proteins across the different venoms. D, Number of true venom molecules, including proteins actively transported to the extracellular space, annotated as allergens, and/or with known venom-related functions, and venom trace molecules, originating primarily from damaged venom gland cells and identified in whole venom extracts. E, Number of true venom molecules identified in the whole venom extracts, with overlapping regions showing shared proteins across the venoms. Ag5 indicates antigen 5; DPPIV, dipeptidyl peptidase IV; Hyal, hyaluronidase; IT, Italy; NZ, New Zealand; PLA1, phospholipase A1.

Of particular interest were the differences observed in the recognition of PLA1 allergens. Patients 2, 12, 47, and 48 exhibited strong reactivity with PLA1 from the 2 *V vulgaris* venom preparations but showed almost no reactivity with PLA1 from *V alascensis* venom or Venomil®. Additionally, for the other clearly PLA1-reactive patients, ie, 52 and 53, reactivity was more pronounced with *V vulgaris* PLA1, while patient 28 showed only a weak signal.

Proteomic Analysis of V alascensis and V vulgaris Venom

V alascensis and *V vulgaris* venoms exhibited similar band patterns in the SDS-PAGE analysis, with prominent bands at approximately 25, 36, 45, and 100 kDa (Figure 5A). Proteomic analysis of the excised bands confirmed that the proteins with the highest intensities within these bands corresponded to the expected allergens: Ag5, PLA1, hyaluronidase (2 isoforms), and DPPIV. All allergens were identified by multiple peptides in all venoms (Figure 5B). Importantly, none of the peptides identified in *V alascensis* and *V vulgaris* venoms showed any amino acid sequence differences compared to the known sequences of *V vulgaris* allergens within the identified peptides.

In the whole venom proteome analysis, 281, 131, and 263 protein groups—each containing proteins that share the same identified peptide sequence(s)—were detected in *V alascensis*, *V vulgaris* NZ, and *V vulgaris* IT venoms, respectively. An overlap across all 3 venoms was observed for 39 protein groups (Figure 5C), of which most are likely venom trace molecules derived from the breakdown of venom gland cells and surrounding tissues or are components of the extracellular matrix [20]. Proteins actively transported to the extracellular space, annotated as allergens, or with known venom-related functions were classified as "true venom molecules" [20-22] and are listed in Table S2. Notably, the proportion of these true venom molecules was higher in the 2 *V vulgaris* venoms than in *V alascensis* venom (Figure 5D). This difference is most likely caused by the preparation methods, as the *V vulgaris* venoms were extracted via capillary methods, whereas *V alascensis* venom was derived from whole venom gland homogenates. Shared true venom molecules across all 3 venoms (Figure 5E) include well-established allergens and intriguing new allergen candidates, such as DOMON domain–containing protein and apolipophorin. Another protein identified in the 2 *V vulgaris* venom preparations that warrants further investigation is icarapin-like protein, a homolog of the major honeybee venom allergen Api m 10 [23-26].

Discussion

VIT with YJV is one of the most effective forms of allergen-specific immunotherapy, providing robust protection against potentially life-threatening systemic reactions [4-6]. Although the high efficacy of VIT already suggests that the currently used therapeutic preparations are well-suited to provide effective immunological protection, the recent taxonomic distinction of *V alascensis* from *V vulgaris* has introduced uncertainty regarding the composition and allergological relevance of venoms used in VIT preparations. While these species are closely related, the inclusion of *V alascensis* venom in key therapeutic formulations underscores the need to better understand its proteomic and allergenic characteristics. Our study addresses this gap by conducting a detailed comparative analysis of *V alascensis* and *V vulgaris* venoms.

In 2010, Carpenter and Glare [14] suggested that the North American yellow jackets formerly treated as belonging to a Holarctic *V vulgaris* (Linnaeus, 1758) actually belong to a different species. Delimitation between these 2 species was based on morphological differences and differences in the nucleotide sequence of the mitochondrial gene CO1. Specifically, they compared specimens from North America to those from Eurasia and New Zealand (where *V vulgaris* is an introduced species [27]) and highlighted differences in the male genitalia of both species. The paper lacks a formal diagnosis of the North American species, and the authors argue that the different shape of the apex of the male digiti in ventral view alone is a clear morphological character for differentiation. Coloration of the propodeum is also discussed. However, owing to intraspecific variation, its value for differentiation is limited. In a later publication, the North American species is keyed out based on differences in coloration, mainly of the head and metasoma [13]. The key only addresses the North American fauna, and, therefore, *V vulgaris* was not included. Differentiation from all remaining North American species of Vespula and related genera is undisputed [13].

Our newly extended analysis of DNA barcode data provides clear support for the differentiation between the North American *V alascensis* and the Eurasian *V vulgaris*. From a nomenclatural perspective, Carpenter and Glare [14] argue compellingly that the North American species should be designated *V alascensis* (Packard, 1870), a name previously considered a synonym of *V vulgaris*.

The most relevant allergens in YJV are PLA1 and Ag5, referred to as Ves v 1 and Ves v 5 in *V vulgaris* venom, respectively [28]. The primary amino acid sequences of *V vulgaris* PLA1 and Ag5 were first published in 1996 [29] and 1993 [30], respectively. Notably, both studies were conducted in the US, suggesting that these sequences are likely derived from *V alascensis* rather than *V vulgaris*. The same applies to numerous other studies on *V vulgaris* venom conducted in the US. Consequently, it can be reasonably assumed that much of the allergological information attributed to *V vulgaris* venom is actually a mixture of data derived from *V alascensis* and *V vulgaris* venom.

However, the mixed nature of these allergological studies likely does not hamper the conclusions drawn or their clinical evidence, as the 2 species are closely related and their venom allergens are, consequently, highly homologous. Proteomic analysis of the major allergens (Ag5, PLA1, 2 hyaluronidase isoforms, and DPPIV) from gel bands revealed no differences between the *V alascensis* and *V vulgaris* venoms, with all identified peptides exactly matching the amino acid sequence information available in the UniProt database for known *V vulgaris* proteins. However, it remains speculative whether this reflects a true distinction between the 2 species or whether it represents a mixture of *V vulgaris* and *V alascensis* entries in the database, considering that the taxonomic reclassification has only been in place since 2010. Of note, more peptides

were identified for the inactive hyaluronidase isoform B than for the active isoform, corresponding to their reported relative abundance in the venom [31,32].

The ratio of venom trace molecules (originating from sources such as damaged cells of the venom gland) to true venom molecules with venom functions [20-22] was higher in *V alascensis* venom than in the 2 *V vulgaris* venom preparations. This difference likely reflects the differing venom extraction methods applied. However, the increased number of venom trace molecules, which likely have minimal allergological relevance, is unlikely to affect the immunological findings of this study, especially considering the qualitative nature of the proteomic analysis and the highly comparable protein profiles of all venoms in the SDS-PAGE analysis. Nevertheless, the proteomic analysis identified proteins that warrant further investigation regarding their potential roles as allergen candidates.

In our study, the analysis of sIgE reactivity and basophil activation was conducted with YJV-allergic patients from Southern Bavaria, Germany, suggesting that these patients are primarily sensitized to either *V vulgaris* or *V germanica* venom.

The ELISA results showed that most YJV-allergic patients exhibited similar sIgE reactivity to *V alascensis*, *V vulgaris* (NZ and IT), and the therapeutic mixture Venomil®. The slightly higher sIgE reactivity observed in some European patients to *V alascensis* venom and Venomil® suggests that there is no disadvantage in terms of IgE epitopes in *V alascensis* compared to *V vulgaris*. Inhibition ELISA and CAP inhibition demonstrated comparable inhibitory capacity for *V alascensis* and *V vulgaris* venoms, again suggesting that both venoms share a similar spectrum of IgE epitopes. This finding further underscores the close immunological relationship between the 2 venoms. Although the high conformity of sIgE reactivity between *V vulgaris* and *V alascensis* venoms observed in this study may not be unexpected, as the relevant allergens of various *Vespula* species were already reported to be almost completely cross-reactive [7,8], providing evidence of this for *V alascensis* is crucial given its inclusion in therapeutic preparations.

In the Western blot analysis, all sera demonstrated comparable reactivity with Ag5 allergens from *V alascensis* venom, *V vulgaris* venoms, and the therapeutic venom mixture. For some sera, which showed Ag5 sIgE reactivity in ImmunoCAP measurements (Table S1), reactivity was absent or very low in the Western blots. However, since this finding was consistent across both *V vulgaris* and *V alascensis* venom, it can most likely be attributed to differences in the sensitivity of sIgE detection between the ImmunoCAP and Western blot techniques. Additionally, sIgE reactivity to the DPPIV and hyaluronidase allergens of both venoms was comparable for most patients. The number of hyaluronidase-reactive patients was higher than expected, as the rate of sensitization to YJV hyaluronidase is typically low [31-33]. However, for 4 of the hyaluronidase-reactive sera (patients 14, 48, 52, and 53), reactivity may be attributed to the recognition of cross-reactive carbohydrate determinants, based on sIgE levels to MUXF3. Notably, patient 12 showed exclusive reactivity with the hyaluronidase band of the therapeutic venom mixture. The reactivity profile of this patient (characterized by recognition of DPPIV and AG5 in all venom samples, PLA1 recognition only in *V vulgaris* venom, and hyaluronidase recognition only in the venom mixture) is particularly interesting. This might suggest a primary sensitization to *V vulgaris* venom, leading to PLA1 recognition of the 2 *V vulgaris* venom preparations, as well as a primary sensitization to *V germanica* venom, leading to the recognition of hyaluronidase in the therapeutic venom mixture, which contains *V germanica* venom. Given that *V vulgaris* and *V germanica* are the main sensitizers to YJV in Germany and that the other species in the venom mixture are native to North America and not present in Germany, this appears to be the most plausible explanation. However, a primary sensitization to another YJV species during a stay in another country cannot be entirely ruled out. For patient 14, reactivity with the hyaluronidase-containing band was lower with the venom mixture than with *V alascensis* or *V vulgaris* venom, a fact most likely reflecting the relative abundance of the primary sensitizing venom within the mixture. In contrast to Ag5, which mostly showed a comparable signal between the venoms, the situation was notably different for the second major allergen, PLA1. Almost all reactive sera demonstrated strong sIgE reactivity with PLA1 (Ves v 1) from the 2 *V vulgaris* venom samples while showing weaker or no reactivity with PLA1 from *V alascensis* venom or the therapeutic venom preparation. This divergent recognition pattern could be attributed to potential sequence differences in specific regions of the *V alascensis* PLA1 that were not detected in the proteomic analysis or may result from protein modifications unique to *V alascensis* PLA1. Notably, CAP inhibition in selected patients with weaker or absent PLA1 recognition in Western blots revealed a heterogeneous picture: patient 45 showed stronger inhibition with *V vulgaris* venom, supporting differences in the epitope repertoire. In contrast, patients 47 and 48 had similar inhibition patterns for both *V alascensis* and *V vulgaris* venom, indicating a comparable IgE-binding capacity of both venoms despite the weal or absent recognition of *V alascensis* PLA1 in immunoblots for these patients. Unfortunately, there is currently no detailed information about IgE epitopes of Ves v 1 or other vespid PLA1 allergens that might shed further light on this observation.

Intriguingly, in the basophil activation tests, *V alascensis* and *V vulgaris* venom exhibited very similar dose-response curves, suggesting that both venoms possess an equivalent ability to induce clinically relevant effector cell activation. Importantly, this observation suggests that the differences in PLA1 recognition observed in Western blot analysis do not influence basophil activation, reinforcing the functional similarity of the venoms in the context of effector cell responses [34]. Despite the high allergenic relevance of PLA1 in YJV allergy [35], this observation, together with the high efficacy of the *V alascensis*–containing therapeutic YJV preparation in European patients, may imply the following: (*1*) the observed differences in PLA1 recognition are not clinically relevant for most patients; and/or (*2*) the immunoblotting results are qualitative and less precise or quantitatively relevant for allergenic responses compared to the basophil activation test and ELISA; and/or (*3*) the clinically relevant response might be predominantly driven by Ag5, which is recognized by approximately 82%-98% of YJV-allergic patients [36,37] and similarly across all venoms in this study.

In summary, given that *V alascensis* venom is included in widely used VIT preparations, its detailed characterization is essential. The high efficacy of these products suggests their suitability for therapeutic use. However, the recent taxonomic distinction between *V alascensis* and *V vulgaris* has emphasized the need to characterize *V alascensis* venom and thus clarify the composition of preparations used in VIT. Despite this reclassification, the findings of our study demonstrate that the venoms of both species share a high degree of immunological similarity. Proteomic and immunological analyses indicate that the differences between the venoms are minimal and unlikely to have significant clinical implications, more so because both induce similar basophil activation profiles, and that VIT preparations containing *V alascensis* venom have led to high cure rates in European patients in recent decades. Considering the relevance of *V alascensis* venom in YJV-allergic patients and its inclusion in several US studies under the name *V vulgaris*, the relevant allergens PLA1, hyaluronidase, DPPIV, and Ag5 should be added to the official WHO/IUIS allergen nomenclature as Ves a 1, Ves a 2, Ves a 3, and Ves a 5, respectively. In conclusion, this study highlights the functional similarity between *V alascensis* and *V vulgaris* venoms in allergic responses. Given the minimal differences observed between the venoms, our findings support the use of *V alascensis* venom as a substitute for *V vulgaris* venom in YJV VIT preparations for European YJV-allergic patients.

Funding

This study was funded in part by a research grant from Bencard Allergie GmbH to SB. The company had no role in the study design; collection, analysis, and interpretation of data; or the decision to submit the article for publication. KFB was supported by the Luxembourg National Research Fund (PRIDE21/16749720/NEXTIMMUNE). BE received study material at a discount from Bühlmann Laboratories.

Conflicts of Interest

JV received third party funding via a grant from the Federal Ministry of Education and Research (BMBF; GBOL III Dark Taxa), outside the submitted work. TB has provided advice to and received honoraria for talks and consulting fees from ALK-Abelló A/S, outside the submitted work. KB has provided consultancy to or has received honoraria for talks or research grants from Thermo Fisher Scientific Inc., MADX GmbH, Blueprint Medicine Corp., ALK-Abelló A/S, Bencard Allergie GmbH, HAL Allergy, BioMarin Pharmaceuticals Inc., Astra Zeneca PLC, and Advarra Inc., outside the submitted work. TCV, SJH, and MFK are employees of Allergy Therapeutics PLC/Bencard Allergie GmbH. EF is the General Director of Entomon S.R.L. MDH has been an employee of Allergy Therapeutics PLC. CH has received payment for lectures from ALK-Abelló A/S, outside the submitted work. CBS-W has provided consultancy to or has received honoraria for talks or research grants from Allergopharma GmbH & Co. KG, Max Zeller Söhne AG, and LETI Pharma GmbH, outside the submitted work. BE has received financial support from Bühlmann Laboratories AG, outside the submitted work. SB has provided consultancy to or has received honoraria for talks or research grants from Thermo Fisher Scientific Inc., Bencard Allergie GmbH, Allergopharma GmbH & Co. KG, LETI Pharma GmbH, and Allergy Therapeutics PLC, outside the submitted work. The remaining authors declare that they have no conflicts of interest.

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Manuscript received December 19, 2024; accepted for publication May 26, 2025.

Simon Blank

Center of Allergy and Environment (ZAUM)
Technical University of Munich and Helmholtz Munich
Ingolstädter Landstraße 1
85764 Munich, Germany
E-mail: simon.blank@tum.de