Assessing the allergenic relevance of *Vespula alascensis* venom: Implications for venom immunotherapy

Running title: Allergenic relevance of *V. alascensis* venom

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Author contributions

BOS and KFB performed experiments, analyzed data, and wrote the manuscript. CK, BS, and SK performed experiments. JV and RSP performed the taxonomic evaluation and wrote the manuscript. KB and UD supervised the recruitment of patients, analyzed data, and revised the manuscript. TCV and EF supervised the preparation of venom extracts and revised the manuscript. SJH, MFK, and MDH initiated the study, discussed the data, and revised the manuscript. TB, CH, and CBS-W supervised the study, analyzed data, and revised the manuscript. GD supervised the proteomic analysis, discussed the data, and revised the manuscript. BE recruited patients, supervised basophil activation testing, analyzed data, and revised the manuscript. SB planned and supervised the study, analyzed data, and wrote the manuscript.

Abstract

Background: Venom immunotherapy (VIT) is an effective, causative treatment for yellow jacket venom (YJV)-allergic patients, often using venom mixtures of different species. Recent taxonomic reevaluation 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 venom immunotherapy (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 basophil activation tests. Patients' sIgE reactivity was assessed by ELISA, inhibition ELISA, and Western blot. Additionally, proteomic analysis of the venoms was conducted.

Results: Basophil activation tests showed highly similar activation profiles for *V. alascensis* and *V. vulgaris* venoms. Moreover, ELISA and inhibition ELISA analyses revealed comparable overall sIgE reactivity for both venoms. In contrast, Western blotting demonstrated similar sIgE profiles for antigen 5 allergens, though phospholipase A1 allergens were differentially detected. Proteomic analysis revealed no differences in the major allergens of the two 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.

Keywords: allergen; allergen-specific immunotherapy; cross-reactivity; Hymenoptera venom allergy; venom immunotherapy; *Vespula alascensis*; *Vespula vulgaris*; yellow jacket venom

Summary box

What do we know about this topic?

Taxonomic reevaluation revealed that widely used yellow jacket venom immunotherapy (VIT) preparations contain *Vespula alascensis*, a North American species, instead of *Vespula vulgaris* venom. A detailed venom characterization is essential, as its allergenic relevance 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 *Vespula alascensis* and *Vespula vulgaris* venoms in allergic responses. It highlights the importance of *V. alascensis* sensitization for European patients, supporting its inclusion in yellow jacket VIT preparations for Europe.

Abbreviations

Ag5, antigen 5

BAT, basophil activation test

ELISA, enzyme-linked immunosorbent assay

PLA1, phospholipase A1

sIgE, specific immunoglobulin E

VIT, venom immunotherapy

WB, Western blot

YJV, yellow jacket venom

Introduction

Yellow jacket (*Vespula* spp.) venom (YJV) allergy is a leading cause of severe anaphylaxis [1-3]. However, effective and causative treatment is possible through venom immunotherapy (VIT), achieving between 91% and 99% curative outcomes [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 this genus.

Nevertheless, these species are closely related, exhibiting a high degree of homology and, consequently, significant cross-reactivity among the relevant allergens [7, 8]. The official WHO/IUIS Allergen Nomenclature list currently includes allergens from the yellow jacket species *V. flavopilosa, V. germanica, V. maculifrons, V. pensylvanica, V. squamosa, V. vidua,* and *V. vulgaris* [9]. Phylogenetic analyses show that, within the genus *Vespula*, the American species *V. squamosa* and *V. vidua* are quite distantly related to the remaining 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 two taxonomic groups. The Ag5 allergens, with sequences known for all WHO/IUIS-listed species, show 93-98% sequence identity within the subgenus *Paravespula* and 71-74% identity when compared to those of the subgenus *Vespula*. 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 other 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 preparations 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* venom) [15, 16] or Venomil® Wasp (mixture of *V. germanica*, *V. alascensis*, *V. maculifrons*, *V. pensylvanica*, and *V. squamosa* venom) [17], contain *V. alascensis* rather than *V. vulgaris* venom within the venom formulation mixture.

V. alascensis and *V. vulgaris* are indeed 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 United States - 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* venom.

Methods

Venoms

For the preparation of *V. alascensis* venom extract, 600 hand-dissected venom sacs were homogenized in 30 mL β-alanine-acetic acid buffer (25 mM β-alanine, 75 mM NaCl, 13 mM KCl, pH 4.6 (adjusted using acetic acid)) using a tissue grinder and then centrifuged 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) were provided lyophilized by Entomon (Florence, Italy). Venomil® Wasp was acquired from Allergy Therapeutics (Worthing, UK). Protein determination was done by bicinchoninic acid assay (BCA) (Thermo Fisher Scientific, MA, USA).

Patients

Blood and/or serum samples of 53 YJV-allergic patients (32 female & 21 male; mean age 50.1 years) and 5 non-allergic controls from Southern Bavaria (Germany) were included in the study. Diagnosis of YJV allergy was based on a combination of clinical history and skin testing and/or sIgE measurement to venoms and molecular allergens. Clinical data of patients 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

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

Basophil activation test

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

Measurement of sIgE reactivity

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

Proteomic analysis

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

Results

Taxonomic reevaluation of *V. alascensis* and *V. vulgaris*

Results of the analyses of DNA sequence data in Carpenter and Glare 2010 [14] are clear, showing that the North American specimens are different from the Eurasian/New Zealand specimens (Figure 1 in Carpenter and Glare 2010). However, the specimen sampling was very limited (only two respectively five specimens), and the analyses lacked some standard methods and metrics.

Results from comparing the total of 514 CO1 (cytochrome oxidase subunit I) DNA barcode sequences (169 *V. alascensis*, 345 *V. vulgaris*, supplementary Data S1) show that the two species can be molecularly differentiated from each other (supplementary Results S1). Both species form an individual BIN in BOLD [19] (when corrected for misidentifications) each 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 five non-allergic controls.

The basophil activation (% $CD63^+$ basophils) in 12 responders with YJV allergy showed 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 non-responder (Figure S1). The non-allergic controls exhibited no basophil activation in response to any of the tested venoms (Figure S1).

Specific IgE reactivity of YJV-allergic patients

The sIgE reactivity to the different *Vespula* venoms was first assessed with ELISA using sera from 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 from New Zealand and Italy, and the therapeutic venom mixture Venomil®. Notably, one patient (P33) exhibited a positive signal almost exclusively with *V. alascensis* venom and Venomil®. Interestingly, for several patients, including P6, P11, P24, and P40, the reactivity was slightly higher to these two venoms compared with the two *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 16 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 compared to *V. alascensis* venom, the differences were not statistically significant.

To gain more detailed insights into sIgE specificity at a component-resolved level, Western blot (WB) analyses were performed using 16 sera (Figure 3C). For all sera clearly recognizing antigen 5 (Ag5) allergens in WB (P2, P12, P21, P28, P36, P46, P50, and P51), 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 (e.g. P21, P28 and P46). Only one patient (P53), who exhibited weak Ag5 reactivity, showed distinctly weaker binding to Ag5 in Venomil®. It is important to note that Venomil® contains venom from five species, as opposed to a single species, to cover a broader epitope spectrum.

Hyaluronidase (Hyal) sIgE recognition was similar among individual patients 19, 45, 46, 47, 48, 49, 51, 52, and 53, with P45 and P46 showing only weak signal intensities. While patient 12 reacted exclusively to Hyal from Venomil®, patient 14 showed higher reactivity to Hyal from the other three venoms.

Dipeptidyl peptidase IV (DPPIV) sIgE reactivity (P2, P12, P19, P47, P48, and P52) 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).

Of particular interest were the observed differences in the recognition of phospholipase A1 (PLA1) allergens. Patients P2, P12, P47, and P48 exhibited strong reactivity with PLA1 from the two *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, P52 and P53, reactivity was more pronounced with *V. vulgaris* PLA1, while P28 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 SDS-PAGE analysis, with prominent bands at approximately 25, 36, 45, and 100 kDa (Figure 4A). Proteomic analysis of the excised bands confirmed that the proteins identified with the highest intensities within these bands corresponded to the expected allergens: Ag5, PLA1, Hyal, and DPPIV. All allergens were identified by multiple peptides (Figure 4B), except for the inactive Hyal isoform B, which was identified by only a single peptide in all venoms (data not shown). 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. 39 protein groups overlap across all three venoms (Figure 4C). The majority of these proteins 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]. Notably, the proportion of these "true venom molecules" was higher in the two *V. vulgaris* venoms compared to *V. alascensis* venom (Figure 4D). 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 three venoms (Figure 4E) include well-established allergens and intriguing new allergen candidates, such as DOMON domain-containing protein and apolipophorin. Another protein identified in the two *V. vulgaris* venom preparations that would deserve further investigation is icarapin-like protein, a homolog of the major honeybee venom allergen Api m 10 [23-26].

Discussion

VIT with YJV represents 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. Therefore, our study addresses this gap by conducting a detailed comparative analysis of *V. alascensis* and *V. vulgaris* venoms.

In 2010, Carpenter and Glare suggested that the North American yellow jackets formerly treated as belonging to a Holarctic *V. vulgaris* (Linnaeus, 1758) actually belong to a different species [14]. Delimitation between these two 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 solely the different shape of the apex of the male digiti in ventral view is a clear morphological character for differentiation. Colorations of the propodeum are also discussed. However, due to intraspecific variation, its value for differentiation is limited. In a later publication, the North American species is keyed out based on coloration differences mainly of head and metasoma [13]. This key only treats 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] compellingly argue that the North American species should be designated as *V. alascensis* (Packard, 1870), a name previously considered a synonym of *V. vulgaris*.

The most relevant allergens in yellow jacket venom 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 United States, 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 actually reflects 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 and their clinical evidence, as the two species are closely related and their venom allergens are consequently highly homologous. Proteomic analysis of the major allergens - Ag5, PLA1, Hyal, and DPPIV - from gel bands revealed no differences between *V. alascensis* and *V. vulgaris* venom, with all identified peptides matching exactly 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 two species or whether it represents a mixture of *V. vulgaris* and *V. alascensis* entries in the database, considering that the taxonomic reclassification occurred only in 2010.

The ratio of "venom trace molecules," originating amongst others from damaged cells of the venom gland, to "true venom molecules" with venom functions [20-22], was higher in *V. alascensis* venom compared to the two *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, this analysis identified proteins that warrant further investigation regarding their potential roles as allergen candidates.

In this 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 experiments demonstrated a comparable inhibitory capacity of *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 two 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 described to be almost completely cross-reactive [7, 8], providing evidence of this for *V. alascensis* is crucial given its inclusion in therapeutic preparations.

In 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 was consistent across both *V. vulgaris* and *V. alascensis* venom, this observation can most likely be attributed to differences in the sensitivity of sIgE detection between ImmunoCap and Western blot techniques.

Additionally, the sIgE reactivity to DPPIV and Hyal allergens of both venoms was comparable for most patients. The number of Hyal-reactive patients was higher than expected, as the sensitization rate to YJV Hyal is typically low [31-33]. However, for four of the Hyal-reactive sera (P14, P48, P52, and P53), reactivity may be attributed to the recognition of cross-reactive carbohydrate determinants, as assessed by sIgE levels to MUXF3. Notably, one patient (P12) showed exclusive reactivity with the Hyal band of the therapeutic venom mixture, which may suggest that this patient is primarily sensitized to *V. germanica* venom, a component of the therapeutic formulation. For another patient (P14), reactivity with the Hyal-containing band was lower with the venom mixture compared to *V. alascensis* or *V. vulgaris* venom, a fact most likely reflecting the relative abundance of the primarily sensitizing venom within the mixture. In contrast to Ag5, which showed mostly 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 two *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. Unfortunately, no detailed information about IgE epitopes of Ves v 1 is currently available which might shed further light on this observation.

Intriguingly, in 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 indicates that the differences in PLA1 recognition observed in Western blot analysis do not appear to influence the activation of effector cells, reinforcing the functional similarity of the venoms in the context of allergic responses [34]. This may imply (A) that the observed differences in PLA1 recognition are not clinically relevant, at least for the majority of patients, (B) a low

immunological dominance of this allergen, and/or (C) that the clinically relevant response might be predominantly driven by Ag5, which is recognized by approximately 82-98% of YJV-allergic patients [35, 36] and showed comparable recognition across all venoms in this study.

In summary**,** given that widely used VIT preparations include *V. alascensis* venom, 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 to clarify the composition of preparations used in VIT. Despite this reclassification, the findings of this study demonstrate that the venoms of the two 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. Particularly as both venoms induce similar basophil activation profiles in patients and curative treatment of European patients using VIT preparations containing *V. alascensis* venom has been achieved with high efficiency for the past decades. Considering the relevance of *V. alascensis* venom in YJV-allergic patients and its inclusion in several U.S. studies under the name *V. vulgaris*, the major allergens PLA1 and Ag5 should be added to the official WHO/IUIS allergen nomenclature as Ves a 1 and Ves a 5, respectively. In conclusion, this study highlights the importance of sensitization to *V. alascensis* for European patients and supports its inclusion in VIT preparations for Europe.

References

1. Worm M, Höfer V, Dölle-Bierke S, Bilo MB, Hartmann K, Sabouraud-Leclerc D, et al. Occupational anaphylaxis-Data from the anaphylaxis registry. Allergy. 2024;79(3):702-710.
2. Worm M, Moneret-Vautrin A, Scherer K, Lang R, Fernandez-Rivas M, Cardona V, et al. First European data from the network of severe allergic reactions (NORA). Allergy. 2014;69(10):1397-404.
3. Perez-Codesido S, Rosado-Ingelmo A, Privitera-Torres M, Pérez Fernández E, Nieto-Nieto A, Gonzalez-Moreno A, et al. Incidence of Fatal Anaphylaxis: A Systematic Review of Observational Studies. J Investig Allergol Clin Immunol. 2022;32(4):245-260.
4. Müller U, Helbling A, Berchtold E. Immunotherapy with honeybee venom and yellow jacket venom is different regarding efficacy and safety. J Allergy Clin Immunol. 1992;89(2):529-35.
5. Rueff F, Vos B, Oude Elberink J, Bender A, Chatelain R, Dugas-Breit S, et al. Predictors of clinical effectiveness of Hymenoptera venom immunotherapy. Clin Exp Allergy. 2014;44(5):736-46.
6. Kranert P, Forchhammer S, Volc S, Stenger F, Schaller M, Fischer J. Safety and Effectiveness of a 3-Day Rush Insect Venom Immunotherapy Protocol. Int Arch Allergy Immunol. 2020;181(2):111-118.
7. Hoffman DR. Allergens in Hymenoptera venom. XVI: Studies of the structures and cross-reactivities of vespid venom phospholipases. J Allergy Clin Immunol. 1986;78(2):337-43.
8. King TP, Alagon AC, Kuan J, Sobotka AK, Lichtenstein LM. Immunochemical studies of yellowjacket venom proteins. Mol Immunol. 1983;20(3):297-308.
9. Goodman RE, Breiteneder H. The WHO/IUIS Allergen Nomenclature. Allergy. 2019;74(3):429-431.
10. Lopez-Osorio F, Pickett KM, Carpenter JM, Ballif BA, Agnarsson I. Phylogenetic relationships of yellowjackets inferred from nine loci (Hymenoptera: Vespidae, Vespinae, *Vespula* and *Dolichovespula*). Molecular Phylogenetics and Evolution. 2014;73(190-201.
11. Perrard A, Lopez-Osorio F, Carpenter JM. Phylogeny, landmark analysis and the use of wing venation to study the evolution of social wasps (Hymenoptera: Vespidae: Vespinae). Cladistics. 2016;32(4):406-425.
12. Carpenter J, Kojima J. Checklist of the species in the subfamily Vespinae (Insecta: Hymenoptera: Vespidae). Natural History Bulletin of Ibaraki University. 1997;1:51-92.
13. Kimsey LS, Carpenter JM. The Vespinae of North America (Vespidae, Hymenoptera). Journal of Hymenoptera Research. 2012;28:37-65.
14. Carpenter JM, Glare TR. Misidentification of *Vespula alascensis* as *V. vulgaris* in North America (Hymenoptera: Vespidae; Vespinae). American Museum Novitates. 2010;3690:1-7.
15. ALK-Abello Ltd, *Alutard Wasp Venom Maintenance*. 2023, ALK-Abello Ltd: Berkshire.
16. ALK-Abello Ltd, *Alutard Wasp Venom Initial*. 2023, ALK-Abello Ltd: Berkshire.
17. Feindor M, Heath MD, Hewings SJ, Carreno Velazquez TL, Blank S, Grosch J, et al. Venom Immunotherapy: From Proteins to Product to Patient Protection. Toxins (Basel). 2021;13(9):616.
18. Eberlein B, Krischan L, Darsow U, Ollert M, Ring J. Double positivity to bee and wasp venom: improved diagnostic procedure by recombinant allergen-based IgE testing and

basophil activation test including data about cross-reactive carbohydrate determinants. J Allergy Clin Immunol. 2012;130(1):155-61.
19. Ratnasingham S, Wei C, Chan D, Agda J, Agda J, Ballesteros-Mejia L, et al. BOLD v4: A Centralized Bioinformatics Platform for DNA-Based Biodiversity Data. Methods Mol Biol. 2024;2744:403-441.
20. Van Vaerenbergh M, Debyser G, Devreese B, de Graaf DC. Exploring the hidden honeybee (*Apis mellifera*) venom proteome by integrating a combinatorial peptide ligand library approach with FTMS. J Proteomics. 2014;99(169-78.
21. Grosch J, Hilger C, Bilo MB, Kler S, Schiener M, Dittmar G, et al. Shedding Light on the Venom Proteomes of the Allergy-Relevant Hymenoptera *Polistes dominula* (European Paper Wasp) and *Vespula* spp. (Yellow Jacket). Toxins (Basel). 2020;12(5):323.
22. Grosch J, Lesur A, Kler S, Bernardin F, Dittmar G, Francescato E, et al. Allergen Content of Therapeutic Preparations for Allergen-Specific Immunotherapy of European Paper Wasp Venom Allergy. Toxins (Basel). 2022;14(4):284.
23. Jakob T, Rauber MM, Perez-Riverol A, Spillner E, Blank S. The Honeybee Venom Major Allergen Api m 10 (Icarapin) and Its Role in Diagnostics and Treatment of Hymenoptera Venom Allergy. Curr Allergy Asthma Rep. 2020;20(9):48.
24. Vega-Castro A, Rodríguez-Gil D, Martínez-Gomariz M, Gallego R, Peña MI, Palacios R. Api m 6 and Api m 10 as Major Allergens in Patients With Honeybee Venom Allergy. J Investig Allergol Clin Immunol. 2022;32(2):116-123.
25. Blank S, Seismann H, Michel Y, McIntyre M, Cifuentes L, Braren I, et al. Api m 10, a genuine *A. mellifera* venom allergen, is clinically relevant but underrepresented in therapeutic extracts. Allergy. 2011;66(10):1322-9.
26. Lund A, Dorn B, Jakob T, Christensen LH, Jabs F, Spillner E. A Fully Human Monoclonal Antibody Isolated From a Beekeeper Targets the Immunodominant IgE Epitope of Api m 10. J Investig Allergol Clin Immunol. 2024;0.
27. Donovan BJ. Occurrence of the common wasp, *Vespula vulgaris* (L.) (Hymenoptera: Vespidae) in New Zealand. New Zealand Journal of Zoology. 1984;11(4):417-427.
28. Blank S, Korošec P, Slusarenko BO, Ollert M, Hamilton RG. Venom Component Allergen IgE Measurement in the Diagnosis and Management of Insect Sting Allergy. J Allergy Clin Immunol Pract. 2024; Online ahead of print.
29. King TP, Lu G, Gonzalez M, Qian N, Soldatova L. Yellow jacket venom allergens, hyaluronidase and phospholipase: sequence similarity and antigenic cross-reactivity with their hornet and wasp homologs and possible implications for clinical allergy. J Allergy Clin Immunol. 1996;98(3):588-600.
30. Lu G, Villalba M, Coscia MR, Hoffman DR, King TP. Sequence analysis and antigenic cross-reactivity of a venom allergen, antigen 5, from hornets, wasps, and yellow jackets. J Immunol. 1993;150(7):2823-30.
31. Jin C, Focke M, Leonard R, Jarisch R, Altmann F, Hemmer W. Reassessing the role of hyaluronidase in yellow jacket venom allergy. J Allergy Clin Immunol. 2010;125(1):184-90 e1.
32. Seismann H, Blank S, Braren I, Greunke K, Cifuentes L, Grunwald T, et al. Dissecting cross-reactivity in Hymenoptera venom allergy by circumvention of alpha-1,3-core fucosylation. Mol Immunol. 2010;47(4):799-808.
33. Grosch J, Eberlein B, Waldherr S, Pascal M, Dorn B, San Bartolomé C, et al. Comparative Assessment of the Allergenicity of Hyaluronidases from *Polistes dominula* (Pol d 2), *Vespula vulgaris* (Ves v 2), and *Apis mellifera* Venom (Api m 2). Toxins (Basel). 2024;16(11):498.
34. Eberlein B. Basophil Activation as Marker of Clinically Relevant Allergy and Therapy Outcome. Front Immunol. 2020;11:1815.

35. Blank S, Bazon ML, Grosch J, Schmidt-Weber CB, Brochetto-Braga MR, Bilo MB, et al. Antigen 5 Allergens of Hymenoptera Venoms and Their Role in Diagnosis and Therapy of Venom Allergy. Curr Allergy Asthma Rep. 2020;20(10):58.
36. Schmidle P, Blank S, Altrichter S, Hoetzenecker W, Brockow K, Darsow U, et al. Basophil Activation Test in Double-Sensitized Patients With Hymenoptera Venom Allergy: Additional Benefit of Component-Resolved Diagnostics. J Allergy Clin Immunol Pract. 2023;11(9):2890-2899.e2.

Figure legends

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

Figure 2. Basophil activation tests with *Vespula* spp. venoms. Basophils from yellow jacket venom-allergic patients from Germany were exposed to different concentrations of *V. alascensis* venom, *V. 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, Italy; NZ, New Zealand.

Figure 3. sIgE reactivity of yellow jacket venom-allergic patients from Germany with the different *Vespula* spp. venoms. (A) sIgE immunoreactivity of patients in ELISA. (B) Inhibitory capacity of *V. alascensis* and *V. vulgaris* (IT) venom on sIgE immunoreactivity against the respective other venom. Results are shown for selected patients exhibiting pronounced sIgE reactivity to the venoms. (C) sIgE immunoreactivity of patients in Western blot. Ag5, antigen 5; DPPIV, dipeptidyl peptidase IV; Hyal, hyaluronidase; IT, Italy; NZ, New Zealand; PLA1, phospholipase A1.

Figure 4. Proteomic analysis of the different *Vespula* spp. venoms. (A) SDS-PAGE analysis of the different *Vespula* venoms. Alphanumeric labels within the gel indicate protein bands excised for mass spectrometric analysis. (B) Peptides from the proteins identified within the

bands with the highest intensity with their positions within the protein sequences. (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," primarily originating from damaged venom gland cells, 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, antigen 5; DPPIV, dipeptidyl peptidase IV; Hyal, hyaluronidase; IT, Italy; NZ, New Zealand; PLA1, phospholipase A1.