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Allergy School on Insect Venom Allergy and Mastocytosis

11 - 13 April 2019
Groningen, The Netherlands

ABSTRACT BOOK

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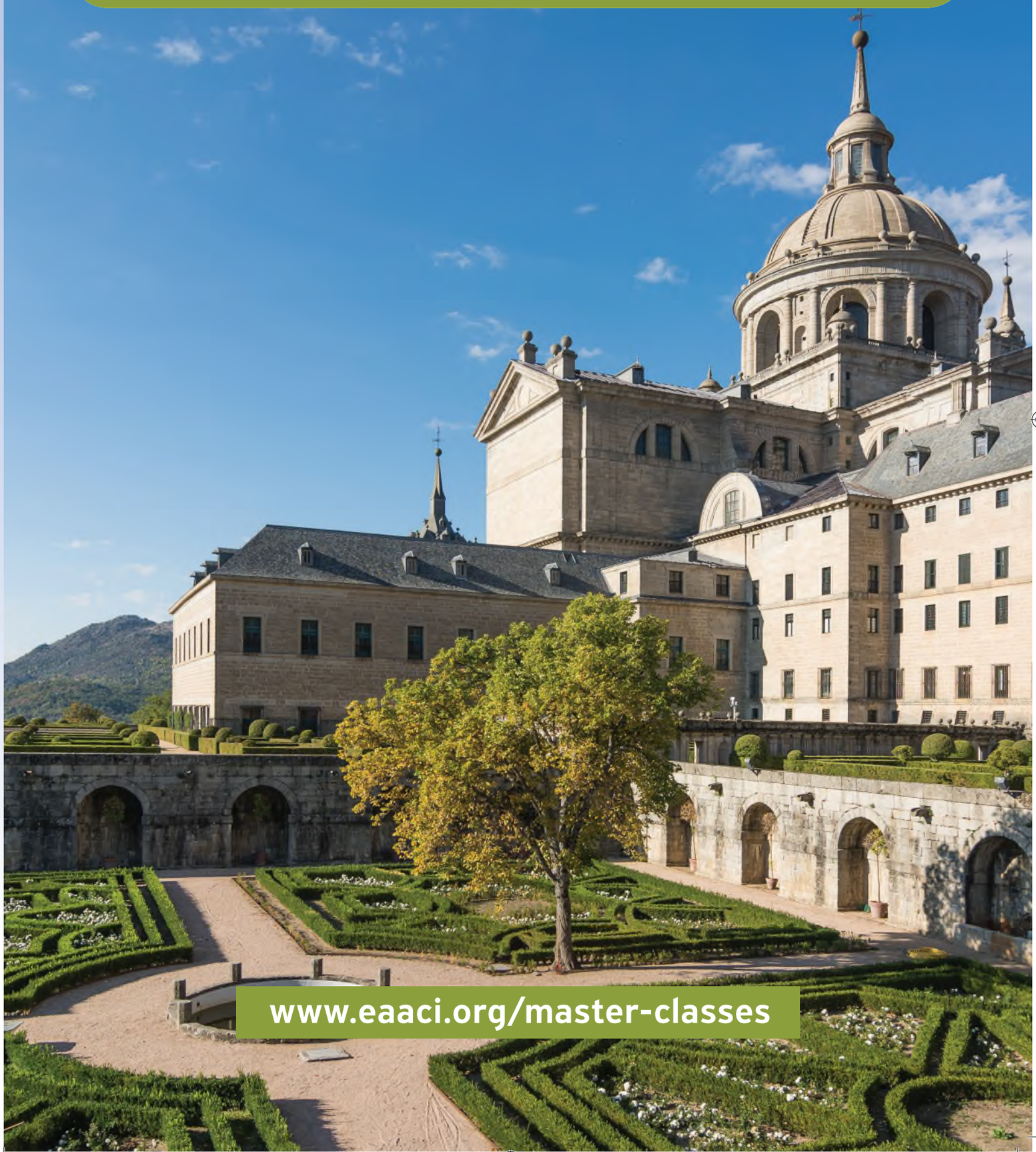

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- 3 – 4 May 2019
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Allergy School on Insect Venom Allergy and Mastocytosis

- 11 – 13 April 2019
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EAACI
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PAAM 2019

17 – 19 October 2019
Florence, Italy

PAAM

6th Pediatric Allergy and Asthma Meeting

www.eaaci.org/paam2019

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GENERAL INFORMATION

CME Accreditation

An application has been made to the UEMS-EACCME® for CME accreditation of this EAACI Allergy School. The CME letter and the Certificate of Attendance can be downloaded after completing the survey which will be sent to you by e-mail after the school. **Please make sure you scan your badge before entering each session room, in order to obtain the CME credits.**

Potential Conflicts of Interest Declaration

Please refer to the relevant event page under the "Meetings" tab on www.eaaci.org for a full conflict of interest declaration, provided by the organising committee and faculty members.

Organising Committee

Hanneke Oude Elberink, Local Organising Chair
Darío Antolín-Amérigo, Local Organising Secretary
Christoph Schrautzer, Insect Venom Hypersensitivity WG Board Member

Poster Information

Posters can be mounted from 11:00 on Thursday, 11 April 2019 and should be removed after the last poster session on Saturday, 13 April 2019. Please make sure to remove the poster and all poster-mounting material from the board. The organisers will remove posters not taken down on time and will not take any further responsibility for the material.

Meeting venue

UMCG -The University Medical Center Groningen
Hanzeplein 1
9713 GZ Groningen, The Netherlands
Phone: +31 (50) 361 61 61
Website: www.umcg.nl

Accommodation

Hotel NH Groningen
Hanzeplein, 132
9713GW Groningen, The Netherlands
Phone: +31 50 584 8181

Contact Details

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ISMA 2019

28 – 30 November 2019
Amsterdam, The Netherlands

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International Symposium on Molecular Allergology

International Symposium on Molecular Allergology

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EAACI Allergy School

Insect Venom Allergy and Mastocytosis

11 - 13 April 2019

Groningen, the Netherlands

Scientific Programme

Thursday, 11 April 2019

12:00 - 14:00	Registration and Light Lunch
14:00 - 14:15	Welcome address *Hanneke Oude Elberink, The Netherlands | Dario Antolin Amerigo, Spain | Christoph Schrautzer, Austria*
14:15 - 15:30	**Session I - What is allergy?** *Chairs: Hanneke Oude Elberink, The Netherlands | Christoph Schrautzer, Austria*
14:15 – 15:30	The Mast cell- IgE paradox, from homeostasis to anaphylaxis *Speaker: Stephen Galli, United States*
15:30 - 16:00	Coffee break
16:00 - 18:00	**Session II - Natural history of hypersensitivity reactions to stings and quality of life: who is at risk and risk perception** *Chairs: Dario Antolin Amerigo, Spain | Stephen Joseph Galli, United States*

16:00 - 16:15	The impact of sting reactions on quality of life *Hanneke Oude Elberink, The Netherlands*
16:15 - 16:45	Hymenoptera allergy: let's start from the beginning *Arantza Vega, Spain*
16:45 - 17:30	Natural history of systemic reactions in children and adults *Dario Antolin Amerigo, Spain*
17:30 - 18:00	Who is really in needs for an EAI and/or VIT? *Hanneke Oude Elberink, The Netherlands*
18:30 – 20:30	Welcome reception

Friday, 12 April 2019

09:30 - 11:30	**Session III - Diagnostic Tools** *Chairs: Beatrice Biló, Italy | Franziska Ruëff, Germany*
09:30 - 10:15	Allergen venom components for the selection of the venom - sensitivity matters! *Markus Ollert, Luxembourg*
10:15 - 10:45	The additional value of cellular tests in insect venom allergy *Peter Korošec, Slovenia*
10:45 - 11:15	Dealing with cross-reactivity: how to detect the right venom for the right patient *Thilo Jakob, Germany*
11:15 – 11:30	A21/O01 - Fluorescent Labelling Of Major Honeybee Allergens Api M 1 And Api M 2 With Quantum Dots And Development Of Multiplex Basophil Activation Test *Ana Koren, Slovenia*
11:30 - 12:00	Coffee break
12:00 - 13:30	**Session IV - Risk factors and Mastocytosis** *Chairs: Hanneke Oude Elberink, The Netherlands | David González De Olano, Spain*
12:00 - 12:30	Alpha-tryptasemia: is there a link with instect venom allergy, mastocytosis and mast cell activation syndrome? *Vito Sabato, Belgium*
12:30 - 13:15	Mastocytosis as a risk factor for insect venom allergy *Patrizia Bonadonna, Italy*
13:15 – 13:30	A32/O02 - Pulling The Trigger In Clonal Mast Cell Disorders: Are Hymenoptera Stings The Only Actors? *Gustavo Jorge Molina Molina, Spain*

13:30 - 14:30 Lunch & Poster discussion Session

Poster Walk 1:P01 – P07
Chair: Franziska Ruëff, Germany

A09/P01 - Predictors Of Severe Anaphylactic Reactions In Patients With Hymenoptera Venom Allergy
Maria Chapsa, Germany
A10/P02 - Systemic Mastocytosis In A 5 Year Old Child Presenting With Hypovolemic Shock, Succeeded By Severe Anaphylaxis To Fentanyl
Inger F Bocca-Tjeertes, The Netherlands
A14/P03 - Insect-Venom Elicited Anaphylaxis, A Prospective Cohort Study From The European Anaphylaxis Registry
Wojciech Francuzik, Germany
A15/P04 - Do We Need Premedication With Omalizumab In Patients With Systemic Mastocytosis Having Venom Immunotherapy?
Asli Gelincik, Turkey
A20/P05 - Omalizumab In Immunotherapy With Hymenoptera Venom
Cristiana Ferreira, Portugal
A34/P06 - Evaluation Of Systemic Mastocytosis With 3 Cases
Betül Ayse Sin, Turkey
A40/P07 - Risk Factors In Hymenoptera Venom Allergy
Svetlana Shvets, Russia

Poster Walk 2: P08 – P14
Chair: Markus Ollert, Luxembourg

A18/P08 - Contribution Of Component Resolved Diagnosis In Hymenoptera Venom Allergy
Asli Gelincik, Turkey
A22/P09 – Sensitization To Bee Venom In Non-allergic Beekeepers
Ana Margarida Mesquita, Portugal
A24/P10 - Molecular Diagnosis And Beyond Unmet Needs In Rush Immunotherapy For Hymenoptera Venom – Single Center Experience In Albania
Mehmet Hoxha, Albania
A27/P11 - Contribution Of Molecular Diagnosis Of Bee Venom Allergic Patients With Systemic Reactions During Venom Immunotherapy
Tatiana Lourenço, Portugal
A28/P12 - Omalizumab In Immunotherapy With Hymenoptera Venom-Case Report
Mara Fernandes, Portugal
A29/P13 - Precision Medicine And The Tryptase Framework Of Wasp Venom IgE-Sensitization In Mastocytosis
Douwe De Boer, The Netherlands
A30/P14 - Soluble FcεRI Is A Potential Biomarker Of IgE Mast Cell Desensitization During Chemotherapy Treatment Of Allergic Cancer Patients
Sherezade Moñino-Romero, Austria

Poster Walk 3: P15 – P21
Chair: Vito Sabato, Belgium

A19/P15 - The Need Of Bee Venom Immunotherapy Reintroduction Due To Unsuccessful 5-Year-Lasting Treatment In Adolescent Boy
Ewa Cichocka-Jarosz, Poland
A25/P16 - Multicenter Study Of Clinical Relevance Of Recombinant Allergen Api M 1 And Ves V 5 Determined By IgE Multiplex Test ImmunoCAP ISAC
Urska Bidovec-Stojkovic, Slovenia
A31/P17 - Systemic Mastocytosis With Low Serum Tryptase: A Challenging Diagnosis
Tiago Azenha Rama, Portugal
A33/P18 - Kounis Syndrome: A Thought-Provoking Case Report
Francesca Rizzo, Italy
A37/P20 - Three Is A Charm!
Toon Ieven, Belgium
A39/P21 - Hymenoptera Species: Who's Eating And Stinging?
Arantza Vega, Spain

14:30 - 16:15 **Session V - Venom immunotherapy**
Chair: Christoph Schrautzer, Austria | Markus Ollert, Luxembourg- waiting for acceptance

14:30 - 15:00 Venom immunotherapy across the world
Beatrice Biló, Italy

15:00 - 15:30 When can we stop venom immunotherapy?
Franziska Ruëff, Germany

15:30 - 16:00 How about VIT in Mastocytosis patients?
David González De Olano, Spain

16:00 – 16:15 A38/O03 - Mastocytosis And Anaphylaxis To Hymenoptera Venom: A Single Center Cohort Study
Christine Breynaert, Belgium

16:15 - 16:30 Coffee break

16:30 - 18:20 **Session VI - Practical workshops**

Preliminary Room Topic 1: Mastocytosis as separate disease entity
Moderator: Patrizia Bonadonna, Italy| Hanneke Oude-Elberink, The Netherlands

Room 17 Topic 2: Different treatment schedules and dealing with side effects
Moderator: Dario Antolin Amerigo, Spain

Room 18 Topic 3: Difficult cases
Moderator: Carmen Moreno-Aguilar, Spain

16:30-17:00	**Workshop round 1**
17:00-17:10	Rotation break
17:10-17:40	**Workshop round 2**
17:40-17:50	Rotation break
17:50-18:20	**Workshop round 3**
19:00 - 22:00	Dinner

Saturday, 13 April 2019

08:30 - 10:30	**Session VII - Behind the scenes of insect hypersensitivity** *Chairs: Kymble Martin Spriggs, Australia | Arantza Vega Castro, Spain*
08:30 - 09:15	Insect stings and bites: not only Hymenoptera *Hanneke Oude Elberink, The Netherlands*
09:15 - 10:00	Mechanisms of allergy: how does early and long VIT protection work *Mohammed Shamji, United Kingdom*
10:00 - 10:15	A36/O04 - Sting-Challenge Demonstrated Tolerance In Patients Undergoing Ant Venom Specific Immunotherapy, Validating New Centre Approach *Kymble Martin Spriggs, Australia*
10:15 - 10:30	A26/O05 - Absence Of Th2 Cell Suppression After Induction Of Venom Immunotherapy In Wasp-Venom Allergic, Indolent Systemic Mastocytosis Patient *Merel C. Onnes, The Netherlands*
10:30 - 11:00	Coffee break
11:00 - 12:30	**Session VIII - Open issues** *Chairs: Christoph Schrautzer, Austria | Markus Ollert, Luxembourg*
11:00 - 11:30	Efficacy and safety of an accelerated outpatient protocol for venom immunotherapy *Christoph Schrautzer, Austria*
11:30 - 12:00	How to deal in an optimal way with the complexity of bee venom allergy? *Markus Ollert, Luxembourg*
12:00 - 12:15	A23/O06 - Predictors Of Severe Cardiovascular Honey-Bee Sting Reaction With Absence Of Skin Symptoms In Patients With Normal Baseline Serum Tryptase Levels *Peter Kopac, Slovenia*
12:15 – 12:30	Open questions and future strategies in insect venom allergy *Chairs: Christoph Schrautzer, Austria | Markus Ollert, Luxembourg*

12:30 - 12:45 Closing remarks

Hanneke Oude Elberink, The Netherlands | Dario Antolin Amerigo, Spain | Christoph Schrautzer, Austria

ABSTRACTS

Friday, 12 April 2019
Oral Abstract Presentations

O01 - Fluorescent Labelling Of Major Honeybee Allergens Api M 1 And Api M 2 With Quantum Dots And Development Of Multiplex Basophil Activation Test

Ana Koren[1], Mojca Lunder[2], Peter Molek[2], Peter Kopac[1], Abida Zahirovic[2], Pia Gattinger[3], Rudolf Valenta[3], Irene Mittermann[3], Peter Korosec[1]

1. University Clinic of Respiratory and Allergic Diseases Golnik, Golnik, Slovenia
2. University of Ljubljana, Faculty of Pharmacy, Ljubljana, Slovenia
3. Medical University of Vienna, Department of Pathophysiology and Allergy Research, Vienna, Austria

Background
Api m 1 and Api m 2 are two major allergens of honeybee venom. Basophil activation test (BAT) is *in vitro* approach for evaluation of the biological relevance of IgE antibodies. Labelling of recombinant allergens with fluorescent probes could represent a new approach for multiplex assessment of allergenic activity with flow cytometry.

Materials and methods
nApi m 1 (Latoxan, France) and rApi m 2 (from Medical University of Vienna) were conjugated to Qdot® 705 or 800 ITK™ Amino (PEG) Quantum Dots and Qdot® 705 or 800 ITK™ Carboxyl Quantum Dots. IgE reactivity of Qdot-labelled allergens was assesed with immunodot assay using of rApi m1 or rApi m2 sIgE-positive sera of honeybee allergic patients. Allergenic activity was assesed with BAT using CD123-PE/HLA-DR-APC/CD63-FITC labelled antibodies. Qdot 705 was measured with 670LP (exc. 488nm) and Qdot 800 was measured with 780/60 (exc. 488nm). Finally, usefulness of Qdot-labelled allergens for multiplex BAT analysis was tested in 12 bee venom-allergic patients and in 3 non-allergic controls. The stimulation of whole blood with amino Qdot 705-Api m 1 and amino Qdot 800 Api m 2 was done in separate tubes, then samples were merged, antibody labelled and analyzed as multiplex.

Results
Both Amino and Carboxyl Qdot-labelled nApi m 1 and rApi m2 showed positive and specific IgE reactivity evaluated with immunoblotting. We then tested weather Qdot labelled allergens are able to induce activation of the basophils in honeybee allergic patients. We demonstrated that only Amino but not Carboxyl Qdot-labelled nApi m 1 and rApi m 2 are able to activate basophils, suggesting that allergenic activity is preserved only in case of Amino Qdot-labelling. Furthermore, we showed that Qdot 705 Amino-labelled Api m 1 and Qdot 800 Amino -labelled Api m 2 could be used in multiplex analysis in which basophil subpopulations and their activations were

analysed according to the binding of fluorescent allergens. Multiplex BAT results were concordant with BAT result in 11/12 patients and 3/3 controls. Moreover, there were also comparable CD63 dose-response curves between labelled allergens used in multiplex approach and conventional BAT.

Conclusion
Quantum Dot labelling of allergens does not affect IgE reactivity; however IgE crosslinking and allergenic activity is preserved only in case of labelling with Amino (PEG) Qdot. Fluorescent labelling of venom components represents a new approach for multiplex BAT testing in *Hymenoptera* venom allergy.

O02 - Pulling The Trigger In Clonal Mast Cell Disorders: Are Hymenoptera Stings The Only Actors?

Gustavo Jorge Molina Molina, Paula Galván, Johana Gil Serrano, Jenny Tatiana Verdesoto, Moisés Labrador Horrillo, Anna Sala Cunill, Olga Luengo, Victoria Cardona, Mar Guilarte

Allergy Section. Department of Internal Medicine. Hospital Universitari Vall d'Hebron, Barcelona, Spain

Background
The implication of hymenoptera venom as a trigger of anaphylaxis in patients with clonal mast cells disorders (c-MCD) is well known. Currently, most anaphylaxis clinical guidelines recognize the need of assessing for an underlying c-MCD in case of hymenoptera sting and in idiopathic anaphylaxis but not so in case of other elicitors.
OBJECTIVE: To study the characteristics and triggers of anaphylaxis in patients with c-MCD, with special focus on hymenoptera sting anaphylaxis.

Materials and methods
Patients with a diagnosis of systemic c-MCD fulfilling the WHO 2016 criteria followed at our department from 2007 to 2018 were included. Anaphylactic reactions were carefully evaluated regarding their clinical characteristics, including triggers, severity of the reaction and skin involvement. Baseline serum tryptase (sBT) levels were recorded. The Spanish Network on Mastocytosis (REMA) score to assess the probability of systemic c-MCD (≥2) was performed. A subgroup of patients with hymenoptera sting anaphylaxis without c-MCD was selected as control group (H-A) and compared to those with hymenoptera sting anaphylaxis with c-MCD (H-SM).

Results
Data from 59 patients with a diagnosis of systemic c-MCD were collected. Anaphylaxis lead to c-MCD diagnosis in 26 patients (49%). The most frequent triggers were drugs (34.5%), hymenoptera stings (31%) and foods (15.5%). In 19% of patients no specific trigger could be identified (idiopathic). REMA score was ≥2 in 15/26 (58%) patients. The majority (87.5%) of H-SM developed a grade III anaphylaxis after hymenoptera sting compared to 50% of H-A. An inverse relationship between involvement of skin during anaphylaxis and having an underlying c-MCD was found (Fisher exact test 0.0012; $p < 0.01$). sBT was elevated (>11.4 mcg/dL) in 21/26 (81%) c-MCD patients. 3/5 patients with normal

sBT had as a trigger a hymenoptera sting, while 2/5 were idiopathic. In patients with drug or food induced anaphylaxis, c-MCD would have been missed in 50% of cases if sBT had not been assessed.

Conclusion
The absence of cutaneous manifestations during anaphylaxis due to hymenoptera sting is a sign suggestive of c-MCD. Moreover, sBT determination should be performed in all patients with an anaphylaxis, independently of the type of triggering agent in order not to miss mast-cell disorders.

O03 - Mastocytosis And Anaphylaxis To Hymenoptera Venom: A Single Center Cohort Study

Toon Ieven[1], Anne-Marie Kochuyt[2], Rik Schrijvers[2,3], Dominique Bullens[4,3], **Christine Breynaert**[4,3]

1. University Hospitals Leuven, Department of Internal Medicine, Leuven, Belgium
2. University Hospitals Leuven, Department of General Internal Medicine (Allergy and Clinical Immunology), Leuven, Belgium
3. KU Leuven Department of Microbiology, Immunology and Transplantation, Allergy and Clinical Immunology Research Group, Leuven, Belgium
4. University Hospitals Leuven, Department of Pediatrics (Pediatric Allergology), Leuven, Belgium

Background
Patients with mastocytosis have an increased risk of severe anaphylaxis after hymenoptera stings.

Materials and methods
A retrospective analysis was performed on a cohort of patients with a diagnosis of mastocytosis from January 1990 to January 2018 in a single tertiary referral center. After informed consent, data were collected from the medical records on demographics, clinical history of anaphylaxis, sensitization to insect venom and venom immunotherapy (VIT).

Results
103 patients with a diagnosis of mastocytosis were included [female: n=52 (50.5%), age at time of inclusion: 28.9 years (7.4–53.0)]. 34/103 (33.0%) patients [3 cutaneous mastocytosis (CM)/31 systemic mastocytosis (SM)] indicated having been stung over the course of their lifetime. 6/34 patients (17.6%) experienced a large local reaction, 12/34 (38.2%) anaphylaxis and 15/34 (44.1%) no reaction. Of the 103 patients, n=29 [28.2%, serum basal tryptase (SBT) level 23.3 ng/ml (14.5-72.7)] had a history of anaphylaxis [indolent SM (ISM)/monoclonal mast cell activation syndrome (MMAS)/smouldering SM (SSM)/aggressive SM (ASM) respectively n=24/3/1/1]. 11/29 patients experienced anaphylaxis after a hymenoptera sting [hymenoptera venom allergy (HVA) group; MMAS/ISM without skin lesions (ISM^-)/ISM with skin lesions (ISM^+) respectively n=2/8/1, number of episodes 2 (1-3), age at first symptoms 42.1 years (38.4-55.1)]. 18/29 patients suffered anaphylaxis due to another cause [non-HVA group; number of episodes 2 (1-4), age at first symptoms 32.1 years (22.9-42.2; p=0.024 compared to the HVA

group)]. In the HVA group, 3/11 had a positive major criterion for the diagnosis of SM vs. 14/18 in the non-HVA group (p=0.028). In contrast, c-kit D816V mutation was positive in 10/18 in the non-HVA group versus 10/10 in the HVA group (p=0.013). In the HVA group, anaphylaxis was the main symptom leading to the diagnosis of SM (11/11, 100%) vs. 8/18 (44.4%) in the non-HVA group (p<0.01). In the HVA group, 11/11 had documented hypotension after the sting, 9/11 loss of consciousness and 8/11 had no skin symptoms. All were started on VIT [yellow jacket venom (n=9), honeybee venom (n=1) or both (n=2)].

Conclusion
In our cohort, patients with mastocytosis suffering anaphylaxis after hymenoptera stings, had lower SBT levels, often lack typical skin lesions and rarely have the major bone marrow criterion for diagnosis of mastocytosis, compared to patients with mastocytosis and anaphylaxis due to another cause.

Friday, 12 April 2019
Poster Discussion Session
13:30 – 14:30

P01 - **Predictors Of Severe Anaphylactic Reactions In Patients With Hymenoptera Venom Allergy**

Maria Chapsa, Henriette Rönsch, Mathias Langner, Stefan Beissert, Andrea Bauer

Department of Dermatology, University Hospital Dresden, Dresden, Germany

Background
Severe anaphylaxis (SA) in hymenoptera venom allergy (HVA) has been associated with a number of risk factors. Baseline serum tryptase (BST), presence of mastocytosis and older age are well-established risk factors, whereas other factors including sex, personal health issues (comorbidities, concurrent medication) and anaphylaxis-associated findings (e.g. time interval between sting and onset of symptoms (TI), skin symptoms) have been also proposed to be taken into account for individual risk assessment. However, their impact on the severity of the anaphylactic reaction is poorly defined and discussed controversially. The aim of this study was to evaluate risk factors of SA due to hymenoptera field stings.

Materials and methods
A total of 500 patients, who referred to our department for the diagnosis of HVA over a period of 11 years (2007-2018), were included in this retrospective single-center observational cohort study.

Results
Six significant indicators and risk factors for SA were identified (P<0,05): short TI, absence of urticaria/angioedema (U/A) during anaphylaxis, older age, male sex, elevation of BST and diagnosis of indolent mastocytosis. Moreover, BST elevation was significantly related to the absence of U/A and to older age. No relationship could be established between SA and comorbidities, concurrent cardiovascular medication, concentration of venom-specific IgE, threshold of skin tests or the

severity of the systemic reaction during the buildup phase of venom immunotherapy (bpVIT).

Conclusion
Apart from BST and older age, male sex, short TI (<5min) and absence of U/A are also indicators of SA. Cardiovascular concomitant diseases in general are not correlated with the SA. Future studies should examine the association of specific severe cardiovascular diseases (e.g. coronary heart disease, cardiomyopathy) with SA.
Moreover, absence of U/A after field sting in combination with elevated BST constitutes a highly significant indicator of SA, presumably because of the high risk of concurrent presence of an indolent mastocytosis.
Finally, patients with a SA after field sting do not have an elevated risk for systemic reactions during the bpVIT in comparison to the patients with mild anaphylaxis and therefore, they do not require additional preventive measures.

P02 - Systemic Mastocytosis In A 5 Year Old Child Presenting With Hypovolemic Shock, Succeeded By Severe Anaphylaxis To Fentanyl

Inger F Bocca-Tjeertes, Hanneke N Oude Elberink, Bouwe Molenbuur, Aline B Sprikkelman

UMCG, Groningen, The Netherlands

Background
Mastocytosis is characterized by the clonal expansion and accumulation of mast cells (MCs) in different tissues and organs. In children, cutaneous mastocytosis, or typical maculopapular cutaneous lesions (TMCL/urticaria pigmentosa), is the most common form of mastocytosis, with a prevalence of 13 in 100.000, and resolution in many in puberty. Systemic mastocytosis (SM) is very rare in children. However, it is more likely in children with a persistent serum tryptase level of >20ng/mL, or those with symptoms of explosive diarrhea, syncope, as well as recurrent anaphylaxis reactions. Precautions are taken for procedural anesthetics if SM is suspected. In these cases, histamine releasing opioids, like morphine, are preferably replaced by fentanyl or any other synthetic opioid.

Case report
A 5 year old boy was referred to our hospital. At age six months, the patient was referred to a dermatologist for lesions on his forehead, consistent with TMCL, confirmed by a skin biopsy. During his entire life, he frequently suffered from diarrhea. At age three he was seen by a pediatrician for failure to thrive. At this point, serum tryptase was 42.6ng/mL. The consulted gastro-enterologist concluded there was no SM. Tryptase was 47.1ng/mL. At age five, the patient suffered from an anaphylactic shock following diarrhea for which he had to be resuscitated. During transfer to pediatric intensive care, morphine was administered intravenously, which triggered severe hypotension. Therefore, renewed intubation was performed using fentanyl provoking again severe hypotension. Tryptase rose to >200ng/mL. A few weeks later, without any complication, bone marrow biopsy (BMP) was performed under general anesthesia using propofol and ketamine after administration of H1 and H2-blockers intravenously. BMP revealed abnormal morphology of MCs (>25% spindle shaped), an activating mutation at codon 816 of

KIT, and the expression of CD25 in MCs, but no aggregates of >15 mast cells (major criterion). Hereby, meeting all minor criteria for SM. In follow-up the patient is doing well with H1 and H2-blockers combined with nalcrom.

Conclusion
SM should be considered in all children with a persistent serum tryptase >20ng/mL. In this case, severe delay was most likely due to lack of knowledge. Anaphylaxis to synthetic opioids is rarely seen, but possible and all anesthetics should be administered in a highly controlled setting in children with SM, preferably after premedication.

P03 - **Insect-Venom Elicited Anaphylaxis, A Prospective Cohort Study From The European Anaphylaxis Registry.**

Wojciech Francuzik[1], Sabine Dölle-Bierke[1], Franziska Ruëff[2], Claudia Pföhler[3], Kathrin Scherer Hofmeier[4], Margitta Worm[1]

1. Charité – Universitätsmedizin Berlin, corporate member of Freie Universität Berlin, Humboldt-Universität zu Berlin, and Berlin Institute of Health, Berlin, Germany
2. Department of Dermatology and Allergology, Klinikum der Universität München, München, Germany
3. Department of Dermatology, Saarland University Hospital, Homburg / Saar, Germany
4. Department of Dermatology, University Hospital Basel, Basel, Switzerland

Background
Insect-venom elicited anaphylaxis is a common hypersensitivity reaction which may be life-threatening.

Materials and methods
Using the data from the European Anaphylaxis Registry (11596 cases in total) we identified insect-venom elicited anaphylaxis cases (n = 4482) and analyzed these in comparison to anaphylaxis elicited by other elicitors (n = 7114).

Results
The data show that 68.57% of all insect elicited cases were elicited by yellow jackets, followed by bees (21.86%). The insect venom elicited cases occurred mostly in outdoor places (44.65%) patients' homes (12.87%) or urban places (9.616%).
Skin, gastrointestinal and respiratory symptoms occurred less frequently in insect elicited cases of anaphylaxis, whereas cardiologic symptoms (with hypotension, collapse, and loss of consciousness) were more frequent. Intramuscular adrenaline (as a first-line therapy) was administered significantly less often in insect venom elicited cases (4.04%, $p < 0.0001$). The mortality rate in insect anaphylaxis was comparable (0.156%) to other cases (0.295%, $p = 0.174$).
Patients who experienced insect-venom anaphylaxis were older ($p < 0.0001$), more often had concomitant mastocytosis ($p < 0.0001$) and cardiologic conditions ($p < 0.0001$) and females more often had concomitant thyroid diseases and less often suffered from a food allergy or atopic dermatitis.

Conclusion
Symptoms of insect venom anaphylaxis are distinctively different from other reactions, indicating that the therapy of insect elicited cases of anaphylaxis should be considered separately. Indeed we observed different therapeutic patterns in insect elicited cases of anaphylaxis (more antihistaminics but fewer corticosteroids, bronchodilators, and surprisingly - adrenaline). This indicates that the management of insect-venom induced anaphylaxis may be improved and is especially required in patients with concomitant cardiologic conditions and these with hyperreactive mast cells.

15 P04 - Do We Need Premedication With Omalizumab In Patients With Systemic Mastocytosis Having Venom Immunotherapy?

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Background
In systemic mastocytosis (SM) insect stings are one of the most important causes of anaphylaxis. Recent literature presented that the use of omalizumab as a premedication may decrease adverse effects occurred during venom immunotherapy (VIT) although consensus statement is needed.

Materials and methods
In this case series, we reported demographic and clinical characteristics of 9 patients diagnosed as SM and Hymenoptera venom allegy and presented seven VIT receiving patients with or without omalizumab premedication.

Results
4 patients were female (44.4%) and the mean age was 49.6±10.7 years. Bone marrow biopsies of all patients were compatible with SM. The median tryptase level was 25,8 µg/L (16-150) and c-Kit D816V mutation was positive in 8 patients. Culprit insect types in the history were bee, wasp and both in 5, 1 and 2 patients, respectively and one patient was unaware of the insect type. All patients had grade 4 systemic reactions. Seven patients underwent VIT (4 for bee, 1 for wasp and 2 for both venoms) and two patients refused to receive VIT. Four patients received monthly 150 mg of omalizumab three months before VIT and during the updosing period while in 3 patients VIT started without omalizumab. Among the patients who received omalizumab, one patient (Patient no:7) experienced anaphylaxis during skin prick tests and the other (Patient no:6) during the first dose of VIT and both had to receive adrenaline (Table 1). Therefore, VIT was postponed and three doses of omalizumab was administered prior VIT in patient no 6. The other two patients were diagnosed as mastocytosis prior to VIT and therefore received omalizumab. The remaining three patients were diagnosed as mastocytosis after the updosing period of VIT and did not receive omalizumab and had no reactions during VIT. Six patients received clustered schema in updosing and one patient had conventional VIT (Patient no:6). In one patient (Patient no:6) who was pretreated with omalizumab before VIT, a grade 4 reaction occurred in the 6th week of VIT.

However, in the 3rd year of treatment a trouble-free bee sting was witnessed.

Conclusion

Omalizumab may be considered as a premedication in patients who experience reactions during skin tests and VIT but systemic reaction may develop in patients under omalizumab premedication and precaution should be considered during VIT in every systemic mastocytosis patient.

Characteristics of the patient

No of patients	**Age**	**Gender**	**Types of venom in history**	**Grade of reactions in history**	**VIT**	**Side effects during VIT**	**Prick tests**	**Total IgE kU/L**	**Spesific IgE kU/L**	***Tryptase µg/L***	**C-Kit D816V mutation**	**Omalizumab**
1	46	Male	Bee	4	Bee	No reaction	Bee + Wasp -	62,6	Bee :0,27 Wasp:0,04	76,7	+	-
2	44	Male	Unknown	4	Bee and wasp	No reaction	Bee + Wasp -	55	Bee :1,13 Wasp:1,35	17,2	+	-
3	34	Male	Bee	4	Refused by patient	-	Bee - Wasp -	-	Bee :0,75 Wasp:0,01	24	+	-
4	52	Female	Bee	4	Bee	No reaction	Bee + Wasp -	-	Bee :0,01 Wasp:0,01	150	+	+
5	50	Female	Wasp	4	Wasp	No reaction	Bee - Wasp -	55	Bee :0,05 Wasp:1,76	27	+	-
6	39	Male	Bee	4	Bee	Systemic	Bee + Wasp -	219	Bee :1,46 Wasp:0,39	25,8	-	+
7	59	Female	Bee and wasp	4	Bee and wasp	No reaction	Bee + Wasp +	13,7	Bee :0,001 Wasp:0,001	29,3	+	+
8	70	Female	Bee	4	Bee	No reaction	Bee + Wasp -	16	Bee :10,1 Wasp:1,1	16	+	+

No of patients	Age	Gender	Types of venom in history	Grade of reactions in history	VIT	Side effects during VIT	Prick tests	Total IgE kU/L	Spesific IgE kU/L	*Tryptase µg/L*	C-Kit D816V mutation	Omalizumab
9	54	Male	Bee and wasp	4	Refused by patient	-	Bee - Wasp -	29,6	Bee :3,6 Wasp:1,17	16,1	+	-

P05 - **Omalizumab In Immunotherapy With Hymenoptera Venom**

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Background
Specific immunotherapy (VIT) is the established therapeutic option in patients who experience allergic reactions due to hymenoptera stings. Sistemic reactions may occur with VIT, preventing its progression. Omalizumab (OMZ) can be used in combination with VIT, in order to prevent systemic adverse reactions.

Materials and methods
We report 4 successful cases of tolerance to bee VIT after pre and concomitant treatment with OMZ.

Results
All patients are beekeepers, had normal basal tryptase levels, started VIT using an ultrarush schedule with antihistamine pre-treatment and had severe systemic reactions during VIT. The first case is a 43-year-old female, healthy, with a history of a grade II reaction according to Mueller's classification; the second and third patients are first patient´s children: a 16 year-old male and a 19 year-old female, both healthy, that experienced a grade IV and III reaction; the fourth patient is a 33-year-old male, with hypertension under Irbesartan and had a history of a grade III reaction. Omalizumab doses were calculated based on weight and total IgE level. In the first two patients, OMZ was initiated 1 week before VIT in the first administration and 1 hour before in the subsequent ones. In patient 3, OMZ was administrated every 2 weeks during 2 months (1 week before first VIT´s administration) and was maintained 1 week before subsequent ones. In patient 4, OMZ was administrated 1 week before every VIT administrations. These approaches were applied for 6 months. In all patients, VIT tolerance to 100µg was accomplished and no severe systemic reactions occurred after several months of OMZ discontinuation. Patient 3 just recently initiated VIT administration without OMZ and more time is needed to evaluate the result. We started the first administration of pre-treatment with OMZ in a fifth patient: a 39-year-old female, beekeeper, healthy, with a history of a grade III reaction who also developded an anaphilaxis reaction with VIT ultrarush.

Conclusion
Omalizumab seems to be a secure and effective option for those patients who do not tolerate VIT. More studies are needed to establish doses, frequency and duration of treatment.

P06 - Evaluation Of Systemic Mastocytosis With 3 Cases

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Background
Systemic mastocytosis (SM) is a heterogeneous disease which is characterized by the abnormal proliferation of mast cells. It can be divided into various subtypes and phenotypes with different prognoses. Systemic mast cell activation results with anaphylaxis in these patients. Here, we report the clinical characteristics of three SM patients, presenting with anaphylaxis.

Case report
Case 1 was a 40 years old man who was referred to our clinic due to 10 years old history of flushing episodes. The episodes are characterized with generalized redness of the body, headache, weakness, and the last episode was accompanied by syncope. Tryptase level was elevated to 23,8 ng/ml (normal <11,5 ng/ml). KITD816V mutation was identified in blood sample. The bone marrow biopsy was hypercellular, and focal paratrabecular infiltration of atypical mast cells was seen. Case 2 and case 3 (female-35 yo, and female-56 yo) had anaphylaxies several times in their history, without discribing any particular trigger. Case 3 physical examination revealed macular pigmented lesions distributed mainly on the trunk. Serum basal tryptase levels were 38,2 ng/ml and 191 ng/ml, respectively. Skin biopsy was reported as cutaneous mastocytosis for the latter one. Hepatosplenomegaly was detected in case 3. The average time to diagnosis for the patients were average two years.

Conclusion
SM includes a wide spectrum of signs and symptoms and atypical presentation can delay the diagnosis substantially. Skin involvement, anaphylaxis attacks and unexplained osteoporosis should alert physician for mastocytosis. A normal serum tryptase does not exclude the diagnosis of SM and it should be considered in the differential diagnosis of patients presenting with recurrent anaphylaxis without a clear cause.

P07 - Risk Factors In Hymenoptera Venom Allergy

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Background
Increased serum tryptase and/or mastocytosis has been linked to the severity of the reaction after Hymenoptera stings. The aim of the study was to analyse Hymenoptera venom-allergic patients with regard to basal tryptase relation to the severity of sting reactions. Mastocytosis and/or elevated basal serum tryptase may be associated with severe anaphylaxis.

Materials and methods
Of the 93 patients included in this study (Group A), 34 were allergic to Bee venom (BV) and 59 were allergic to Vespula venom (VV) (Table 1). All patients gave a history of systemic allergic reactions to Hymenoptera stings. Based on clinical symptoms, the reactions of the patients were divided into 4 grades of severity according to Mueller classification. Basal serum tryptase was measured in all patients (34 Honey Bee, 59 Vespula). Levels of the mast cell-specific enzyme tryptase and of venom-specific IgE (sIgE) were estimated by ImmunoCAP.

Results
Basal serum tryptase levels were elevated in 16 (17.2%) of the 93 patients. Evidence of cutaneous mastocytosis as documented by skin biopsy was present in 3 of 16 patients (18.8%) – all with a history of severe shock reactions. All patients with elevated tryptase had a history of severe systemic allergic reactions to Hymenoptera stings; no significant correlation, however, between basal serum tryptase and sting reaction severity was observed ($r = 0.174$; $p = 0.099$). There was a correlation of the grade of the initial allergic reaction and venom-specific IgE to Bee venom ($r= 0.296$, $p=0.0114$), but not Vespula venom ($r=0.063$, $p=0.562$).

Conclusion
These results corroborate the elevation of basal serum tryptase as well as mastocytosis as the risk factors for severe or even fatal shock reactions to Hymenoptera stings. Although the efficacy of venom immunotherapy in these patients is slightly reduced, most of them can be treated successfully. Based on currently available data, lifelong venom immunotherapy treatment for these patients is typically considered.

Demographic, clinical and laboratory data on 93 Hymenoptera venom-allergic patients.

Group of patients	Allergic to Bee venom (n 34)	Allergic to Vespula venom (n 59)
Sex (male/female)	18/16	26/33
Grade of reaction I II	 1 1	 8 2

III IV	26 4	28 23
Venom-specific IgE kU\L < 0.35 > 0.35	5 29	13 46
Basal serum tryptase (normal/elevated)	30/2	45/14

P08 - **Contribution Of Component Resolved Diagnosis In Hymenoptera Venom Allergy**

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Background
In Hymenoptera venom allergy, difficulties in diagnosis can be seen in daily life practice and new practical diagnostic methods seems to be promising. In this study, the contribution of component resolved diagnostics (CRD) were evaluated in patients who had a systemic reaction due to a Hymenoptera.

Materials and methods
81 patients from four different centers were included in the study. Prick, intradermal skin test with venom extracts were performed and serum specific IgE levels for whole venoms were measured. sIgEs for Api m1, Api m2, Api m10, Ves v1 and Ves v5 were also evaluated (Euroline DPA-Dx Venom kit 2, Euroimmun, Lubeck, Germany).

Results
Seventeen out of 33 patients with bee venom allergy revealed a positive skin test result and/or a high sIgE level to honeybee venom whereas 16 patients had positivity with both venoms. In 11 out of 17 patients with bee venom allergy, the diagnosis was confirmed with CRD whereas CRD was negative in the remaining 6 patients. In 13 of the bee allergic patients with double positivity to both venoms (13/16), double sensitivity was confirmed with CRD. CRD revealed a sensitivity of 73% in bee venom allergic patients. Seven of 18 patients with wasp venom allergy demonstrated sensitivity only to Vespula spp according to skin tests and/or sIgE levels whereas 11 patients revealed double positivity. Total sensitivity of Ves v1 and Ves v5 was calculated as 88%. Eight of 20 patients with a history of hypersensitivity to both venoms showed double sensitivity with CRD, one patient

revealed
cross-reactivity, seven patients was found sensitive only to bee venom, and finally one patient was sensitive only to Vespula spp. 10 patients were uncertain for the culprit insect type, half of them had double sensitivity and one had cross-reactivity according to CRD.

Conclusion
CRD seems to be more helpful in diagnosing wasp venom allergy than bee venom allergy. It is also promising to differentiate double sensitivity from cross-reactivity and it is valuable in cases where the culprit insect is unknown.

P09 – SENSITIZATION TO BEE VENOM IN NON-ALLERGIC BEEKEEPERS

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Background
Hymenoptera venom allergy is a major cause of anaphylaxis and beekeepers are at particular risk.
OBJECTIVE: To evaluate sensitization to bee venom in beekeepers without any history of systemic reactions to bee stings.

Materials and methods
This cross-sectional study used a questionnaire, skin prick tests (SPT) with common aeroallergens and intradermal tests (IDT) with bee venom (0.1 and 1 m mg/mL) on beekeepers who volunteered to participate during a beekeeping meeting. Written informed consent was obtained.

Results
A total of 64 beekeepers without any systemic reactions to bee stings agreed to participate; 52 (81%) males with median age of 46 (±15) years. Four (6%) reported asthma and 9 (14%) rhinitis. Duration of beekeeping activity was as follows: 5 (8%) under 1 year, 10 (16%) 1 to 2 years, 18 (28%) 2-5 years, 13 (20%) 5-10 years and 18 (28%) longer than 10 years.
Of the total, 38 (59%) had positive IDT. In beekeepers under 1 year, 3 (60 %) had positive IDT and in those longer than 10 years, 9 (50%) had positive IDTs.
In this group, there was no significant association between the estimated mean annual number of stings and sensitization to bee venom.
The beekeepers who wore totally protective suits had less sensitization to bee venom ($p=0.011$). Individuals with more years of beekeeping had a higher number of positive IDT with 0.1 mg/mL ($p <0.05$). In addition, sensitization to cultivated grass pollen and wild grass pollen was associated with a higher number of positive IDT with 0.1 mg/mL and 1 mg/mL ($p=0.001$; $p=0.048$; $p=0.007$; $p=0.028$).

Conclusion
In this group, 59% of the beekeepers were found to be sensitized to bee venom. This is possibly due to the greater exposure to stings. We would infer this could occur with greater number of stings, when no protective suit is worn and a longer period of beekeeping. Regular exposure to bee venom in these individuals may

confer greater tolerance and thus reduce the risk of systemic allergic reactions due to stings. More extensive studies with larger samples and follow-up may help to clarify these issues.

P10 - MOLECULAR DIAGNOSIS AND BEYOND UNMET NEEDS IN RUSH IMMUNOTHERAPY FOR HYMENOPTERA VENOM- SINGLE CENTER EXPERIENCE IN ALBANIA

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Background
Component resolved diagnosis (CRD) as a novel tool is helping in providing correct selection of venom immunotherapy treatment (VIT) formulations and monitoring of such lifesaving treatment. Despite significant number of patients requiring emergency care due to hymenoptera sting allergy in Albania, few undergone VIT. This is first study performed in Albania since CRD availability which limitations mainly include diagnosis and treatment related cost.

Materials and methods
Retrospective analytical-descriptive study providing data from patients records treated with VIT at the Allergology Service at Mother Theresa UHC, Tirana.

Results: 25 cases (56% male), mean age 37.83 years (SD ± 16.52) received VIT for APIS 21 (84%), Vespula 3 (12%); Polister 1 (4%). IDR (Intra Dermal Reaction) sensitization at concentration 0.1 mcg/ml showed Apis 84%, Ves 16% and Pol 12%. CRD performed incidences of sensitivization for antigens were: honeybee i1-75%; rApi m1-25%; rApi m2-33,3%; rApi m10-41,7%; common vasp i3-58,3%; rVes v1- 25%; rVes v5-58,3%. CDD was positive in 25% of cases indicating cross reactivity. Previous VIT patients had an average of 2.69 episodes of generalized systemic reactions (SR) classified according Mueller grading system: 44% - grade 4; 40% - Grade 3; 16% Grade 2. In 12(48%) SR happened on initial phase of VIT; 58% (Mueller Grade 3-4); 5 (20%) had SR during mantenaince phase of VIT (dose I-VIII) with 3 cases of anaphylaxis. Natural sting challenge during VIT happened in 20% cases resulting in SRs 1-grade Mueller lower than prior VIT initiation. There was no statistically significant correlation ($p > 0.05$) between Mueller grading of SR and size of papules/ erythema in IDR test).

Conclusion
Our experience with the molecular components of the hymenoptera venom has been limited, but remains a key to successful treatment and follow-up for VIT. Fully reimbursement of molecular diagnosis and VIT treatment in near future will help decreasing burden of venom allergy disease and improvement in patients quality of life in Albania.

P11 - Contribution Of Molecular Diagnosis Of Bee Venom Allergic Patients With Systemic Reactions During Venom Immunotherapy

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Background
Bee venom (BV) allergy is one of the most common causes of severe anaphylaxis in adults. Venom immunotherapy (VIT) is considered the most effective treatment, but systemic reactions (SR) can occur during it. Molecular diagnosis can improve diagnostic accuracy, but no correlation was identified with SR during VIT. **Aim**: Characterize the sensitization profile by molecular components of pts with anaphylactic reactions to BV under VIT and investigate if SR during VIT are related to different patterns of sensitization.

Materials and methods
Prospective study including 30 pts under VIT for at least 1 year. We considered a group of pts reacting during the ultra-rush (Group A) that was compared with the group with no reactions (Group B). Serum specific IgE (sIgE) for BV (i1) and recombinants: rApi m1, rApi m2, rApi m3, rApi m5 and rApi m10 were evaluated before and after 1 year of VIT by ImmunoCAP (Termofisher Scientific, Uppsala, Sweden). A value>0.35kUA/l was considered positive. All statistical tests were performed with Graph-Pad Prism v5.01.

Results
80%-male, mean age-45 years old (14-70). Group A -10 pts; Group B-20 pts. 4 pts (2 -group A and 2-group B) were drop out during first year of VIT. Before VIT, sIgE to rApi m1 was detected in 86.7%, rApi m2-46.7%, rApi m3-16.7%, rApi m5-43.3% and rApi m10-70%. Positive results to at least one bee venom allergen were detected in 100%. 80% of pts were sensitized to >1 allergen and 13.3% to all allergens. Characterization profile of both groups - median and interquartile range (IQR25/75) before and 1 year after VIT are represented in table 1.There was no statistically significant differences in the profile of both groups before VIT, however we found a significant decrease: p=0.045, p=0.017, p=0.021 to i1, Api m3, Api m10 respectively, in group B 1 year after VIT.

Conclusion
These data showed that 1 year after VIT there was a significant decrease of Api m3 and Api m10 in pts without reactions during VIT, however there was not found association between pts with SR during VIT and there sensitization profile. Nevertheless is important to study a greater number of pts.

	Group A				Group B			
	Median+	IQR25/75+	Median*	IQR25/75*	Median+	IQR25/75+	Median*	IQR25/75*
i1	8.58	2.52/ 26.68	7.37	1.62/65.88	10.05	2.53/24.93	5.14	1.92/17.10
rApi m1	3.65	0.85/15.94	2.55	0.95/28.91	1.87	0.45/11.11	2.01	0.42/6.66
rApi m2	0.74	0.02/3.46	0.63	0.05/9.25	0.03	0/1.61	0.11	0/0.70
rApi m3	0.17	0.02/1.14	0.08	0.02/2.45	0.03	0/0.23	0.02	0/0.08
rApi m5	0.29	0.02/2.85	0.85	0.03/3.39	0.16	0/2.25	0.13	0.01/1.43
rApi m10	1.98	0.20/2.80	1.116	0.21/5.30	0.43	0.11/5.28	0.45	0.07/1.98

Table 1. Characterization of sensitization profile of group A and B before and 1 year after VIT (+ before VIT; * 1 year after VIT)

P12 - OMALIZUMAB IN IMMUNOTHERAPY WITH HYMENOPTERA VENOM- CASE REPORT

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Background

In Europe the prevalence of systemic reactions with hymenoptera sting varies between 0.3-7.5%, being higher in beekeepers. Hymenoptera venom immunotherapy (VIT) provides protection in 80-100% of the cases. Allergic reactions may occur with VIT especially during the initiation with ultra-rush, preventing its progression. Omalizumab can be used in combination with ITV, in order to reduce allergic reactions.

Materials and methods

A 53-year-old man, beekeeper in his free time, goes to the emergency room with nasal obstruction, rhinorrhea, dyspnea and facial erythema beginning 10 minutes (min) after a bee sting on the right index finger. At the emergency room, 3 hours after the sting, he was hemodynamically stable, eupneic, without bronchospasm, with nasal obstruction and facial erythema. He was treated with systemic steroids, clemastine, and ranitidine, with improvement. The tryptase was 16.6 ug/L.

Immunoallergology workup revealed bee venom specific IgE> 100 kUA /l and positive intradermal skin test for bee venom extract at concentration of 0.01 µg/mL. Bee venom ultra-rush was started with pretreatment with clemastine and montelukast but it was interrupted by anaphylactic reaction 30 min after administration of 10 µg of bee venom. He was treated with made adrenaline, methylprednisolone and bronchodilators, with improvement. He repeated new ultra-rush with pretreatment with montelukast and antihistamine for 15 days and had a new reaction 30 min after administration of 10 µg of venom: erythema on the face and neck,that regressed after corticosteroid and ranitidine ev. On the same day he repeated the administration of 10 µg, with reappearance of skin complaints and edema of the uvula on observation. He made hydrocortisone, ranitidine and aminocaproic acid ev, with improvement. A new ultra-rush was performed under omalizumab, maintaining antihistamine and daily montelukast. Initially, he did 2 administrations of omalizumab, 7 days and 1 hour before the ultrarush, with onset of erythema of the face and nasal obstruction. He subsequently performed 4 doses of 300 mg omalizumab with a 15-day interval. On the 7th day he restarted ultra-rush with good tolerance.

Conclusion
Omalizumab has been used in association with IT in the control of allergic reactions with good results. The authors describe a clinical case in which the use of omalizumab successfully allowed the progression of ultra-rush with hymenoptera venom.

P13 - Precision Medicine And The Tryptase Framework Of Wasp Venom IgE-Sensitization In Mastocytosis

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Background
Mastocytosis patients are at high risk if they are IgE-sensitized to insect venom, while tryptase measurements are very useful or even required to perform a clinical follow-up of both the status of mastocytosis and anaphylaxis. One of the challenges is to distinguish a chronically elevated tryptase level (TL *evl*) due to mastocytosis from an anaphylactic TL *evl*. As a decreased tryptase level (TL *dcr*) below the basal level (TL *bas*) may follow an anaphylactic TL *evl* and the long-term TL *bas* may vary in time, the challenge is to distinguish a TL *evl* and TL *dcr* from the TL *bas*. The goal of this study is to establish the TL *bas* and the individually biological variation (CV*i*) of tryptase in mastocytosis patients as well as to identify abnormal tryptase fluctuations in general and the provoking IgE-sensitization in particular.

Materials and methods
Tryptase data of mastocytosis patients, which were obtained routinely for their

follow-up, were collected retrospectively and included if > 6 data points were available. Iterative polynomial regression fitted the patients' data points in a model, each time adjusted after outlier exclusion. Outlier exclusion was based on using the Median Absolute Deviation set at 4.5. Using the model, the combined total correlation of variation (CV*t*) of the CVs of each observed data point was calculated. The analytical CV (CV*a*) was set at 5.7% and the unforeseen CV (CV*u*) at 0%. The CV*i* was defined as CV*i* = square root of [$(CVt)^2 - (CVa)^2 - (CVu)^2$]. All tryptase measurements and those for IgE sensitizations were performed by the ImmunoCAP assay.

Results
Median number of datapoints per patient was 10 and the period of follow-up 7.1 yr. The range of CV*i* of the mastocytosis patients (n = 47) was 0.6-16.6% (95% CI 4.9-6.9%; median 5.1%). Although the CV*i* was stable and small in general, the range within the group was broad and abnormally distributed; As in some patients the TL *bas* significantly varied in time also, the use of a grouped TL *bas* and CV*i* was not justified. Personalized TL *bas* and CV*i* were needed to recognize abnormal fluctuations. Based on outlier calculation single or multiple abnormal fluctuations were identified in 13 patients, which at least in one case could be attributed to wasp venom IgE-sensitization.

Conclusion
Personalized TL *bas* and CV*i* are required to recognize abnormal fluctuations in tryptase levels. Therefore, precision medicine should be part of the tryptase framework in mastocytosis in general and of IgE-sensitization in mastocytosis in particular.

P14 - Soluble FcεRI Is A Potential Biomarker Of IgE Mast Cell Desensitization During Chemotherapy Treatment Of Allergic Cancer Patients

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Background
IgE-desensitization to chemotherapy in allergic cancer patients inhibits mast cell (MC) activation and protects against anaphylaxis in a high proportion of patients but no biomarkers exist to predict at risk candidates and there is little understanding of the events leading to successful desensitizations. A soluble isoform of the high affinity IgE receptor (sFcεRI) has been identified in atopic

individuals and its role is thought to protect against anaphylaxis, and it is not known whether sFcεRI could modulate the desensitization process. We sought to understand the effect of desensitization on sFcεRI production *in vitro* and monitor serum sFcεRI titers *in vivo* in cancer desensitized patients.

Materials and methods
Murine MCs expressing the human IgE-binding FcεRIα were sensitized with serum from platin allergic patients and stimulated with anti-human IgE. β-hexosaminidase and sFcεRI were measured after activation and desensitization. Ovarian and colon cancer patients (n= 14) with severe allergic reactions to oxaliplatin and carboplatin were evaluated at the time of rapid desensitization and serum sFcεRI, IgE, and tryptase titers were measured before and after desensitization.

Results
Desensitization significantly inhibited β-hexosaminidase release and sFcεRI production in humanized MCs, in contrast to activation. Two groups of patients were identified based on their baseline sFcεRI titers. Patients with sFcεRI titers > 2 ng/mL (n= 5) presented increased sFcεRI and IgE titers, and a significant decrease in tryptase levels following successful desensitization. In contrast, patients (n=9) with sFcεRI titers < 2 ng/mL showed increased IgE and tryptase levels, whereas sFcεRI remained unchanged. One patient reacted during desensitization in that group.

Conclusion
Desensitization inhibited sFcεRI production in humanized mast cells in vitro, which correlated with inhibition of β-hexosaminidase release, providing novel insight into the mechanism of IgE-desensitization. In cancer desensitized patients higher serum sFcεRI was associated with decreased tryptase and protection from allergic reactions. Measurement of sFcεRI may provide a new biomarker of protection against drug-induced reactions during desensitization.

P15 - The Need Of Bee Venom Immunotherapy Reintroduction Due To Unsuccessful 5-Year-Lasting Treatment In Adolescent Boy

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Background
Venom immunotherapy (VIT) in children rarely requires reintroduction.

Case report
The 12-years-old boy was accepted for the reintroduction of bee venom immunotherapy (B-VIT). In the past, due to anaphylactic shock after bee sting in V 2011, he was treated according to the guidelines with B-VIT (Pharmalgen for 1-year, next Alutard) from March 2012 to October 2017. In the course of B-VIT in I-V 2017 large local reactions (LLR) and late mild systemic reactions due to venom injections were observed, while natural four subsequent bee stings were tolerated well. After detailed work-up, in June 2017, Giardia lamblia infestation was diagnosed and treated with metronidazole with recovery. Within further course of VIT till October 2017 he tolerated well both subsequent venom injections, and three

natural field stings. After 5 years of treatment, in October 2017, we decided to stop B-VIT. In 2018 he was stung three times by bee: in V and in VII in thumb with the normal reaction, without necessity of symptomatic treatment. For the third time he was stung by bee in the left cheek in VIII 2018. Fifteen minutes later, despite taking immediately oral antihistamine and glucocorticosteroid, he presented with vomiting, general urticaria, somnolescence. In GP office massive urticaria, facial edema, dysartia, BP 100/60, HR 50/min, exacerbated vesicular sound over the lungs were observed. He was given dexamethasone, phenazolinum iv. Ambulance staff found RR 16, BP 90/60 (regularly 100/60), HR 80/min, Sat 99%, normal vesicular sound; they refused to transport the boy to the hospital. Patient stayed under GP's supervision for three hours, and then he was transported by parents to the clinic. At the admission he presented well, with normal vital signs and parameters. Only oral antihistamines were ordered. Since that time he was not stung by bee. There are still bee-hives in the house vicinity. In subsequent diagnostics in August 2018 laboratory results were as follows: 1. sIgE to: BV extract 24.1 kU/l, Api m 1 7.75 kU/l, Api m 10 12.1 kU/l, 2. baseline serum tryptase 4.79 kU/l. In November 2018 he restarted B-VIT with Pharmalgen given as ultrarush protocol by Birnbaum. At the dose of 30 mcg (cumulative dose 61 mcg) he reacted with general urticaria and pruritus, with no other general symptoms. Double dose of cetirizine and one dose of dexamethasone i.m. were introduced.

Conclusion
Now, B-VIT is continued according to cluster protocol with up-dosing of 10-20 mcg every two weeks to achieve maintenance dose of 150 mcg. No immediate or late reactions were observed, though each medical visit is stressful for the patient.

P16 - Multicenter Study Of Clinical Relevance Of Recombinant Allergen Api M 1 And Ves V 5 Determined By IgE Multiplex Test ImmunoCAP ISAC

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Background
ImmunoCAP ISAC (ISAC) is an advanced diagnostic tool for the assessment of complex cases. Two major venom components honeybee rApi m1 and yellow jacket rVes v5 are also included on this microarray. We evaluated ISAC results for those two components and its possible clinical relevance.

Materials and methods
Specific IgE to rApi m1 and rVes v5 were analyzed in all subjects, which were routinely tested with ISAC from 2012 to 2017 at University Clinic Golnik, Slovenia or at Faculty of Medicine Plzen, Czech Republic. Results were compared with singleplex ImmunoCAP (CAP) assay and evaluated weather they are clinically

relevant.

Results
Positive results for rApi m1 and/or rVes v5 were observed in 342 (11.4%) out of 3001 ISAC tested subjects. 232(67.8%) of 342 subjects were sensitized for rVes v5, 83 (24.3%) for rApi m1 and 27 (7.9%) for both allergens. Positive ISAC results from 93 (27.2%) subjects were clinically evaluated and compared with CAP. Honeybee venom allergy was confirmed in 5.4% (5/93) subjects, yellow jacket venom allergy in 23.7% (22/93), and both in one subject. Twelve of those patients (43%) experienced anaphylactic reactions while 16 (57%) had large local reaction. Concordance between ISAC and CAP results was 90.3% (84/93) for rApi m1 and 97.8% (91/93) for rVes v5. Discordance for rApi m1 was present in 9 subjects; 8 were negative with ISAC, but positive with CAP, one was positive with ISAC, but negative with CAP. Discordance for rVes v5 was demonstrated only in 2 subjects; in both ISAC was positive and CAP negative. There was a significant correlation between semi-quantitative ISAC and quantitative CAP measurements, both for rApi m1 ($R=0.79$, $p<0.0001$) and rVes v5 ($R=0.69$, $p<0.0001$).

Conclusion
In ISAC microarray, positive rApi m1 and rVes v5 results are frequent, reaching approximately 10-15% in the Middle Europe geographic region. The results were confirmed with standard CAP assay, both according to the positivity/negativity and semi-quantitative/quantitative levels, with higher matching for rVes v5 than for rApi m1. The sensitization was relevant in one third of the subjects (half with anaphylactic sting reactions), what obviously suggests that every positive subject should be clinically evaluated.

P17 - Systemic Mastocytosis With Low Serum Tryptase: A Challenging Diagnosis

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Background
Mastocytosis are a heterogenous group of diseases, characterized by clonal mast cell (MC) accumulation on several organs and systems. Serum tryptase (sbT) is mostly used as a MC proliferation marker and not as an activation marker. In the past, a normal sbT was used as a surrogate marker to exclude systemic mastocytosis (SM), in patients with mastocytosis in the skin (MIS). We aim to show how challenging the diagnosis and staging of SM may be in patients with a normal sbT, while showing that quite different clinical presentations may be seen in patients with similar sbT.

Case report

Clinical case 1.
A 21 years old female patient with persistent allergic rhinitis, with a onset of MIS at 2 years of age, was referred to us due to severe diarrhea starting 2 months before. The patient had a recent endoscopic study in which mastocytosis lesions were found. She had no history of anaphylaxis and her only trigger for MC mediator release symptoms was emotional stress. Blood tests showed an iron deficiency anemia and sbT rounding 8 ng/mL. Bone marrow (BM) study did not show MC aggregates, and MC were negative for CD25, CD2, and c-KIT D816V mutation. She was started on sodium cromoglycate and her gastrointestinal symptoms started improving a few weeks later. Diarrhea ceased completely after three months.

Clinical case 2.
A 44 years old female non-atopic patient with morbid obesity, depression, with onset of MIS at 35 years of age, was referred to us due to multiple anaphylactic episodes (1-2 yearly), starting 12 years before. The patient also complained of frequent pyrosis and sporadic diarrhea. Triggers for anaphylaxis included NSAIDs, several antibiotics and emotional stress. Blood tests showed sbT rounding 11 ng/mL. BM study showed atypical MC that did not form aggregates, immunophenotypically positive MC for CD25 and CD2 with c-KIT D816V mutation restricted to MC. Previous medication was suspended and she was started on sodium cromoglycate and proton pump inhibitors. She has not had anaphylaxis ever since.

Conclusion
Both cases were diagnosed with indolent MS with low BM MC burden. This diagnosis is often quite difficult, due to the histologically absent/low number of MC aggregates. Despite their similar good response and prognosis, these cases showed rather different clinical presentations and daily life impairments.

P18 - Kounis Syndrome: A Thought-Provoking Case Report

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Background
Kounis syndrome is defined as the co-incidental occurrence of an acute coronary syndrome with hypersensitivity reactions following an allergic event, which could be triggered by many mediators, including hymenoptera venom. Three different variants have been defined: type I in patients without risk factors or coronary lesions, in which the allergic event may induce either coronary artery spasm without increased cardiac enzymes or coronary artery spasm progressing to acute myocardial infarction; type II in patients with pre-existing atheromatous disease previously quiescent or symptomatic, in whom acute hypersensitive reactions may induce coronary artery spasm with or without plaque erosion or rupture, culminating in acute myocardial infarction; type III has been defined in patients

with preexisting coronary disease and drug eluting coronary stent thrombosis.

Materials and methods
A 61 years old male, affected by mild hypertension, was brought to the Emergency Room (ER) with symptoms of anaphylactic shock after a wasp sting. Five minutes after the sting, the patient experienced tachycardia, rash, abdominal pain, dyspnea, chest pain and visual blurring, briefly followed by syncope; when the medical aid arrived at the scene a few minutes later, the patient was half-conscious and both epinephrine and methylprednisolone were administered.
During the medical assistance in the ER, a significant elevation in cardiac enzymes (troponin I blood level was eight times the upper limit of normal) and ECG nonspecific repolarization were shown. Immediate coronary angiography was anyway performed and revealed an 80% stenosis in the left circumflex artery and the patient underwent coronary angioplasty. In addition, to exclude neurological causes of syncope, a brain CT was performed and didn't show any pathological findings.

Results
One month later, the patient underwent allergologic examination, which showed: level of serum tryptase at the upper limit of normality, presence of specific IgE against wasp venoms and positivity for wasp in intradermal tests. Therefore, the patient started venom immuno-therapy for wasp (Polistes).

Conclusion
This case report describes a probable case of type II Kounis syndrome, in which the allergy workup has been performed with a significant delay; although it is not a rare disease, Kounis syndrome diagnosis is easily overlooked. The prescription of VIT in these cases is mandatory, also to avoid cardiac o cardiological effects of epinephrine injections.

P19 - Anaphylaxis Related To Hymenoptera Sting: The Relevance Of Laboratory Testing And Bone Marrow Biopsy

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Background
Hymenoptera venom anaphylaxis may be related to a mast cell disorder.

Case report
A 40-year-old man in June 2012 presented sudden loss of hearing and vision and marked hypotension one hour after a wasp sting He was given intramuscular adrenaline with prompt benefit. Over the following years until the summer of 2018 he reported frequent bites by unspecified hymenoptera without either cutaneous or

systemic manifestations, as he promptly took antihistamine and corticosteroid therapy at each episode. In September 2018, following a wasp sting, despite the usual therapy, he had general malaise with chills and subsequent loss of consciousness, and it was admitted to the nearest hospital and the symptomatology regressed only after having practiced therapy with epinephrine. He was referred to our allergy clinic and prick, intradermal tests were performed with Bee venom, Wasp, Polistes and Hornet. A local skin reaction was documented only for Wasp and Polistes that occurred after a few minutes from the tests and regressed after 48 hours, but no systemic symptoms were observed specific IgE for Bee, Wasp and Horsefly and their respective recombinants confirmed hypersensitivity to Wasp with slight positivity for Yellow Jacket 0.21 KU/l, Dolichovespula maculata 0.11 KU/l and Polistes 1.54 KU/l, rVespv5 and rPold5 respectively of 0.23 and 1.35 KU/l, and the remaining negative.

Conclusion
Should this be the case for other investigations? Yes, in the diagnostic hypothesis of an underlying mast-cell disorder. In fact, despite the patient did not report a wide range of mast-cells mediator symptoms, he had a syncope without urticaria and angioedema, moreover the specific IgE did not show particularly high titers. Therefore, his the assay of the serum tryptase 44.90 µg/L and the RT-PCR for c-KIT mutation D816V in peripheral blood gave a positive result. Finally, the bone marrow biopsy confirmed our suspicion of indolent systemic mastocytosis, showing nodular lymphocyte aggregates with CD117+, CD25+ and CD30+ mast cells at the periphery and scattered eosinophilic granulocytes.

P20 - Three Is A Charm!

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Background
Patients with mastocytosis have an increased risk for severe anaphylactic reactions after hymenoptera stings.

Case report
A 42-year old female patient was admitted to the ER due to loss of consciousness and hypotension (85/40 mmHg) 4 hours after a honeybee sting and 15 minutes after eating a cake and drinking alcohol. An acute serum tryptase, one hour after the event, was 59.6 µg/L (basal serum tryptase (SBT) 12.0 µg/L). Specific serum

IgE (sIgE) for honeybee venom (HBV) (Phadia Thermofisher) was 3.77 Ua/ml two months after the sting and intradermal skin testing with HBV was positive at 1 µg/ml. During the next months, the sIgE for HBV decreased. No immunotherapy for HBV was started due to the low sting risk and long interval between sting and reaction. Eight months later, the sIgE for HBV was 0.38 Ua/ml, and again 1 year later, sIgE for HBV was < 0.10 Ua/ml. SBT decreased to a semi-normal level of 10.8 Ua/ml. The reaction was considered as a non-specific mast cell release and an adrenalin auto-injector was prescribed.
In 2017, 8 years later, this patient presented again immediately after a honeybee sting with dizziness. Parameters were stable and rescue medication was administered. Acute serum tryptase was 8.6 µg/L. Allergologic work-up 14 days later showed following results: SBT 10.8 µg/L, sIgE HBV 6.63 kU/L (Api m1 0.46 kU/L, Api m10 < 0.10 kU/L), sIgE yellow jacket venom (YJV) < 0.10 kU/L. A D816V KIT-mutation was detected in peripheral blood (0.015%). Mastocytosis work-up revealed a diagnosis of indolent systemic mastocytosis (3 minor criteria ($CD25^+$, spindle shaped and D816V KIT^+ mast cells). Bone densitometry showed osteoporosis (T-score -2.2 and -2.7 for L2-L4 and femur respectively). Two months later, patient had a YJ sting: she experienced a large local reaction and presyncope, with recovery after taking antihistamines. Three weeks later, during hair bleaching, she experienced dizziness, dyspnea and loss of consciousness with hypotension (60/40 mmHg) and an acute tryptase level 34 µg/L. Two weeks later, sIgE for YJV was 0.40 kU/L (Ves v1 0.45 kU/L, Ves v5 < 0.10 kU/L), HBV 2.40 kU/L (Api m1, Api m2, Api m5 and Api m10 <0.10 kU/L, Api m3 1.00 kU/L, Bromelain <0.10 kU/L). Venom immunotherapy (VIT) for YJV was started and 6 months later, VIT for HBV was associated.

Conclusion
This case illustrates the need to rule out an underlying mastocytosis in patients with severe and atypical reactions after hymenoptera stings and shows the rapid decrease of sIgE in these patients.

P21 - Hymenoptera Species: Who's Eating And Stinging?

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Background
Vespula, *Vespa* and *Polistes* behaviour differs in their habits and feeding source. The knowledge of the feeding habits of vespids could help to identify the insect responsible of the sting in a food environment. This could provide an important support in Hymenoptera venom allergy diagnosis.

Materials and methods
A prospective observational study was performed in Spain, from June 2017 to January 2019. Pictures of Hymenoptera species in a food environment were

collected and identified by an entomologist. Insect, kind of food and place were recorded.

Results
One hundred and three images corresponding to 50 insects were analysed. The identified insects were 37 *Vespula (germanica* and *vulgaris*), 5 *Vespa* (1 *crabro*, 4 *velutina*), 4 *Polistes* (2 *dominula*, 2 *gallicus*), 2 *Cerceris* spp., 1 *Bombus terrestris* and 1 *Apis mellifera*. Foods associated with the insects were carbohydrates in 20 cases (7 of them were alcoholic beverages) and proteins in 30. Only vespine species were found on proteic food (meat, fish, seafood).
Most of the insects were in restaurant or home terraces (64%). Other places were countryside (7), swimming pool (3) and indoor (4).
All *Vespa* species were found in north-west Spain. There were no differences concerning *Vespula* and *Polistes* in the rest of regions.

Conclusion
Vespula was the hymenopteran mainly associated with food environments in our country (74%). Though *Polistes* spp. were present, they only were found in connection with carbohydrates (alcoholic beverages or fruit).

P22 - Pit Latrines And Latrodectism: An Additional Risk Factor For Spider Bites In Rural South Africa.

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Background
The incidence, prevalence and the entire epidemiology of spider bites by the latrodectus species has been well studied in South Africa (SA) in the early 1990's and later reviewed in the early 2000's. Risk factors that were found to be directly linked with cases involving latrodectus species included occupations such as construction workers, agricultural workers, municipal and utility workers, domestic workers, and entomologists.

Case report
According to the SA nationwide census of 2011, it was discovered that in SA 57% of the population used flush toilets (16 million people), 31% pit toilets (16 million people), 3% chemical toilets (1.5 million people), and 2% bucket toilets (1 million people). The dark, moist, filthy and insect predominant nature of pit toilets serves as an ideal habitat for spiders. We report 3 cases of latrodectus spider bites that occurred while using a pit latrine in the rural South Eastern part of SA, with one case resulting in a severe case of latrodectism requiring antivenom administration.

Conclusion
In view of the above, pit latrines should be considered as a potential risk factor for spider bites in rural South Africa. Although rare, but some cases may be severe, with a potential of leading to death; hence government officials need to be informed, and an intervention strategy be put in place.

O04 - Sting-Challenge Demonstrated Tolerance In Patients Undergoing Ant Venom Specific Immunotherapy, Validating New Centre Approach.

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Background
Native ant *Myrmecia pilosula* [Jack Jumper Ants (JJA)] are responsible for a significant burden of hymenoptera-associated allergic disease in south-eastern Australia. In some areas a population sensitisation prevalence of ~3%; with a 12-month exposure and sting rate ~50% higher than that of honeybees; and significantly more severe anaphylactic phenotype. Although randomised clinical trial (RCT) data has established clear efficacy for venom specific immunotherapy, until recently it had not been used significantly outside of the state of Tasmania. Our state venom immunotherapy centre in Victoria is the first institution to implement this program at scale outside Tasmania. We report on the validation of our service by successful live-sting challenge of our initial cohort of patients.

Materials and methods
M pilosula venom-allergic patients were established on allergen specific immmunotherapy according to a locally adapted 6-week modified-semi-rush protocol to a 50 mcg per month maintenance dose of standardised Tasmanian *M pilosula* venom (Royal Hobart Hospital, Tasmania). After 12 months of therapy patients underwent sting challenge with live, locally collected, mainland ants - with two sequential stings being applied to the forearm over a 30min period followed by 2 hours observation. Antihistamines were avoided prior, but were provided 30mins after second sting (in line with likely real-world behaviour) to treat residual symptoms.

Results
55 participants with prior history of clinically severe systemic JJA allergy, and demonstrable sensitisation, who completed > 12months of venom specific immunotherapy.
Baseline Tryptase ranged from 1.0 - 23.3 mcg/L
Following dual sting challenge:
0/55 (0%) experienced severe objective systemic reactions.
No adrenaline or other treatment for severe systemic reactions were required.
All participants (55/55, 100%) experienced local erythema, pain & swelling at the sting sites.
9/55 (16%) experienced mild subjective systemic symptoms.
3/55 (5%) experienced mild objective distal cutaneous symptoms.
All non-local symptoms resolved withint the 2 hour observation period.

Conclusion
Our state centre processes are validated, replicating efficacy shown in prior RCTs. Tasmanian sourced *M pilosula* venom is effective in inducing profound clinical tolerance and protecting against severe allergic reactions in patients previously severely allergic to the geographically distinct mainland *M pilosula* ants.

O05 - **Absence Of Th2 Cell Suppression After Induction Of Venom Immunotherapy In Wasp-Venom Allergic, Indolent Systemic Mastocytosis Patients.**

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Background
Wasp venom allergy (WVA) is a frequent manifestation of indolent systemic mastocytosis (ISM). In the general WVA population venom immunotherapy (VIT) induces a long-term clinical tolerance to wasp venom (WV) that lasts after cessation of VIT. However, in ISM patients this protection after cessation of VIT is absent, suggesting a different underlying immunological response to VIT in ISM patients.

Materials and methods
Specific IgE (sIgE) for WV, Ves v 1, and Ves v 5, as well as WVsIgG4 were determined in serum of ISM and non-ISM WVA patients before and after induction of VIT. WV-specific Th cell responses were analysed by characterizing CFSE labelled PBMCs cultured in the presence of dialyzed, heat inactivated wasp venom (dhiWV) extract. *Ex vivo* expression of IL-4, IFNγ, IL-10, FOXP3, IL-9 and IL-17 in WV-activated Th cells was analysed by flow cytometry.

Results
WV-specific IL-4 producing Th cells were detected in both WVA patient groups. ISM patients show significantly lower serum levels of WV- and Ves v 5-sIgE. During VIT both patient groups showed induction of Ves v 5-sIgE, and WV-sIgG4. In WVA patients without ISM numbers of WV-specific IL-4 and IL-9 positive Th cells are suppressed by VIT. Remarkably, this Th2 cell suppression was not observed in WVA patients with ISM.

Conclusion
This is the first study on an underlying WV-specific Th2 response in WVA ISM patients. ISM patients remarkably show no Th2 cell suppression after induction of VIT, which stands in contrast to the non-ISM WVA population. This difference may either be a potential explanation for the lower clinical effectiveness of VIT in ISM patients or may be the result of a delayed Th2 cell suppression in this population. To further explore these observations data on the long-term immunological effect of VIT in this population are required.

O06 - Predictors Of Severe Cardiovascular Honey-Bee Sting Reaction With Absence Of Skin Symptoms In Patients With Normal Baseline Serum Tryptase Levels

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Background
Severe sting reactions (Muller grade III and IV) are often accompanied with urticaria and angioedema. However, some patients develop prompt cardiovascular symptoms in the absence of skin symptoms. Those high risk patients are suspected for the underlying clonal mast cell disease, regardless of baseline tryptase levels. Therefore, we sought to investigate the predictors of severe HB sting reactions in patients with normal baseline serum tryptase levels.

Materials and methods
We analyzed clinical factors (age, sex, use of beta blocker agents and angiotensin-converting enzyme inhibitors) and immunological factors (sIgE to HBV, JYV), rApi m 1 and rApi m 10, baseline tryptase levels and basophil CD63 expression to HBV) in 38 patients with severe honey-bee sting reaction with cardiovascular symptoms and absence of skin symptoms and compared it to 225 patients with Muller reaction grade III and IV with skin symptoms. In all patients baseline tryptase was < 11.4 ug/ml. We ascertained predictors of anaphylaxis without skin symptoms using penalized logistic regression.

Results
Patients with absence of skin symptoms were older in comparison with patients with skin symptoms (median 50 years vs 47 years respectively, P:0.038), and had moderately higher baseline tryptase levels (median 4.76 ug/ml vs 3.93 ug/ml respectively, P:0.002) and basophil response at and 1 mcg/ml (median 86.4% vs 76.4% respectively, P:0.007). There was no difference in sex, use of beta blocker agents and angiotensin-converting enzyme inhibitors. However only baseline tryptase levels was independent predictor for anaphylaxis without skin symptoms (P:0.015; OR (95% CI) 1.237 (1.042-1.471)).

Conclusion
Minor increase in baseline serum tryptase is independent predictor for severe HB sting reaction without skin symptoms. The mechanistic background for this minor but clinically important tryptase changes are currently unknown and thus further studies are urgently needed.

SPEAKERS' DOCUMENTS

Only to be used for individual study purposes

HYMENOPTERA ALLERGY: Let's start from the beginning

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RD16/0006/0023

INTRODUCTION

Despite the big advances in hymenoptera venom allergy (HVA) diagnosis, to date the single best predictor of the outcome of a future sting is the history of reaction to a previous sting. Clinical history allows to know the risk of a future sting reaction in a certain person; to decide if we should prescribe venom immunotherapy (VIT); the risk of developing systemic reactions with the treatment; and the risk of relapse after finishing VIT. Clinical history is a first-class tool, available to any allergist to get information about many aspects of the disease.

1. **Risk of sting reaction:** The development of HVA is related to stings frequency. Patients' work and habits are of great importance. Rural habitat and the number of stings suffered along life are risk factors of HVA development. Between 14-42% of beekeepers can suffer an allergic reaction to bee venom. A short interval between a previous well tolerated sting and the next one increases the risk of a systemic reaction to a subsequent sting. Recurrence of systemic reactions is determined by previous reaction severity.
2. **Risk of sting severity:** Severe systemic reactions were significantly more common in older and male patients. The fatality rate is higher than in children and young adults. Another variables associated to severe reactions are vespid venom allergy, cardiovascular disease, elevated basal serum tryptase, existence of systemic mastocytosis (SM) or clonal mast cell activation syndrome (cMCAS), ACE inhibitors or beta-blockers intake. The Spanish Network on Mastocytosis has developed a scoring system based on patient gender, the clinical symptoms observed during anaphylaxis and serum baseline tryptase to predict for the presence of both MC clonality and SM among individuals who suffer from anaphylaxis. It's a simple and useful screening tool to identify those patients at risk of presenting mastocytosis or clonal mast cell activation syndrome. A score

> 2 correlates to a positive bone marrow biopsy in 89% of the patients. Indolent SM without skin lesions associated with insect-induced anaphylaxis display a marked male predominance, absence of urticaria or angioedema and the presence of cardiovascular symptoms such as hypotension, leading to loss of consciousness.

3. **Double positive results: Influence of alcohol intake.** Sensitization to CCDs and clinically irrelevant double (honeybee and wasp) IgE reactivity are common among Hymenoptera venom allergic patients who drink alcohol. A simple questionnaire about alcohol consumption could be useful when interpreting levels of specific IgE in these patients.
4. **Risk of systemic reactions with venom immunotherapy:** The most important risk factor is treatment with honeybee venom: there is a 3.1- to sixfold higher risk for systemic adverse events. Mastocytosis has been reported as a risk factor itself for side effects during administration of VIT. Tryptase basal value is not and independent variable, but it was reported a significant association between the increase in serum tryptase on the first day of VIT and future systemic adverse reactions during VIT (risk ratio, 7.6).
5. **Risk of relapse after VIT:** Elderly, bee venom allergy, cMCAS or SM has been associated to a risk of systemic reaction after an insect sting during or after the VIT. VIT conferred a full protection in the majority (86%) of re-stung patients, although this percentage is slightly smaller than that reported in patients without SM.
6. **Quality of life:** Patients with hymenoptera venom allergy experience impairment in quality of life that can be measured using QoL specific questionnaires, at the diagnosis, during VIT or after an sting challenge.
7. **Insect identification:** Knowledge of sting circumstances may help to identify the stinging insect. Insect behaviour and biology help to identify the culprit hymenoptera. Human food is mainly attractive for *Vespula spp*. However, honeybees, *Polistes* and *Vespa* spp. are also attracted by sweets and fruits and act in a human enviroment.

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DIFFICULT CASES

CARMEN MORENO AGUILAR

HURS

ARADyAL
Instituto de Salud Carlos III

CASE 1

DAVID AGAINST GOLIATH

MALE 42

- Social background: Farmer, high exposure to stings. No reaction to wasps.
- Clinical background: Hemochromatosis. Hypertension (Amlodipine/Olmesartan). Seasonal pollinic rhinoconjunctivitis.

MALE 42

July 2017

Bee sting on forehead skin

MALAISE
DYSPNEA
SYNCOPE

HYPOTENSION

iv Methylprednisolone

Primary Emergency Set

RECOVERY

MALE 42

Day	Dose (µg)	Lapse (min)	
1	10	15	Well tolerated
	20		• Flushing • Hand palm erythema • Dizziness • Hypotension (72/42) • Tryptase: 17
	20		Good response to Epinephrine
8	50		
	50		

MALE 42

The Journal of Allergy and Clinical Immunology

Alvarez Towse 2010

	Outcome	Value
Sex	Male	+1
	Female	-1
Symptoms	Pruritus-urticaria-angioedema absence	+1
	Pruritus-urticaria-angioedema	-2
	Syncope	+3
Tryptase	< 15 µg/L	-1
	> 25 µg/L	+2

+4

REMA score <2: Low chance for clonality
REMA score >2: High chance for clonality

Sensitivity: 0,92
PPV: 0,89

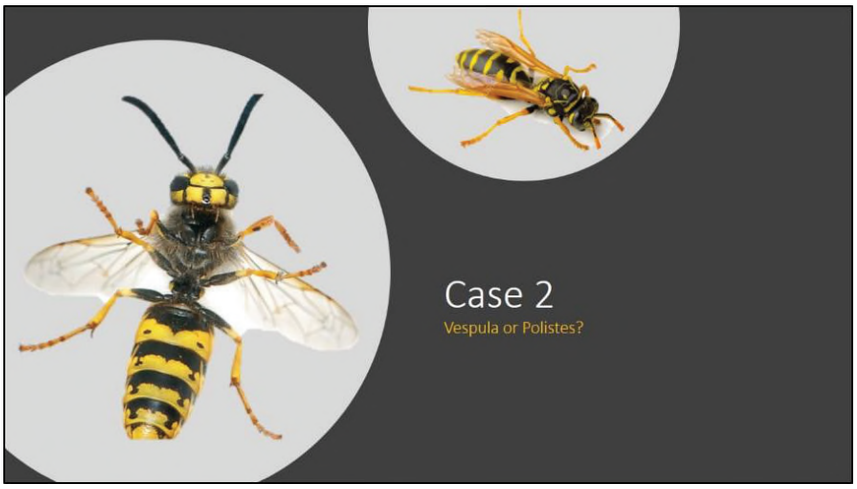
Specificity: 0,81
NPV: 0,87

MALE 42

Mastocytosis treatment:
Cromoglicate + Calcium

VIT Pretreatment:
Clorpheniramine + Metilprednisolone

Day	Dose (µg)	Lapse (min)	
1	10	10	Mild flushing
	10		Good response to clorpheniramine
	10		Well Tolerated
8	10		
	10		Well Tolerated
	20		
15	30		
	30		Well Tolerated
	40		
Montly	100		Well Tolerated



Case 2

Vespula or Polistes?

GIRL, 16

No relevant medical background

GIRL, 16

September 2012 :

Sting of unidentified wasp on left forearm.

URTICARIA
LIP SWELLING
ABDOMINAL PAIN
WHEEZES

Epinephrine
Methylprednisolone
Clorpheniramine

Quick recovery

GIRL, 16

	Apis	*Polistes*	rPol d 5	*Vespula*	rVes v 1	rVes v 5
ID (µg/ml)	NEG	0.01		0.001		
IgE (KU/L)	0.10	16.47	0.64	14.89	19.31	0.12
IgG4 (µg/L)	0.00	987		132		
Triptase (µg/L)	3.43					

GIRL, 16

IgE Inhibition Assay

	Vespula	*Polistes*
IgE KU/L	14.89	16.47
	100 µl serum + 200 µl diluent	
	4.02	5.97
	Preincubation 100 µl serum + 20 µg Vespula venom	
	3.02 (36% inhibition)	1,92 (68% inhibition)
	Preincubation 100 µl serum + 20 µg Polistes venom	
	0.62 (86% inhibition)	0.37 (94% inhibition)
	Preincubation 100 µl serum + 10 µg Vespula + 10 µg Polistes	
	0.32 (92% inhibition)	0.42 (93% inhibition)

GIRL, 16

Immnunoterapy with Polistes venom extract was decided.

But… if you don't have the opportunity to perform inhibition, BAT or any discriminative diagnosis technique, you also can protect your patients in cases of a double sensitization to Vespula-Polistes in Southern Europe

Efficacy and safety of an accelerated outpatient protocol for wasp venom immunotherapy

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Abstract

Background: Venom immunotherapy (VIT) is a well-established treatment for the prevention of systemic sting reactions. Although several attempts have been made to shorten protocols for the up-dosing phase of immunotherapy, the conventional protocol requiring 15 weekly injections to reach the maintenance dose remained undisputed for decades.

Objective: The aim of the study was to evaluate an accelerated up-dosing protocol with 8 weekly injections in 7 weeks.

Methods: Seventy-six vespid venom allergic patients with a history of a systemic sting reaction were included. Patients were treated with oral antihistamines one hour prior to injections. The purified depot preparation Alutard SQ vespid venom (ALK-Abelló, Hørsholm, Denmark) was administered with an initial dose of 1µg followed by 5, 10, 20, 40, 60, 80 and 100µg at 1-week-intervals. In total, 73 sting challenges with living wasps were performed, whenever possible, one week after reaching the maintenance phase.

Results: Only 3 (3.9%) patients showed objective symptoms which were mild and limited to the skin and 5 (6.6%) patients developed mild, subjective systemic reactions. 22 (28.9%) patients experienced large local reactions at the injection site. All patients tolerated sting challenges, 31 (42.5%) already within the first two weeks after reaching the maintenance dose.

Conclusion: Our 7-week outpatient protocol proved to be safe and effective, even in patients with cardiovascular disease and antihypertensive treatment. This rapid outpatient up-dosing protocol is practical as well as efficient in terms of time and costs for patients and medical staff, which will lead to a better patient acceptance of VIT.

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Natural history of systemic reactions in children and adults

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Stings by hymenoptera, namely bees, wasps, yellow jackets, hornets and ants, usually cause just local reactions. However, in some cases, they can induce systemic symptoms, and even fatal reactions.1-3
Insect stings by hymenoptera species are very common with data indicating that 56.6%-94.5% of the general population has been stung at least once in their lifetime.4

Reactions to hymenoptera venom are also responsible for decreased quality of life and significant anxiety about future stings.1,3 The results of the quality-of-life questionnaire demonstrated that a well-tolerated sting challenge test improves the quality of life of venom-allergic patients by reducing the anticipatory anxiety associated with the fear of being stung.5,6

In the last years, recombinant technologies have represented a great advance in the diagnosis of hymenoptera venom allergy as they provide a profile of sensitisation to specific allergens. Molecular diagnosis helps to discriminate between true sensitisation and cross-reactivity in multiple-venom sensitisation, which is crucial in order to prescribe the appropriate venom immunotherapy.7,8

Moreover, Systemic reactions (SR) due to hymenoptera venom allergy affect between 2.3% and 2.8% of the rural population in Spain and may lead to potentially life-threatening anaphylaxis.8
In Europe the rate of self-reported severe systemic reactions (SSR) in European epidemiological studies ranges from 0.3 to 7.5% in adults and up to 3.4% in children.1
Severe SSR is less frequent in children and appears to be rare in children of preschool age (<5 years).1

Several risk factors may be taken into account in adults:

1. Age: Elderly patients with hymenoptera venom allergy (HVA) and pre-existing cardiovascular disease have an increased risk of a fatal sting reaction.1
2. Medication: although there is one study which reported angiotensin-converting enzyme inhibitors to confer a higher risk for more severe SSR; however, there is a growing base of evidence that indicates that ACEI do not increase the risk for severe SSR in untreated patients
3. Mastocytosis: although mastocytosis is a risk factor for both the development of HVA and for more severe SSR, Venom Immunotherapy is usually well tolerated by the majority of patients with underlying systemic mastocytosis.1
4. Type of venom in immunotherapy: bee venom has a higher risk of relapse than vespid venom immunotherapy.
5. Type of insect involved: vespid>bee stings. 9
6. Severity of initial systemic reaction: It is agreed that an initial SSR is more prone to produce a higher risk for future SSR.1,10
7. Systemic adverse events during VIT: Different publications had reported a risk of showing a subsequent SR in 16.4%-46% of patients instead of 5.4%-8% who did not suffer a systemic reaction.1
8. Higher specific/total E ratios: are at higher risk of developing systemic reactions.11
9. Delayed administration of epinephrine: increases the probability of anaphylactic reactions regardless of the elicitor.12

10. Time interval between sting and adverse reaction of less than 5 minutes is predictive of severe anaphylaxis. 13
11. A higher The REMA score, indicates patients who are in need of bone marrow biopsy, namely those patients who are suffering SSR in the absence of urticaria/angioedema along with male sex, hypotensive shock, and tryptase levels > 25 ng/ml.14

Conclusions:

Several risk factors have been proposed, analysed and stated as showing a greater extent of subsequent higher risk in future stings. Children usually have a better prognosis but there is a need for future epidemiologic studies which may create the exact picture of future reactions both in children and adults.

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Session VI. Practical Workshops
Venom immunotherapy. Treatment schedules and how to deal with side effects

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Venom immunotherapy (VIT) is the most effective form of specific immunotherapy to date conferring a protection against future stings in over 95% of cases. Hitherto, several relevant queries remain unanswered, namely optimal doses, duration, and means of assessment.1-3

Important progress has been lately made in terms of diagnosis by means of component-resolved diagnosis. Moreover, basophil activation test results in patients with negative serum immunoglobulin E (IgE) and skin prick test confer this technique a promising future, although these outcomes shall be considered with caution.

However, VIT is not absent of adverse reactions, namely, local and systemic adverse reactions which may occur, especially in the buildup phase. Consequently, this treatment shall be supervised by an expert in insect venom allergy, and it is periodically administered in a hospital setting which account with all the required resources to circumvent and to treat any adverse reaction derived from its utilization.3-4

In the workshop, delegates will be able to discover the three build-up protocols of VIT that the Committee on Allergy to Hymenoptera of the SEAIC has published, including a 9-week conventional treatment and clustered 3-4 weeks treatments.
Those build-up protocols were assessed in terms of tolerance and safety in the context of a prospective multicenter study involving 13 hospitals.1

Table 1. Conventional 9-week protocol

Week	Concentration mcg/mL	Dose mL	Dose mcg
1	1	0.1	0.1
2	10	0.1	1
3	10	0.5	5
4	100	0.1	10
5	100	0.2	20
6	100	0.4	40
7	100	0.6	60
8	100	0.8	80
9	100	1	100

Table 2. Cluster 4-week protocol

Day	Concentration mcg/mL	Dose mL	Dose mcg
1	10 100	0.5 0.1	5 10
8	100 100	0.2 0.3	20 30
15	100 100	0.5 0.5	50 50
29	100	1	100

Table 3. Cluster 3-week protocol

Day	Concentration mcg/mL	Dose mL	Dose mcg
1	10	0.5	5
	100	0.1	10
	100	0.2	20
	100	0.2	20
8	100	0.5	50
	100	0.5	50
22	100	1	100

Pretreatment schemes:

SRs involving respiratory and/or cardiovascular symptoms can occur during VIT and occasionally require emergency interventions.4 Therefore, pretreatment with several medications has been used in order to circumvent future SR. Omalizumab and antihistamines are currently being used in different hospitals showing efficacy. Specifically, antihistamines have increased VIT efficacy probably by inhibition of type 2 helper T-cell cytokine production.1,4

There is a need to specify the exact dose and duration of pretreatment in terms of its retrieval after several maintenance dose.

Conclusions:

There are several buildup protocols of VIT. Of note, the committee on Allergy to Hymenoptera of the SEAIC examined 3 different protocols and proved to be safe and well-tolerated in the patients studied, with only 6% experiencing a systemic reaction, ie, 0.8% of all injections given.4 There is debate about the time interval between doses in manteinance protocols, being mainly 4-8 week the most frequently used.

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Allergy

ORIGINAL ARTICLE BIOTECHNOLOGY AND IN VITRO DIAGNOSTICS

Added sensitivity of component-resolved diagnosis in hymenoptera venom-allergic patients with elevated serum tryptase and/or mastocytosis

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Abstract

Background: Anaphylaxis caused by hymenoptera venom allergy is associated with elevation of baseline serum tryptase (sBT) and/or mastocytosis in about 5% of patients. Up to now, no information has become available on single venom allergen sIgE reactivity and the usefulness of component-resolved approaches to diagnose this high-risk patient group. To address the component-resolved sIgE sensitization pattern and diagnostic sensitivity in hymenoptera venom-allergic patients with elevated sBT levels and/or mastocytosis, a panel of yellow jacket and honeybee venom allergens was applied on a widely used IgE immunoassay platform.

Methods: Fifty-three patients with mastocytosis and/or elevated sBT tryptase level and systemic reactions to hymenoptera venoms were analyzed for their IgE reactivity to recombinant yellow jacket and honeybee venom allergens by Immulite3 g.

Results: sIgE reactivity to Ves v 1, Ves v 5, Api m 1 to Api m 4 and Api m 10 was found at a similar frequency in hymenoptera venom-allergic patients with and without elevated sBT levels and/or mastocytosis. However, the use of the recombinant allergens and a diagnostic cutoff of 0.1 kU_A/L allowed the diagnosis of patients with otherwise undetectable IgE to venom extract. The diagnostic sensitivity of yellow jacket venom allergy using the combination of Ves v 1 and Ves v 5 was 100%.

Conclusions: In high-risk patients with elevated sBT levels and/or mastocytosis, the use of molecular components and decreasing the threshold sIgE level to 0.1 kU_A/L may be needed to avoid otherwise undetectable IgE to hymenoptera venom extracts in about 8% of such patients.

Abbreviations
BM, bone marrow; CCD, cross-reactive carbohydrate determinant; CM, cutaneous mastocytosis; CRD, component-resolved diagnosis; HBV, honeybee venom; MIS, mastocytosis in the skin; sBT, baseline serum tryptase; sIgE, specific IgE; SM, systemic mastocytosis; YJV, yellow jacket venom.

Hymenoptera venom allergy is a potentially life-threatening disease mediated by the cross-linking of receptor-bound IgE antibodies on the surface of mast cells and basophils in allergic individuals. In recent large studies on patients with mastocytosis, a higher incidence of severe anaphylaxis following

hymenoptera stings than in the general population was documented (1–3).

Mastocytosis is a heterogeneous disorder characterized by proliferation and accumulation of mast cells in the skin, bone marrow (BM), and other tissues (4, 16). In recent years, an association of hymenoptera venom allergy, especially of severe allergic sting reactions, with mastocytosis was documented in 1–7.9% of patients with hymenoptera venom allergy and mastocytosis (1, 3, 5–10). The most frequent type of sensitization in patients with clonal mast cell disorders is to *Vespidae* (5). In addition, the level of baseline serum tryptase (sBT) in patients with hymenoptera venom is associated with more severe reactions following hymenoptera stings (1, 2).

Tryptase is a mast cell mediator, present in two major forms: alpha and beta. The beta tryptase is stored in mast cell granulae and released during mast cell activation (11). The baseline level of tryptase in serum is closely related to the total load of mast cells in the body (12).

There are several inherent problems in the management of patients with hymenoptera venom-allergic patients with elevated sBT levels and/or mastocytosis, both in diagnosis and in applying immunotherapy.

Patients with elevated sBT levels and/or mastocytosis have lower total IgE levels as compared to the general population (13, 14). When these patients have hymenoptera allergy, negative IgE and negative skin tests appear to be quite common, restricting them from otherwise indicated hymenoptera venom immunotherapy (VIT).

During hymenoptera venom immunotherapy, side-effects are more frequent in patients with mastocytosis, especially in those with yellow jacket venom (YJV) allergy, compared with the general hymenoptera venom-allergic population (15). According to different studies in which sting challenge and/or field sting reactions of yellow jacket venom-allergic patients who have underwent VIT were analyzed, the protection rate of VIT in patients with mastocytosis and/or elevated sBT level varies from 15 to 85% (1, 3, 7, 9, 17, 18), with an average protection rate of 72%. This is a much lower success rate compared to 95% for yellow jacket venom-allergic patients without mastocytosis (19). These findings indicate a lower efficacy of VIT, especially in yellow jacket venom-allergic patients with mastocytosis and/or elevated sBT compared to yellow jacket venom-allergic patients without this diagnosis.

So far, no information has been available on the sIgE reactivity pattern to hymenoptera venom allergen components in patients with elevated sBT levels and/or mastocytosis and history of systemic sting reactions. Because special reactivity pattern might be a potential explanation of the higher susceptibility to develop hymenoptera venom allergy and of reduced efficacy of VIT in mastocytosis patients, here we analyzed the sensitization profiles of those patients with a panel of cross-reactive carbohydrate determinant (CCD)-free yellow jacket and honeybee venom (HBV) allergens on an established sIgE immunoassay platform (20). Component resolution revealed no obvious differences in the reactivity profiles of hymenoptera venom-allergic patients with and without elevated sBT levels and/or mastocytosis. However, increased diagnostic sensitivity was observed when a threshold of 0.1 kU_A/l was used on an allergen-resolved level in patients with increased sBT or mastocytosis and undetectable or low sIgE to hymenoptera venom extract or unclear skin test results.

Methods

Patients

The study group contained 53 patients (26 male/27 female, age 18-76, median age 55) with allergy to hymenoptera venom and increased sBT level and/or mastocytosis, and the control group contained 26 hymenoptera venom-allergic patients (11 male/15 female, age 24–80, median age 57) without increased sBT level and/or mastocytosis.

Diagnosis of hymenoptera venom allergy was based on a combination of a clinical history of an anaphylactic sting reaction, a positive intradermal skin test, and/or positive sIgE levels to hymenoptera venom extracts (HBV, i1, and YJV, i3).

The diagnosis of mastocytosis was made according to WHO criteria (4). Serum tryptase was measured at least 2 weeks after a sting event using a commercial fluorimetric assay (Thermo Fisher Scientific, Uppsala, Sweden) and the threshold set at 11.4 ng/ml. Bone marrow biopsies were conducted in 24 patients and smears examined for the presence of atypical mast cells according to the guidelines (21). Bone marrow mast cells were analyzed for the expression of CD25 by immunofluorescence as described previously (4) and the activating c-kit mutation D816V detected by PCR (22). All patients had given informed written consent, and the study was approved by the local ethics committee.

Allergens

Api m 1, Api m 2, Api m 3, Api m 10, Ves v 1, and Ves v 5 were recombinantly produced as secreted full-length CCD-free proteins in *Spodoptera frugiperda* (Sf9) insect cells and purified by nickel-chelating affinity chromatography as previously described (23–27). Api m 4 was generated by peptide synthesis. All allergens were used for the generation of research prototype allergen immunoassays (Siemens Healthcare Diagnostics, Tarrytown, NY,USA).

Immunoreactivity of patient sera

sIgE reactivity was analyzed on an Immulite2000 platform (Siemens Healthcare Diagnostics) using commercially available assays for HBV (i1) and YJV (i3) (Siemens Healthcare Diagnostics) and clinical research prototype immunoassays for Api m 1, Api m 2, Api m 3, Api m 4, Api m 10, Ves v 1, and Ves v 5 (Siemens Healthcare Diagnostics).

Results

Clinical data of patients

The study group contained 53 patients with elevated sBT levels and/or mastocytosis and a history of hymenoptera

Table 1 Demographic and clinical data of the hymenoptera venom-allergic patients

	Mastocytosis/ sBT group	Normal tryptase group
Total	53	26
Sex		
Male	26	11
Female	27	15
Age		
Mean (SD)	54,4 (14.07)	53.9 (15.7)
Median (range)	55 (18–76)	57 (24–80)
Tryptase ng/ml, mean (SD)	31.9 (37.4)	5.4 (2.1)
Total IgE kU/l, median (range)	56.7 (2.3–8496)	89 (10–2551)
Mastocytosis disorder		
Systemic mastocytosis	17	0
Cutaneous mastocytosis	5	0
Mastocytosis in the skin	4	0
MMAS	1	0
Grade* of allergic reaction		
I	3	6
II	17	5
III	20	13
IV	9	2
unknown	4	0

*According to Ring and Messmer (40).

venom allergy. Of those, 49 had a history of YJV and four patients a history of HBV hypersensitivity. The demographic and clinical data of patients are summarized in Table 1. Fifty-one of the patients had sBT levels of higher than 11.4 ng/ml (Fig. 1). The other two were included into the study group due to a clear diagnosis of cutaneous mastocytosis (CM). Seventeen patients were diagnosed with systemic mastocytosis (SM), and one exhibited an activating c-KIT mutation with monoclonal mast cell activation syndrome (MMAS). Nine additional patients were diagnosed with CM or mastocytosis in the skin (MIS). BM biopsy was performed in 24 patients. The clinical characteristics of the patients of the study group are shown in detail in Table 2.

Diagnostic sensitivity of sIgE to YJV allergens Ves v 1 and Ves v 5

The study group and the control group contained 49 and 25 patients, respectively, for whom yellow jackets were clearly identified as the culprit insect eliciting a systemic allergic reaction. For these patients, the diagnostic sensitivity of YJV extract and of the allergens Ves v 1 and Ves v 5 was addressed (Fig. 2). Using YJV extract, the diagnostic sensitivity in the study group was 91.8% using a cutoff of 0.1 kU_A/l and 87.8% using a cutoff of 0.35 kU_A/l, respectively. Four patients showed sIgE levels below 0.1 kU_A/l. In contrast, in the control group, all patients could be diagnosed using YJV extract and a cutoff of 0.1 kU_A/l (92% with the cutoff of 0.35 kU_A/l).

When using the cutoff of 0.35 kU_A/l, the diagnostic sensitivity of the allergens was unexpectedly low in the study group (63.3% for Ves v 1 and 85.7% for Ves v 5). Decreasing the cutoff to 0.1 kU_A/l, sIgE reactivity with the allergens Ves v 1 and Ves v 5 was found in the study group at a prevalence of 81.6% and 98%, respectively. In stark contrast, in the control group, the diagnostic sensitivity of Ves v 1 and Ves v 5 was 72% and 92%, respectively, regardless of which cutoff was used. Interestingly, in the group of patients with elevated sBT levels and/or mastocytosis, there is a relevant portion of patients exhibiting sIgE levels against the allergens in the range between 0.1 and 0.35 kU_A/l compared to none in the control group. However, by using the combination of the two major YJV allergens Ves v 1 and Ves v 5 and a cutoff of 0.1 kU_A/l, the diagnostic sensitivity could be raised to 100% in the study group and the control group, respectively. Using the traditional cutoff of 0.35 kU_A/L, four patients in the study group (8.2%) with severe systemic reactions would have been completely negative in *in vitro* sIgE measurement, while none in the control group would have been missed.

IgE reactivity to YJV and HBV allergens in patients with and without elevated sBT levels and/or mastocytosis

To examine whether patients of the study group and the control group differ in their IgE reactivity profile to individual allergens, the patients were divided into different groups based on a combination of clinical history, skin test, and sIgE to HBV and YJV extract. Although most of the patients had a systemic reaction after a yellow jacket sting, patients with a double-positive skin test and/or detectable specific IgE to YJV and HBV were classified as double-positive. For sIgE measurements, a cutoff of 0.1 kU_A/l was used, which has previously been established as a suitable lower-end cutoff on the Immulite2000 immunoassay platform (20). The study group contained 29 patients, who were sensitized to YJV only, whereby three patients without detectable sIgE were included due to an anaphylactic reaction of grade II or III after sting by an YJ. Twenty patients were double-positive to YJV and HBV, and only four patients were monosensitized to HBV. The control group consisted of nine patients monosensitized to YJV and 17 with double-positive test results.

All patient populations exhibited comparable reactivity with the YJV major allergens Ves v 1 and Ves v 5. Using the cutoff of 0.1 kU_A/l, 82.8% of the YJV-monosensitized and 80% of the double-positive patients of the study group showed IgE reactivity with Ves v 1 (Fig. 3A,B), which was comparable with 77.8 and 70.6% in the control group (Fig. 3D,E). IgE reactivity to Ves v 5 was detected in 96.6 and 100% of the patients of the study group (Fig. 3A,B) and in 88.9 and 94.1% of patients of the control group (Fig. 3D, E). The IgE reactivity of the different patient groups with the YJV allergens using the cutoffs of 0.1 and of 0.35 kU_A/l is summarized in Fig. 3F. The detailed reactivity profiles of the patients are shown in Table S1.

Table 2 Characteristics of hymenoptera venom-allergic patients with mastocytosis and/or elevated baseline serum tryptase

Pt ID	Age	Sex	Grade of sting reaction*	Serum tryptase (ng/ml)	BM biopsy	BM mast cells CD25+	Activating KIT mutation	Spindle-shaped mast cells	Skin morphology	Mast cell disorder
1	50	M	II	13.7	Neg	Neg	Neg	Neg	Neg	
2	59	M	IV	14.8	n.d.	n.d.	n.d.	n.d.	Neg	
3	26	W	III	22.2	n.d.	n.d.	n.d.	n.d.	Pos	MIS
4†	59	W	II	20.4	n.d.	n.d.	n.d.	n.d.	Neg	SM
5	56	W	I	17.9	Pos	Neg	n.d.	Pos	Neg	SM
6	73	W	II	21.0	n.d.	n.d.	n.d.	n.d.	Neg	
7	31	W	II	18.6	Neg	Neg	Neg	Neg	Pos	CM
8	55	M	III	28.7	n.d.	n.d.	n.d.	n.d.	Neg	
9	44	M	III	73.8	Pos	n.d.	n.d.	Neg	Pos	SM
10	39	W	n.d.	4.8	Neg	Neg	Neg	Neg	Pos	CM
11	63	W	II	13.5	n.d.	n.d.	n.d.	n.d.	Neg	
12	60	M	IV	27.4	Pos	n.d.	n.d.	Neg	Pos	SM
13	46	W	III	42.4	Pos	n.d.	n.d.	Neg	Neg	SM
14	62	W	I	61.3	Pos	n.d.	n.d.	Neg	Pos	SM
15	72	W	n.d.	14.3	n.d.	n.d.	n.d.	n.d.	Neg	
16	76	W	III	11.6	n.d.	n.d.	n.d.	n.d.	Neg	
17	44	M	III	13.0	n.d.	n.d.	n.d.	n.d.	Neg	
18	66	W	III	18.9	Pos	n.d.	n.d.	Pos	Pos	SM
19	59	W	III	177.0	Pos	Neg	Pos	Pos	Neg	SM
20	42	W	II	11.7	n.d.	n.d.	n.d.	n.d.	Neg	
21	41	M	n.d.	52.1	Pos	Pos	Pos	Neg	Pos	SM
22	42	M	III	24.2	n.d.	n.d.	n.d.	n.d.	Pos	MIS
23	55	W	I	11.7	n.d.	n.d.	n.d.	n.d.	Neg	
24	69	W	II	13.1	n.d.	n.d.	n.d.	n.d.	Neg	
25	55	W	III	11.7	Pos	Pos	Pos	Pos	Neg	SM
26	56	M	II	50.4	Pos	Pos	Neg	Pos	Neg	SM
27	66	W	III	149.0	Pos	Neg	Neg	Neg	Pos	SM
28	49	M	III	81.0	n.d.	n.d.	n.d.	n.d.	Pos	MIS
29	66	W	II	11.6	n.d.	n.d.	n.d.	n.d.	Neg	
30	65	W	III	14.0	Neg	Neg	Neg	Neg	Neg	
31†	47	M	IV	13.2	n.d.	n.d.	n.d.	n.d.	Neg	SM
32	44	M	II	7.3	Neg	Neg	Neg	Neg	Pos	CM
33	64	M	III	23.3	n.d.	n.d.	n.d.	n.d.	Neg	
34	68	M	III	11.8	n.d.	n.d.	n.d.	n.d.	Neg	
35	19	M	III	13.6	n.d.	n.d.	n.d.	n.d.	Neg	
36	73	W	II	25.9	n.d.	n.d.	n.d.	n.d.	Neg	
37	53	M	IV	29.7	Pos	Pos	Pos	Neg	Neg	SM
38	46	M	III	14.9	n.d.	n.d.	n.d.	n.d.	Neg	
39	68	W	II	17.4	n.d.	n.d.	n.d.	n.d.	Neg	
40†	46	M	IV	128.0	n.d.	n.d.	n.d.	n.d.	Pos	SM
41	75	M	III	27.5	Neg	Neg	Pos	Neg	Neg	MMAS
42	49	M	IV	12.6	Neg	Neg	Neg	Pos	Neg	
43	70	M	IV	12.7	n.d.	n.d.	n.d.	n.d.	Neg	
44	69	W	III	12.6	n.d.	n.d.	n.d.	n.d.	Neg	
45	55	W	II	16.1	Pos	Pos	Pos	Pos	Neg	SM
46	66	M	IV	11.5	n.d.	n.d.	n.d.	n.d.	Neg	
47	54	M	III	14.9	n.d.	n.d.	n.d.	n.d.	Neg	
48	41	M	II	13.8	Neg	Neg	Neg	Neg	Neg	
49	46	W	II	18.9	Pos	Neg	Neg	Neg	Pos	CM
50	76	W	n.d.	25.0	Pos	Neg	n.d.	Pos	Pos	SM
51	18	M	II	17.2	n.d.	n.d.	n.d.	n.d.	Neg	
52	36	M	IV	38.8	n.d.	n.d.	n.d.	n.d.	Pos	MIS
53	56	W	II	140.0	Pos	n.d.	n.d.	Neg	Pos	CM

BM, bone marrow; CM, cutaneous mastocytosis; MIS, mastocytosis in the skin; MMAS, monoclonal mast cell activation syndrome; n.d., not determined; SM, systemic mastocytosis. Patients 1–29 are monosensitized to YJV, patients 30–49 are sensitized to YJV and HBV, and patients 50–53 are monosensitized to HBV.

*According to Ring and Messmer (40).

†The patient came to clinic with an existing diagnosis of mastocytosis.

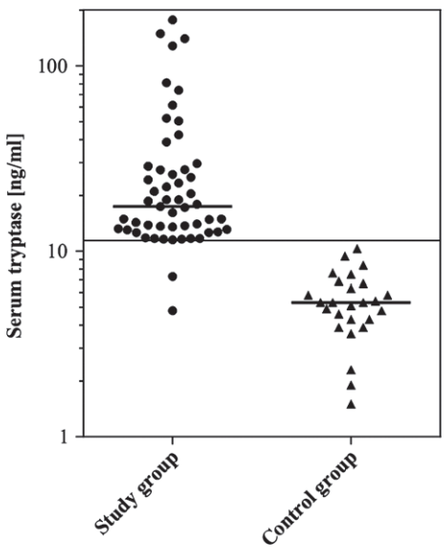

Figure 1 Serum tryptase levels of hymenoptera venom-allergic patients. Serum tryptase levels of the study group (n = 53) and the control group (n = 26) as measured at least 2 weeks after the last episode of a sting reaction. The mean of the study group is 31.93 ± 37.43 ng/ml and of the control group 5.43 ± 2.12 ng/ml. The assay threshold value set at 11.4 ng/ml is represented by a solid line.

Moreover, the IgE reactivity with the HBV allergens Api m 2, Api m 3, Api m 4, and Api m 10 (Fig. 3B,E) was comparable to that described in a former study for patients with a primary sensitization to HBV (28). Except for the HBV major allergen Api m 1, the reactivity was lower compared to other studies most likely reflecting the different patient selection (28–30).

Reactivity profiles of patients with low or undetectable sIgE to hymenoptera venom extract

Among the patients of the study group, four patients (Fig. 4A, patients 12, 27, 28, and 29) with grade II to IV systemic reactions to yellow jacket stings had sIgE levels to YJV below 0.1 kU_A/L. Three of these patients were diagnosed with a mastocytosis disorder, and two additionally exhibited negative intracutaneous skin tests with YJV. All these patients showed sIgE to Ves v 5 (one additionally to Ves v 1) in the range between 0.1 and 0.35 kU_A/l. Two patients with systemic mastocytosis exhibited YJV-specific IgE in the range between 0.1 and 0.35 kU_A/L (Fig. 4A, patients 21 and 25). One of them showed sIgE reactivity to Ves v 1 above 0.35 kU_A/l and one with Ves v 5, respectively. Additionally, one patient of the control group with sIgE to YJV below 0.35 kU_A/l could be clearly diagnosed using Ves v 5 and another one by using Ves v 5 and Ves v 1 (Fig 4A, patients 55 and 62).

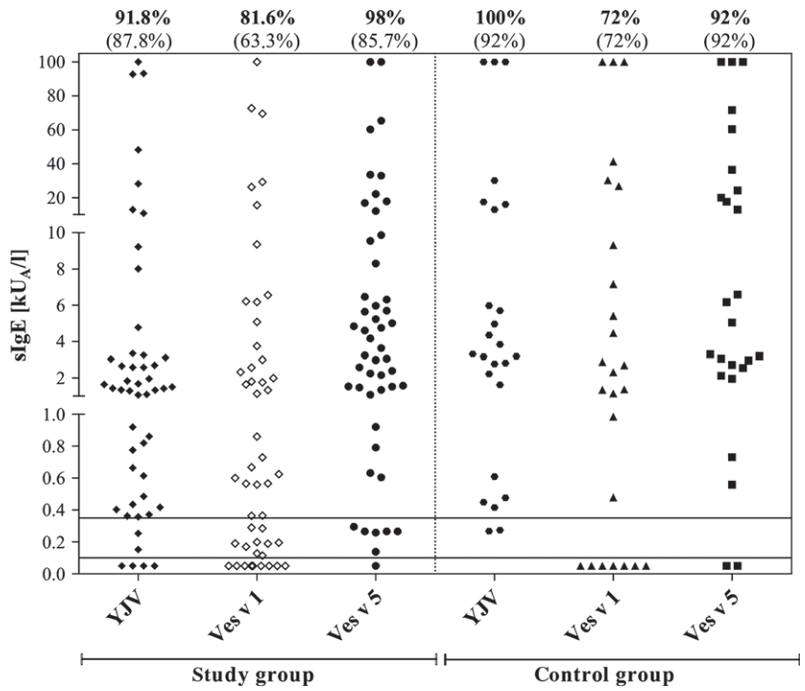

Figure 2 sIgE reactivity of individual sera using extract or recombinant allergens from patients with systemic reactions after YJ stings. IgE reactivity to YJV extract or recombinant YJV allergens (Ves v 1, Ves v 5) of sera from YJV-allergic patients with (study group) and without (control group) elevated sBT level and/or mastocytosis. The lower-end cutoffs of 0.1 kU_A/l and 0.35 kU_A/l are presented as solid lines. Percentages in boldface and in parentheses indicate the IgE reactivity of allergens using the cutoff of 0.1 and 0.35 kU_A/l, respectively.

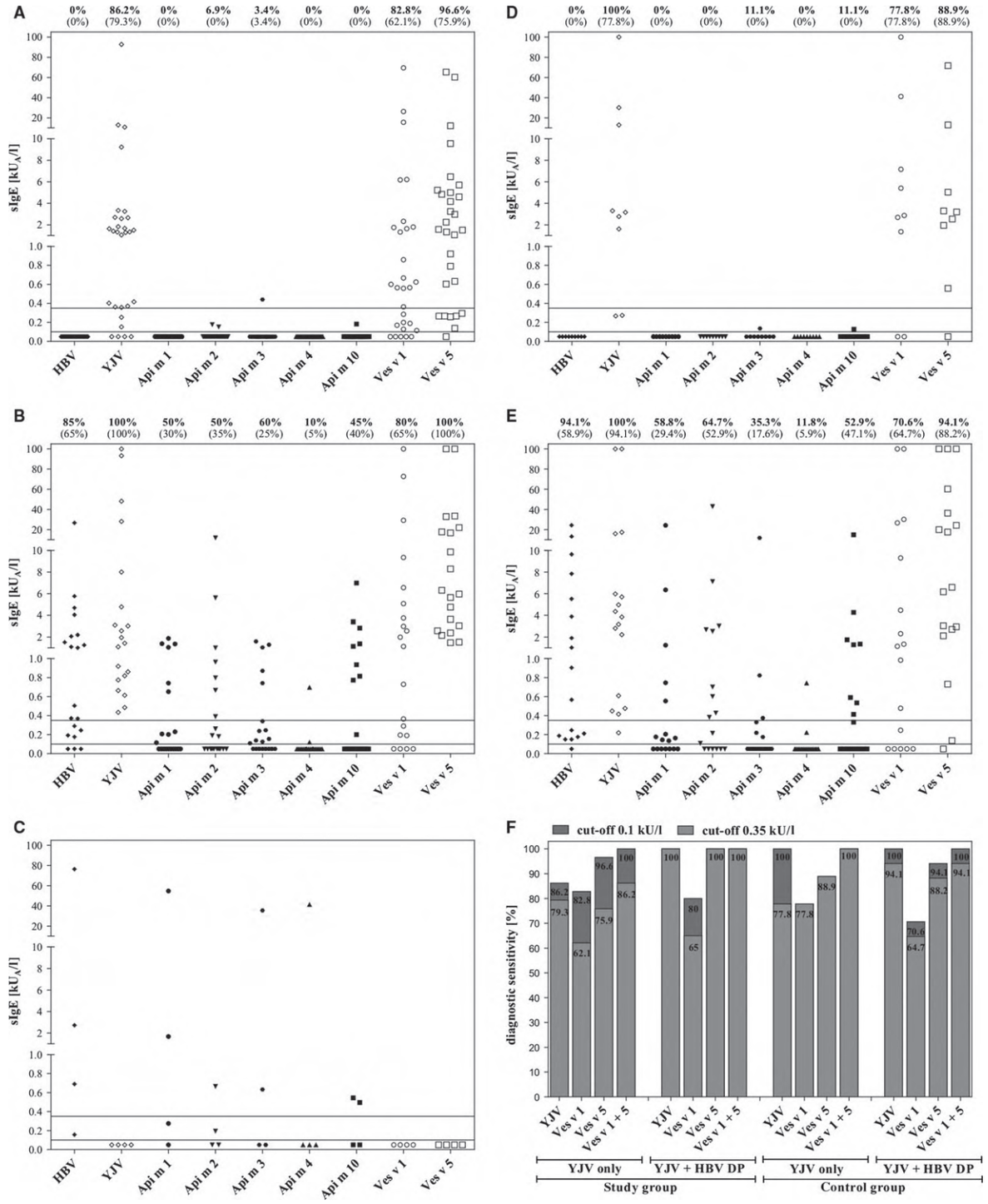

Figure 3 Immunoreactivity of patient sera with individual allergens and venom extracts. IgE reactivity to HBV and YJV allergens and venom extracts of sera from hymenoptera venom-allergic patients with (A–C) and without (D,E) elevated sBT level and/or mastocytosis. (A,D) Patients monosensitized to YJV. (B,E) patients sensitized to YJV and HBV. (C) Patients monosensitized to HBV. The lower-end cutoffs of 0.1 and 0.35 kU$_A$/l are presented as solid lines. Percentages in boldface and in parentheses indicate the IgE reactivity of allergens using the cutoff of 0.1 and 0.35 kU$_A$/L, respectively. (F) Diagnostic sensitivity of sIgE to YJV, Ves v 1, and Ves v 5 using the different cutoffs.

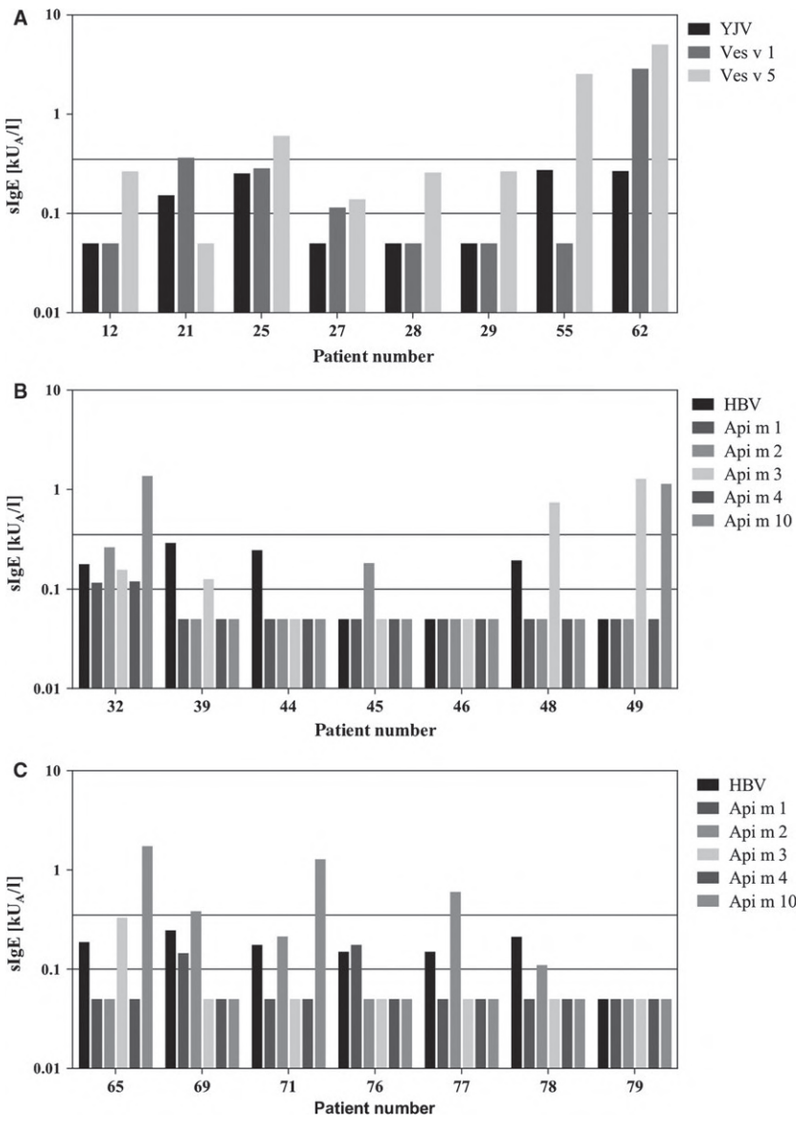

Figure 4 sIgE reactivity using recombinant allergens of individual patients with low or undetectable sIgE to hymenoptera venom extract. (A) Reactivity with YJV allergens (Ves v 1, Ves v 5) of patients with clear-cut clinical history of YJV allergy. (B,C) Reactivity with HBV allergens (Api m 1, Api m 2, Api m 3, Api m 4, Api m 10) of patients with clinical history of YJV allergy and additional evidence for a sensitization to HBV. (B) Patients of the study group with elevated sBT level and/or mastocytosis. (C) Patients of the control group without elevated sBT level and/or mastocytosis. The lower-end cutoffs of 0.1 and 0.35 kU$_A$/L are presented as solid lines.

We additionally analyzed the reactivity profile with HBV allergens of patients of the study group (Fig. 4B) and control group (Fig. 4C) double-positive for YJV and HBV and low or undetectable sIgE to HBV. One patient (patient 49) who had HBV-specific IgE of <0.1 kU$_A$/l (but in a former measurement 0.5 kU$_A$/l) exhibited a significant reactivity with Api m 3 and Api m 10. Five additional patients (patients 32, 39, 48, 65, and 71) with sIgE to HBV between 0.1 and 0.35 kU$_A$/l also exhibited reactivity with Api m 3 and/or Api m 10 with values above 0.35 kU$_A$/L (except patient 39). Interestingly, patient 71 in 2010 had sIgE to HBV of 0.93 kU$_A$/L and patient 48 in 2008 and 2010 of 5.64 and 0.51 kU$_A$/l, respectively, hinting to a history of HBV allergy. Three patients (45, 77, and 78) reacted with Api m 2 only, which might be explained by cross-reactivity with Ves v 2, the homologue from YJV. One patient (44) with low sIgE to HBV and two (46 and 79) with a positive skin test to HBV showed no reactivity with any of the HBV allergens, and only two patients (69 and 76) exhibited a slight reactivity with Api m 1. Patient 69 additionally showed reactivity with Api m 2 above 0.35 kU$_A$/L and, in addition to a history of YJV allergy, showed a mild reaction (grade I) after a honey-

bee sting, indicating that low-level sIgE reactivity might also be of clinical relevance.

Discussion

In this study, we addressed, for the first time, the component resolution of sIgE reactivity to a broad panel of recombinant YJV and HBV allergens of hymenoptera venom-allergic patients with elevated sBT levels and/or mastocytosis. Hymenoptera venom allergy represents the most common trigger for anaphylaxis in patients with mastocytosis (6), and moreover, mastocytosis patients and those with increased sBT levels are at risk for more severe sting reactions (10, 32, 33). The frequency of mastocytosis in patients with hymenoptera venom allergy is in the range between 1 and 7.9% (1, 3, 5, 7), which is substantially higher than in the general population with a range between 0.00125 and 0.07% (7). Five to 19% of patients with mastocytosis suffer from hymenoptera venom allergy (6, 34).

The only causative treatment that is effective in reducing the risk of subsequent systemic reactions in hymenoptera venom-allergic patients is venom immunotherapy (VIT). A prerequisite for VIT is the demonstration of a sensitization by sIgE or skin test. However, proper diagnosis of hymenoptera venom allergy in mastocytosis patients is in some cases problematic because total IgE levels are lower (13) and sIgE and skin tests might be more often negative compared to hymenoptera venom-allergic patients without mastocytosis.

To date, there is only scarce knowledge about the pathogenic mechanisms underlying the association between mastocytosis and hymenoptera venom allergy. In addition, there is a well-documented reduced therapeutic efficacy of VIT in mastocytosis patients (19). One potential hypothesis is that specific sIgE sensitization patterns in addition to special characteristics of the disease account for these phenotypes of higher susceptibility to develop hymenoptera venom allergy and of reduced efficacy of VIT in mastocytosis patients. To date, no data about the sIgE reactivity profiles with particular allergenic components have become available for patients with elevated sBT levels and/or mastocytosis.

Hence, in this study, we examined hymenoptera venom-allergic patients with elevated sBT levels and/or mastocytosis for their sIgE reactivity profiles with recombinant YJV (Ves v 1 and Ves v 5) and HBV (Api m 1-4 and Api m 10) allergens. For the analysis of the particular sIgE reactivity, we used venom allergens that were recombinantly produced in *Spodoptera frugiperda* (Sf9) insect cells and that therefore allow the detection of allergen-specific IgE without the interference of cross-reactive carbohydrate determinants (23, 24, 26, 35), which represent a major concern for the specificity of diagnostic tests in hymenoptera venom allergy (36–38). The recombinant allergens were used for the generation of clinical research prototype immunoassays and analyzed on random-access automated immunoassay platform capable of measuring sIgE (Immulite2000 system, Siemens Healthcare Diagnostics) (20).

The analyses of sIgE reactivity on a component-resolved level revealed no obvious differences in the reactivity profiles of hymenoptera venom-allergic patients with and without elevated sBT levels and/or mastocytosis. This was true not only for the reactivity with the YJV major allergens Ves v 1 and Ves v 5 but also for the reactivity with the HBV allergens Api m 1-4 and Api m 10, thus pointing to the conclusion that the immunologic specificity does not account for the observed differential phenotypic aspects of disease risk, severity, and VIT outcome.

Because most of the patients included in this study had a clinical history of a systemic reaction to YJV (only few to HBV), their reactivity to YJV allergens was of special interest. Interestingly, we found that in stark contrast to the control group, a large portion of patients with elevated sBT levels and/or mastocytosis had sIgE levels against the allergenic components in the range between 0.1 and 0.35 kU_A/l. Only by applying a diagnostic cutoff of 0.1 kU_A/l and a combination of the major allergens Ves v 1 and Ves v 5, we were able to reach a diagnostic sensitivity of 100% in the both patient groups. In contrast, using the cutoff of 0.35 kU_A/l, four patients of the elevated sBT levels and/or mastocytosis group (8.2%, none in the control group) would have been completely negative in sIgE diagnostics and two of these patients also with negative intradermal skin test would not meet the inclusion criteria for VIT, despite having a history of a severe systemic reactions after stings by YJ. This demonstrates the added value of component-resolved diagnosis for patients with undetectable sIgE to venom extract as already shown previously (39). Such an added value of increased sIgE assay sensitivity might be especially important for patients with elevated sBT levels and/or mastocytosis. It is a common finding in those patients that hymenoptera venom extract-specific IgE is negative, which has been primarily attributed to an increased adsorption of IgE to the high-affinity IgE receptors on the surface of the large number of mast cells (1, 14). In addition, we also observed an incomplete representation of Ves v 5-specific IgE by the YJV extract as compared to the recombinant allergen component Ves v 5, a finding that has been described previously by others for the measurements of sIgE in YJV-allergic patients on the ImmunoCAP system (31). Moreover, in two patients with a sIgE level to YJV extract of <0.35 kU_A/l, we observed a similar finding for Ves v 1-specific IgE (patients 21 and 62).

Admittedly, it is a matter of debate which diagnostic cutoff is reasonable for the detection of relevant sIgE sensitization. Our data, however, indicate that it might be advantageous to use the cutoff of 0.1 kU_A/l in patients with low amounts of circulating sIgE due to an overload of mast cells in the body and who are at a particular high risk to suffer from severe or even fatal anaphylactic reaction to another insect sting. It has been demonstrated previously that sIgE concentrations above the 0.1 kU_A/l lower-end threshold value can be measured reproducibly on major sIgE immunoassay platforms such as the ImmunoCAP system or the Immulite2000 system, which was used in this study (20).

In this study, we were also able to detect a significant sIgE reactivity to HBV allergens in patients with low or undetectable sIgE to HBV extract, especially to the allergens Api m 3 and Api m 10, which were previously shown to be

underrepresented in several therapeutic venom preparations (24) and induce lower levels of sIgG4 under VIT with honey bee venom extract (28). These therapeutic venom preparations are also commonly used for skin testing, a fact that even might explain the negative skin test with HBV of most of the patients who are sensitized to those allergens exclusively. However, due to the fact that the vast majority of the study patients were only stung by yellow jackets, the clinical relevance of these sensitizations remains unclear.

In summary, our data demonstrate that although no obvious differences can be found in the sIgE reactivity profile itself with routine or research prototype hymenoptera venom allergens available so far when comparing hymenoptera venom-allergic patients with or without elevated sBT levels and/or mastocytosis, there is a diagnostic advantage and added value of recombinant allergens in combination with a lower-end assay cutoff of 0.1 kU_A/l for the diagnosis of patients with low or undetectable sIgE to venom extract or unclear skin test results, especially for patients with elevated sBT levels and/or mastocytosis.

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Author contributions

JM performed the experiments and analyzed the data; KB coordinated the recruitment of mastocytosis patients, collected and analyzed the data, and provided critical revision of the manuscript; UD coordinated the recruitment of hymenoptera venom-allergic patients, collected and analyzed the data, and provided critical revision of the manuscript; JR coordinated the recruitment of patients and revised the final version of the manuscript; CS-W contributed to the interpretation of data and revised the final version of the manuscript; TG produced recombinant allergens and provided critical revision of the manuscript; SB supervised the study, analyzed the data, designed the figures, and wrote the manuscript; MO initiated and supervised the study, analyzed the data, and wrote the manuscript.

Conflicts of interest

KB has received payments from Thermo Fisher for oral presentations at company seminars and for controlling of company-initiated webinars for integrity. TG is an employee of PLS-Design GmbH. MO reports personal fees from Thermo Fisher, from Siemens Healthcare Diagnostics, and from Hitachi Chemical Diagnostics, and is co-founder of PLS-Design GmbH. The other authors declare that they have no conflict of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Serological data of patients assessed in sIgE reactivity analysis.

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The high molecular weight dipeptidyl peptidase IV Pol d 3 is a major allergen of *Polistes dominula* venom

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Hymenoptera venom allergy can cause severe anaphylaxis in untreated patients. *Polistes dominula* is an important elicitor of venom allergy in Southern Europe as well as in the United States. Due to its increased spreading to more moderate climate zones, *Polistes* venom allergy is likely to gain importance also in these areas. So far, only few allergens of *Polistes dominula* venom were identified as basis for component-resolved diagnostics. Therefore, this study aimed to broaden the available panel of important *Polistes* venom allergens. The 100 kDa allergen Pol d 3 was identified by mass spectrometry and found to be a dipeptidyl peptidase IV. Recombinantly produced Pol d 3 exhibited sIgE-reactivity with approximately 66% of *Polistes* venom-sensitized patients. Moreover, its clinical relevance was supported by the potent activation of basophils from allergic patients. Cross-reactivity with the dipeptidyl peptidases IV from honeybee and yellow jacket venom suggests the presence of exclusive as well as conserved IgE epitopes. The obtained data suggest a pivotal role of Pol d 3 as sensitizing component of *Polistes* venom, thus supporting its status as a major allergen of clinical relevance. Therefore, Pol d 3 might become a key element for proper diagnosis of *Polistes* venom allergy.

Stings of hymenoptera of different species can cause life-threatening IgE-mediated anaphylaxis in venom-allergic patients. The most prominent elicitors of venom allergy in Western and Central Europe are honeybees (*Apis mellifera*) and yellow jackets (*Vespula vulgaris*)[1]. Additionally, allergic reactions to paper wasps, especially to *Polistes dominula*, are common in Southern Europe and the Unites States[2–6]. *Polistes dominula*, known to be domestic in Southern Europe, is an invasive species entering the US (1970s) from the north-east to the west coast (1990s)[7], South Africa (2008)[8] and central Europe (1956)[9]. Therefore, allergy to *Polistes dominula* venom (PDV) will most probably gain importance also in other areas.

The only curative treatment for venom allergy is venom-specific immunotherapy (VIT)[10,11]. To ensure a successful treatment and to avoid the increased risk of side effects, possible *de novo* sensitizations and higher costs, the correct therapeutic venom must be selected[4,12]. To accomplish this, a careful anamnesis is important to identify the insect that elicited the allergic reaction. Due to the number and hard to discriminate phenotypes of insects that can induce allergic reactions, many patients and allergy specialists are not able to correctly distinguish

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between different hymenoptera species such as *Vespula spp*. and *Polistes spp*.[13]. Therefore, clinicians depend on additional diagnostic tests.

The increased knowledge of the composition of hymenoptera venoms has led to major improvements in diagnostic approaches and created the field of molecular or component-resolved diagnostics (CRD) in hymenoptera venom allergy[4,14,15]. In combination with skin testing and venom extract-based specific IgE (sIgE) diagnostics, CRD has created added clinical value for a proper allergy diagnosis. For CRD of hymenoptera venom allergy commercial allergens are available for different test platforms to determine sIgE serum titers[6].

For the diagnosis and discrimination of honeybee venom (HBV)- and yellow jacked venom (YJV)-allergic patients, many commercial allergens are available, allowing for high diagnostic sensitivity and specificity[16–22]. However, for the diagnosis of PDV allergy only one allergen (antigen 5, Pol d 5) is commercially available. Furthermore, only three allergens of PDV have been identified in the past, namely phospholipase A1 (Pol d 1)[23], protease (Pol d 4)[24] and antigen 5 (Pol d 5)[25] from which Pol d 4 is a minor allergen with restricted diagnostic importance (unpublished data). Even though, we have recently shown, that *Polistes* venom is free of cross-reactive carbohydrate determinant-(CCD-)based cross-reactivity[26], available extract-based diagnostic approaches to discriminate between PDV und YJV allergy are hampered by extensive protein cross-reactivity[2,3,27,28].

An increased knowledge of the allergen composition of PDV and the availability of further important PDV components are likely to generate added clinical benefit for proper and advanced diagnostics. Therefore, our study aimed to identify and immunologically characterise additional major allergens of PDV. The here described allergen Pol d 3 is a homologue of the prominent dipeptidyl peptidase IV (DPP IV) allergens Api m 5 and Ves v 3 from HBV and YJV[29]. Extensive sIgE reactivity and the ability to activate basophils from allergic patients clearly support the role of Pol d 3 as major allergen of PDV as well as its potential to be a key element for molecular diagnostic approaches.

Results

Identification of Pol d 3. Immunoblots using PDV and pooled sera of PDV-allergic patients (n = 5) revealed sIgE reactivity with several venom components (Fig. 1). Prominent bands at approximately 23 and 31 to 40 kDa were identified by mass spectrometry as the known allergens antigen 5 (Pol d 5), protease (Pol d 4) and phospholipase A1 (Pol d 1), respectively. Moreover, prominent sIgE reactivity was obtained with a high molecular weight protein of approximately 100 kDa. The protein was subjected to *de novo* sequencing by tandem mass spectrometry. The five identified peptides (Fig. 2) yielded hits in a database search of predicted proteins coded by the recently published genome of *Polistes dominula*[30] and identified the sIgE-reactive protein as venom dipeptidyl peptidase IV (GenBank accession XP_015174445). An additional matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) analysis of the protein band then led to a sequence coverage of approximately 44% with the predicted sequence.

The nucleotide sequence (GenBank accession XM 015318959) of the newly identified allergen codes a mature protein of 751 amino acids and a calculated molecular weight of 86.2 kDa. The discrepancy between the calculated and the apparent molecular mass most likely is due to posttranslational modification by glycosylation as suggested by the presence of 4 or 3 N-linked glycosylation sites based on the binary profile or on average surface accessibility, respectively[31]. The protein belongs to the dipeptidyl peptidase IV superfamily, known to cleave dipeptides from the N-terminus of peptidic substrates, including many chemokines, neuropeptides, peptide hormones and venom peptides[32,33]. Therefore, it is a homologue of the well-established allergens Api m 5 (HBV) and Ves v 3 (YJV)[29]. The identity on protein level with Api m 5 and Ves v 3 is 54.0% and 76.6%, respectively. According to its allergenic properties and the homology to YJV Ves v 3 the new PDV allergen was assigned as Pol d 3.1010 in the WHO/IUIS Allergen Nomenclature Database[34].

Recombinant expression and characterisation of Pol d 3. For recombinant expression, the coding region of Pol d 3 was amplified from PDV gland cDNA. Recombinant production was achieved by baculoviral infection of Sf9 insect cells and Ni^{2+}-affinity chromatography yielded soluble recombinant Pol d 3 from culture supernatants with an apparent molecular weight of approximately 100 kDa as shown by Coomassie staining and reactivity with an antibody reacting with the V5-epitope tag added for recombinant expression (Fig. 3). Moreover, correct folding of the allergen was supported by enzymatic DPP IV activity (Supplementary Fig. S1) as well as by circular dichroism (CD) spectroscopy (Supplementary Fig. S2). For comparable analyses the homologous allergens from HBV and YJV, Api m 5 and Ves v 3, were produced as described previously[29] (Fig. 3).

Reactivity with *Galanthus nivalis* agglutinin (GNA) (reactive with terminal 1,2-. 1,3- and 1,6-linked mannose residues) proves the presence of N-linked glycans (Fig. 3). However, staining with rabbit polyclonal horseradish peroxidase (HRP) antiserum, specific for α-1,3-core fucosylation, the structure responsible for CCD-based cross-reactivity, demonstrates the lack of CCDs (Fig. 3), as shown previously for other allergens produced in Sf9 insect cells[18,35–37].

Furthermore, staining with a polyclonal rabbit Api m 5 antiserum shows reactivity with Api m 5 but also with Ves v 3 and Pol d 3, suggesting cross-reactivity between the three DPP IV hymenoptera allergens. Cross-reactivity was confirmed by sIgE-reactivity of pooled sera of PDV-allergic patients with all three allergens (Fig. 3).

Activation of basophils from venom-allergic patients by Pol d 3. To address the capability of Pol d 3 to cross-link receptor-bound IgE and, thus, to activate effector cells, basophil activation tests (BATs) were performed (Fig. 4) as previously described[28,38]. First, 13 patients from Spain (from the area of Barcelona) with history of an allergic reaction to PDV and/or YJV were analysed for their reactivity with Pol d 3 and Ves v 3 (Fig. 4a). Three patients showed basophil activation only by Pol d 3 (patients 3, 7, 8), two only by Ves v 3 (patients 1, 11) and four by both allergens (patients 4, 9, 10, 13). Interestingly, for most of the patients these data nicely match the data of skin tests and/or sIgE levels to the venom extracts (i3, i77) and allergen components (i209, i210, i211)

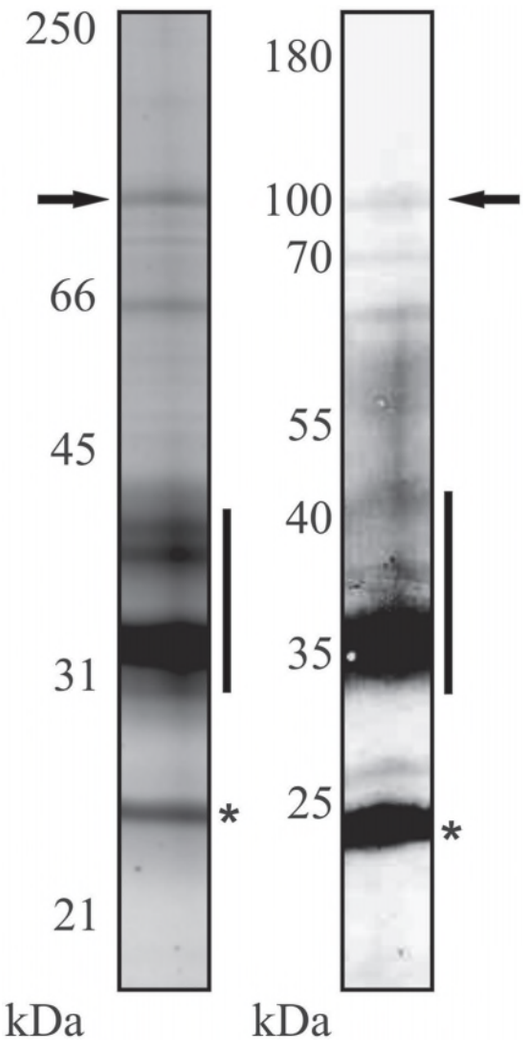

Figure 1. Detection of Pol d 3 in *Polistes dominula* venom. SDS-PAGE and protein staining (SyproRuby staining) of PDV (left) and sIgE-immunoreactivity of pooled sera from PDV-allergic patients with PDV in Western blot (right). The arrow indicates the 100 kDa band that was subjected to tandem mass spectrometry and MALDI-TOF analyses. Asterisks indicate the band that was identified as Pol d 5 and black bars the area of the gel/blot in which Pol 1 and Pol d 4 were identified. Shown are parts of the gel. Blot and full-length gels are shown in Supplemental Fig. S3. The kDa values correspond to the protein marker (not shown) which can be found in Fig. S3.

(Table 1). Therefore, for most patients BATs with the DPPs IV would have been able to identify the insect(s), the patient most likely shows primary sensitization to. For patient 4, showing stronger basophil activation by Pol d 3 compared to Ves v 3, but higher sIgE levels to YJV compared to PDV, clinical information is too scarce to identify the allergy-eliciting venom. Moreover, patient 13 exhibits basophil activation by both allergens but much higher sIgE level to PDV compared to YJV extract and a negative skin test to YJV. However, for this patient basophil activation by Pol d 3 is much stronger compared to Ves v 3, and Ves v 3-reactivity is most likely due to cross-reactivity. For patients 9 and 10 who show comparable activation patterns by both allergens, also clinical data suggest allergy to both venoms.

In order to address cross-reactivity of Pol d 3 in BAT, 15 patients from Germany (from the area of Munich) with allergy to YJV and/or HBV were analysed for their reactivity with Pol d 3, Api m 5 and Ves v 3 (Fig. 4b). Five patients showed basophil activation by Pol d 3 (patients 15, 16, 18, 19, 28) six by Api m 5 (patients 15, 17, 18, 21, 27, 28) and seven by Ves v 3 (15, 18, 19, 20, 24, 26, 28). Again, in most cases activation patterns by Api m 5 and Ves v 3 match sIgE and skin test data, indicating allergy to HBV, YJV or both (Table 1). Three of the Pol d 3-reactive patients, showed basophil activation by all three allergens (patients 15, 18, 28) and one by Pol d 3 and Ves v 3 (patient 19). Only patient 16 showed exclusive basophil activation by Pol d 3, but the overall activation (including the positive control) was very low.

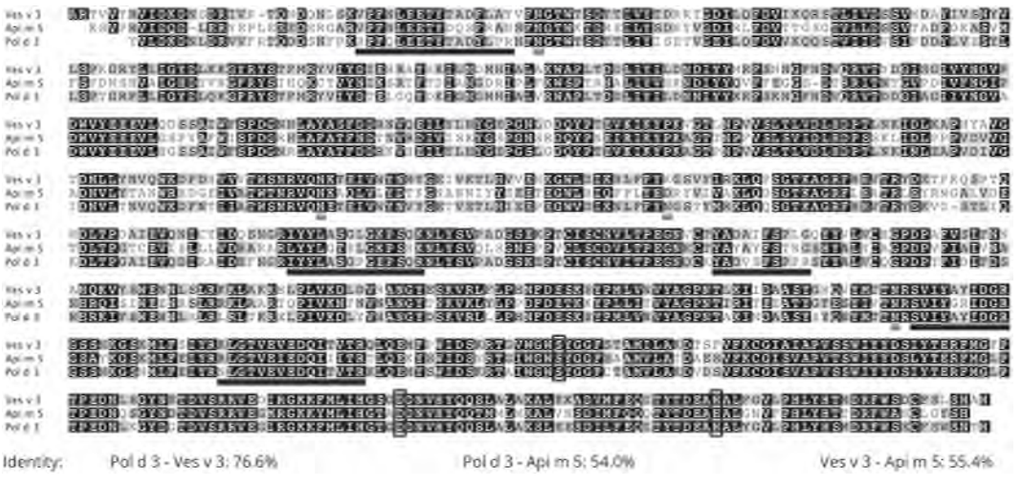

Figure 2. Alignment of Pol d 3 with Ves v 3 and Api m 5. Mature amino acid sequences of Pol d 3 (*Polistes dominula*), Ves v 3 (*Vespula vulgaris*) and Api m 5 (*Apis mellifera*) are shown. Black shaded amino acids are identical between all three proteins, gray shaded amino acids are shared by two proteins and amino acids not shaded are unique to the individual protein. Peptides identified by tandem mass spectrometry are underlined in black and potential N-glycosylation sites in gray. The residues involved in the conserved active center of the enzymes are represented boxed. Overall amino acid identity between the different proteins is stated in percent.

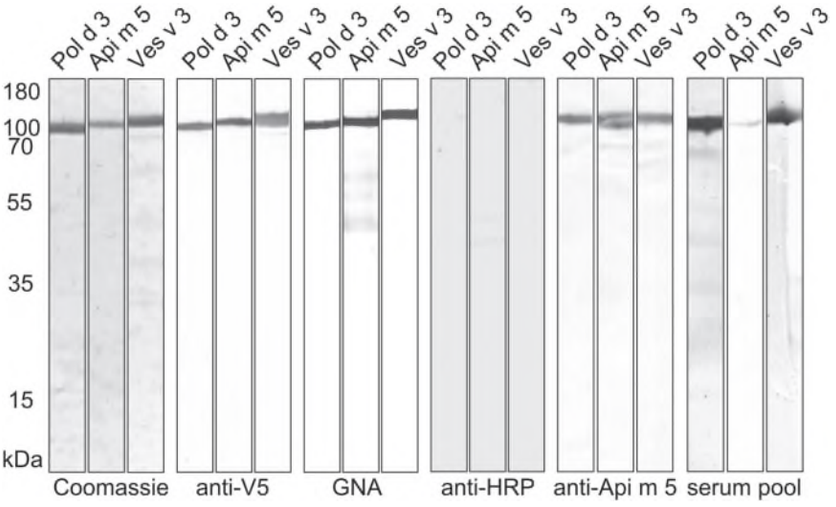

Figure 3. Recombinant expression and characterisation of Pol d 3. SDS-PAGE and Western blot analyses of Pol d 3 recombinantly produced in Sf9 insect cells in comparison with the HBV and YJV homologues Api m 5 and Ves v 3 either by Coomassie blue staining or anti-V5 epitope antibody, GNA (*Galanthus nivalis* agglutinin), anti-HRP antiserum, anti-Api m 5 antiserum and pooled sera of PDV-allergic patients. Shown are parts of one or more gels and blots and full-length gels and blots are given in Supplemental Fig. S3.

sIgE-reactivity of Pol d 3. The sIgE to the newly identified Pol d 3 was addressed by ELISA. 24/30 of the patients from Spain (from the area of Cordoba) were diagnosed with PDV allergy by a combination of clinical history, skin test and sIgE levels to venom extracts and allergen components (Supplementary Table S1). For the remaining 6 patients (10, 11, 15, 23, 26, 30) diagnostic results were less clear and, hence, allergy to PDV and YJV cannot be excluded. In the group of Spanish patients 20/30 (66.7%) exhibited pronounced sIgE-reactivity with Pol d 3 (Fig. 5a).

In order to address sIgE cross-reactivity of Pol d 3 in HBV- and YJV-allergic patients from Germany (from the area of Munich), reactivity with the recombinant allergen was assessed. The patient groups were selected either for primary sensitization to HBV or YJV by detailed clinical characterisation (Supplementary Table S1). Nevertheless, in these groups allergy to both species cannot be fully excluded. However, since PDV allergy in Germany is virtually not present, primary sensitization to PDV can be excluded with high probability. 9/28 (32.1%) of HBV- and 14/22 (63%) of YJV-allergic patients showed reactivity with Pol d 3, respectively (Fig. 5a).

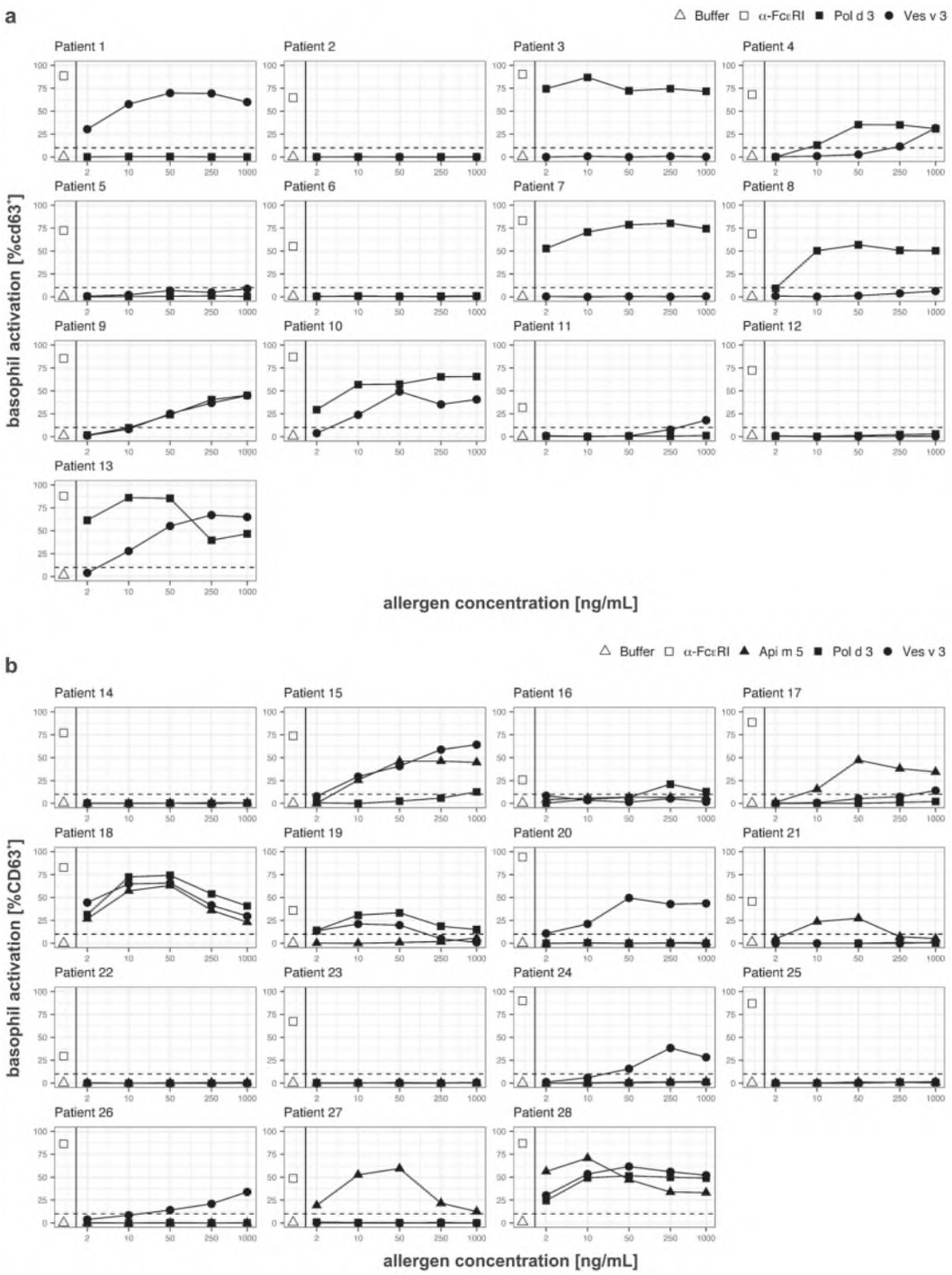

Figure 4. Basophil activation tests of (**a**) PDV- and/or YJV-allergic patients from Spain (area of Barcelona) and (**b**) HBV- and/or YJV-allergic patients from Germany with recombinant DPP IV allergens Pol d 3, Ves v 3 or Api m 5. Basophils were exposed to different concentrations of the DPP IV allergens. Additionally, stimulation with anti-FcεRI antibody (positive control) and plain stimulation buffer (negative control) is shown. Activation is shown as percentage of $CD63^+$ out of total basophilic cells. The cut-off of the assay (10%) is represented as dotted line.

Interestingly, most of the HBV- and YJV-allergic patients with sIgE to Pol d 3 also exhibited sIgE to the homologous allergens from HBV (Api m 5) (Fig. 5b) and YJV (Ves v 3) (Fig. 5c), respectively. Only for one patient with HBV allergy and for 3 YJV-allergic patients with very weak reactivity to Pol d 3, reactivity with the homologous allergens was slightly below the cut-off of the ELISA.

Patient ID	Skin test[1] (i.c.) PDV	Skin test[1] (i.c.) YJV	Skin test[1] (i.c.) HBV	tIgE [kU/L]	sIgE PDV (i77) [kU_A/L]	sIgE YJV (i3) [kU_A/L]	sIgE HBV (i1) [kU_A/L]	sIgE Pol d 5 (i210) [kU_A/L]	sIgE Ves v 5 (i209) [kU_A/L]	sIgE Ves v 1 (i211) [kU_A/L]	sIgE Api m 1 (i208) [kU_A/L]
1	neg.	0.001	neg.	55.5	n.d.	***4.32***	0.01	0.00	0.00	***5.16***	n.d.
2	1	1	neg.	237	***15.3***	***10.6***	0.02	***36.1***	***23.5***	0.07	0.00
3	0.1	0.1	neg.	12.3	***1.07***	***0.35***	0.02	***0.85***	**0.27**	**0.17**	0.00
4	n.d.	n.d.	n.d.	45.3	***0.59***	***1.78***	***2.81***	0.00	0.03	***1.75***	**0.18**
5	neg.	0.1	neg.	84	**0.30**	***1.54***	0.02	0.01	**0.12**	***1.58***	0.00
6	0.001	0.1	neg.	291	***15.9***	***3.53***	0.02	***7.88***	***5.53***	***2.16***	0.00
7	0.0001	0.1	neg.	305	***35.6***	***20.0***	**0.10**	***14.4***	***2.07***	***24.1***	0.00
8	0.01	0.1	0.1	168	***4.34***	***3.55***	***1.16***	***1.9***	***1.21***	***3.93***	***0.35***
9	0.1	0.01	neg.	53.5	***1.98***	***2.11***	0.02	***1***[2]	***1.2***[2]	n.d.	**<0.3**[2]
10	0.01	0.0001	neg.	313	***42.3***	***22.4***	**0.16**	***17.5***	***9.76***	***22.8***	0.02
11	n.d.	n.d.	n.d.	257	**0.14**	***0.88***	0.08	0.00	***0.89***	0.00	0.00
12	1	neg.	neg.	74.6	**0.18**	**0.27**	0.00	**0.23**	**0.18**	0.08	0.00
13	0.1	neg.	neg.	278	***12.6***	***3.09***	0.01	***1.83***	***1.27***	***3.33***	0.00
14	n.d.	0.01	neg.	22	n.d.	***11.9***	***1.08***	n.d.	***12.1***	0.00	0.00
15	n.d.	0.1	0.01	33.1	n.d.	***0.79***	***0.58***	n.d.	**0.20**	***0.88***	0.03
16	n.d.	0.01	0.0001	60	n.d.	***1.45***	***16.2***	n.d.	**0.23**	***0.43***	***1.39***
17	n.d.	0.0001	0.0001	91.6	n.d.	***19.4***	***7.52***	n.d.	***1.82***	***12.7***	**0.31**
18	n.d.	0.01	0.1	35.4	n.d.	***0.46***	***0.73***	n.d.	**0.18**	**0.31**	**0.10**
19	n.d.	0.001	0.1	202	n.d.	***34.2***	***0.59***	n.d.	***34.2***	***7.90***	**0.22**
20	n.d.	0.1	0.1	740	n.d.	***3.26***	***1.48***	n.d.	***2.66***	***0.94***	0.01
21	n.d.	0.001	0.0001	47	n.d.	***0.94***	***15.7***	n.d.	***1.90***	0.02	***3.66***
22	n.d.	neg.	0.001	21.7	n.d.	<0.1	***6.60***	n.d.	0.01	0.03	***2.50***
23	n.d.	0.001	neg.	197	n.d.	***9.30***	***0.43***	n.d.	***6.72***	***4.71***	0.02
24	n.d.	0.1	0.001	41.2	n.d.	***1.61***	***12.6***	n.d.	***0.55***	***6.07***	***4.66***
25	n.d.	0.01	0.1	181	n.d.	***2.52***	***0.71***	n.d.	***7.80***	0.06	0.06
26	n.d.	0.0001	neg.	68.6	n.d.	***14.0***	***0.58***	n.d.	***7.56***	0.08	0.01
27	n.d.	neg.	0.0001	568	n.d.	**0.12**	***>100***	n.d.	0.07	0.04	***>100***
28	n.d.	0.0001	0.0001	65.8	n.d.	***1.29***	***3.29***	n.d.	***1.66***	0.02	***0.66***

Table 1. Clinical data of patients analyzed in basophil activation test. Patients with a systemic reaction after an insect sting (grade I to IV according to Ring and Messmer[46]) were included for BAT. Patients 1 to 13 are from Spain (from the area of Barcelona) and allergic to PDV and/or YJV and patients 14 to 28 are from Germany (from the area of Munich) and allergic to YJV and/or HBV. sIgE and tIgE levels were determined using the UniCAP 250 system (Thermo Fisher Scientific). bold/italic: sIgE $\geq$ 0.35 kU_A/L; bold: sIgE between 0.1 and 0.35 kU_A/L. n.d., not determined; neg., negative. [1]For intradermal skin tests the lowest venom concentration [µg/mL] that gave a positive result is displayed. [2]Measured with ImmunoCAP-ISAC (Thermo Fisher Scientific).

Discussion

Polistes dominula is one of the main elicitors of hymenoptera venom allergy in Southern Europe as well as in parts of the United States. Moreover, *Polistes dominula* is a very invasive species spreading from the warmer to the more moderate climate zones and is therefore likely to gain importance also in other areas. However, compared to other species such as *Apis mellifera* or *Vepula vulgaris*, the knowledge of the composition of important PDV allergens on a molecular level is restricted. Additionally, to date the availability of diagnostic tools for the discrimination between PDV and YJV allergy is very limited. Therefore, our study aimed to identify and immunologically characterise novel important allergens of PDV to extend the available repertoire of venom allergens for analyses on a molecular level.

In this study, we were able to identify the sIgE-reactive 100 kDa allergen of PDV as dipeptidyl peptidase IV and homologue of the well-established DPP IV allergens Api m 5 and Ves v 3 of HBV and YJV, which were shown to represent relevant allergens of clinical importance[18,29,39]. Protein sequence identity with the corresponding allergens of HBV and YJV is 54% and 76.1%, respectively. Due to its allergenic properties and homology to the YJV allergen Ves v 3 the new PDV allergen was assigned as Pol d 3.1010 to the IUIS/WHO allergen nomenclature database[34].

In order to immunologically characterise Pol d 3, the protein was recombinantly produced as soluble and properly folded protein in Sf9 insect cells. Lectin-staining confirmed that Pol d 3 is a glycoprotein, a fact that explains the difference between the calculated molecular mass of the polypeptide chain of 86.2 kDa and the apparent molecular mass of approximately 100 kDa observed in SDS-PAGE and immunoblot analyses. However, recombinant Pol d 3 was devoid of CCD-reactivity as shown previously for other allergens produced in Sf9 insect cells[29,35–37]. Intriguingly, the reactivity of Pol d 3 with a polyclonal Api m 5-specific antiserum[40] hinted to a pronounced protein-based cross-reactivity of the DPP IV allergens of the different hymenoptera species. This cross-reactivity was further confirmed by the reactivity of a serum pool from Pol d 3-reactive PDV-allergic patients with the DPP IV allergens Api m 5 and Ves v 3.

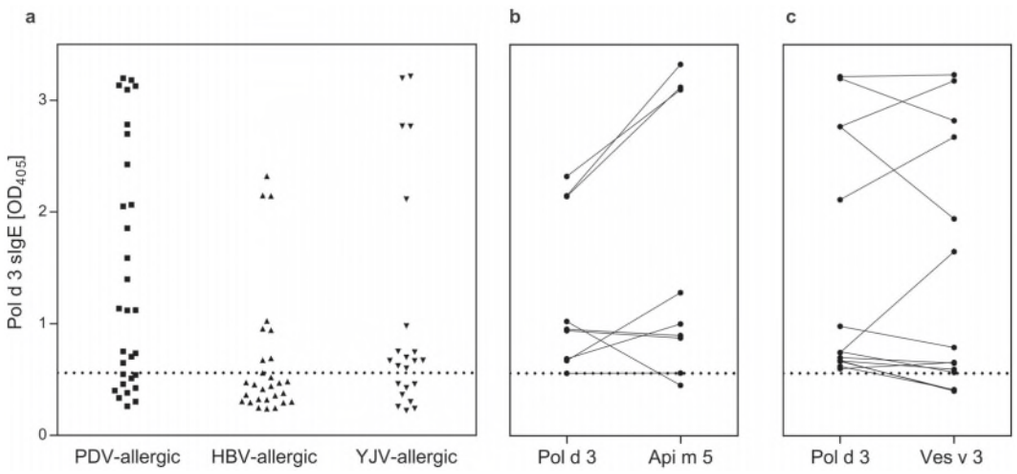

Figure 5. sIgE reactivity of individual hymenoptera venom-allergic patients with recombinant DPP IV allergens in ELISA. (**a**) sIgE immunoreactivity of PDV- (n = 30), HBV- (n = 28) and YJV-allergic patients (n = 20) with Pol d 3. (**b**) Comparative sIgE immunoreactivity of Pol d 3-positive HBV-allergic patients with Pol d 3 and Api m 5. (**c**) Comparative sIgE immunoreactivity of Pol d 3-positive YJV-allergic patients with Pol d 3 and Ves v 3. The lower end cut-off of the ELISAs is represented by dotted lines.

To access the relevance of Pol d 3 as allergen, sIgE-reactivity of PDV-allergic patients with this newly identified PDV component was addressed. Thereby, over 66% of PDV-allergic patients exhibited pronounced sIgE to Pol d 3. This is in a comparable range found for the reactivity of either HBV- or YJV-allergic patients with the corresponding DPP IV allergens Api m 5 and Ves v 3, respectively. In previous studies, it was demonstrated that 58.3% to 61.7% of HBV-allergic patients and 57% of YJV-allergic patients show sIgE to Api m 5 and Ves v 3, respectively[18,29,39]. These data clearly suggest that Pol d 3 represents a major allergen of PDV. As *Polistes dominula* and *Vespula vulgaris* coexist in Spain and are difficult to discriminate, systemic reactions due to both insects cannot be excluded in the Spanish patient population.

Additionally, 32% of HBV- and 63% of YJV-allergic patients exhibited sIgE to the new PDV allergen. The majority of these patients additionally showed reactivity with the homologous allergen of HBV (Api m 5) or YJV (Ves v 3), indicating that the sIgE to Pol d 3 is a result of extensive protein-based cross-reactivity. Moreover, the lower degree of cross-reactivity between Pol d 3 and Api m 5 compared to Pol d 3 and Ves v 3 most likely reflects the lower sequence identity between the PDV and HBV allergen and, thus, of less conserved IgE epitopes.

So far phospholipases A1 (Pol d 1 and Ves v 1) and antigens 5 (Pol d 5 and Ves v 5) are well established cross-reactive allergen pairs of PDV and YJV[2,3,28,41]. Although it is likely that also the hyaluronidases of PDV and YJV are cross-reactive, no reliable data exist. Moreover, at least YJV hyaluronidase seems to be of limited clinical relevance[37,42]. In this study DPP IV allergens were identified as novel pair of cross-reactive major allergens, responsible for the frequently observed double-positive sIgE test results with PDV and YJV. Additionally, our analyses provide for the first time a molecular basis for the observed cross-reactivity between PDV and HBV[43]. Therefore, DPP IV allergens might be pan-allergens, present in various hymenoptera venoms. Also, the presence of DPPs IV in many snake venoms[44] suggests functions of this enzyme class in the venoms of phylogenetically distinct species.

The capacity of Pol d 3 to activate effector cells and to initiate an allergic response was assessed by basophil activation testing and proved its role as potent allergen. In the Spanish patient group the basophil activation patterns by Pol d 3 and Ves v 3 matched the data of skin testing and sIgE levels to allergen extracts and components. For the German patients, this was also the case for the basophil activation data obtained with Api m 5 and Ves v 3. However, in this patient group Pol d 3 clearly demonstrated to be cross-reactive also in BAT. For these patients PDV allergy can be excluded with high probability since it is virtually not present in Germany. Therefore, Pol d 3-reactivity in BAT in these patients most likely is due to cross-reactivity. This is further supported by the fact that most Pol d 3-reactive patients additionally show basophil activation in response to Ves v 3 (and Api m 5). Only one patient showed weak basophil activation in response to Pol d 3 only but also very low general activation according to the positive control. However, if these results are of clinical relevance is difficult to determine since diagnostic sting provocation testing is ethically not justifiable. Nevertheless, the data demonstrate that BATs with recombinant cross-reactive major allergens represent a helpful tool to identify the allergy-eliciting venom as shown previously[28].

To date, in clinical practice the discrimination between allergy to PDV and YJV is quite challenging. In contrast, for the discrimination between allergy to YJV and HBV an extended component-resolved diagnostics has evolved and has shown to provide added benefit for clinical decisions[4]. It was proposed that PDV and YJV allergy should be discriminated by measurement of the level of sIgE to phospholipases A1 (Pol d 1 and Ves v 1) and antigens 5 (Pol d 5 and Ves v 5)[3]. However, in addition to the YJV allergens, so far only Pol d 5 is available for routine diagnostics. In a former study, we were able to demonstrate that for most of the patients, for whom the allergy-relevant venom was clearly identified, the sIgE level with the appropriate antigen 5 (Pol d 5 or Ves v 5)

indeed was higher[28]. However, this does not hold true for all patients. Moreover, for many patients the sIgE levels are in a very comparable range, hence, results will be difficult to interpret in many cases.

Of course, it would be of major interest to identify species-specific marker allergens that would allow a reliable and easy discrimination between PDV and YJV allergy. However, all identified major allergens of the two venoms, including phospholipases A1, antigens 5 and dipeptidyl peptidases IV, exhibit a high degree of cross-reactivity. Hence, the development of a component-resolved diagnostic approach, comparable to that for the discrimination between HBV and YJV allergy is difficult to realize, in our opinion. Nevertheless, the extension of the available panel of allergen components also for routine diagnostics of PDV (and YJV) allergy will generate added value for advanced diagnostics. Certainly, the combination of the results of sIgE measurements to more than one major allergen will help to create a clearer diagnostic picture and to facilitate diagnostic decisions also in vespid venom allergy. Therefore, the newly identified major allergen of PDV, Pol d 3, together with its counterparts of YJV and HBV, might become a key element for molecular diagnostics of hymenoptera venom allergy. Moreover, the detailed knowledge of the allergen composition of different insect venoms will help to understand the immunological mechanisms of venom allergy and therapeutic outcome.

Methods

Patients. Blood and/or sera of 108 patients with allergy either to PDV, HBV and/or YJV were analysed. 65 patients were from the area of South Bavaria (Munich, Germany), 30 patients were from the area of Córdoba, Spain and 13 patients were from Barcelona, Spain. As PDV allergy is almost not present in Germany, allergic reactions to this species can be excluded with high probability and the German patients were allergic to YJV and/or HBV. Patients from Córdoba were primarily allergic to PDV and patients from Barcelona were allergic to PDV and/or YJV. As *Polistes dominula* and *Vespula vulgaris* coexist in Spain and are difficult to discriminate, systemic reactions due to both insects cannot be excluded.

The diagnosis of venom allergy was based on a combination of clinical history of an allergic sting reaction, a positive intradermal skin test, and/or positive sIgE levels to PDV, YJV and/or HBV (i77, i3, i1) and allergen components (i208, i209, i210, i211) (UniCAP250; Thermo Fisher Scientific, Uppsala, Sweden).

All patients had given informed written consent to draw additional blood samples. The study was approved by the ethics committee of the Faculty of Medicine of the Technical University of Munich (protocol number 5478/12), the ethics committee for clinical research of Reina Sofía University Hospital Cordoba (protocol number BLA-VIT-2015-01) and the ethical committee for clinical investigation of the Hospital Clinic of Barcelona (protocol number 2011/6605). All patients were recruited from clinical routine and the obtained data are not part of another study reported elsewhere. All experiments were performed in accordance with relevant guidelines and regulations.

Protein biochemistry. Pol d 3 was identified by tandem mass spectrometry analyses on a MALDI-TOF instrument. A detailed description is given in the Supplementary Methods.

Cloning and recombinant production of venom dipeptidyl peptidases IV. The coding region of Pol d 3 was amplified from *Polistes dominula* venom gland cDNA and plasmids coding for Api m 5 and Ves v 3 were generated as described previously[29,45]. Cloning and recombinant production in *Spodoptera frugiperda* (Sf9) insect cells is described in detail in the Supplementary Methods.

Immunoreactivity of patient sera with recombinant dipeptidyl peptidases IV. sIgE immunoreactivity of sera with the recombinant venom DPPs IV was assessed by ELISA. A detailed description of the ELISA is given in the Supplementary Methods. The lower end functional cut-off, indicated as dotted lines, was calculated as the mean of the negative controls summed with 3 times the standard deviation (SD) of the mean and additionally 10% of the resulting value.

Basophil activation test. Basophil activation tests were performed in 15 YJV- and/or HBV-allergic patients, and in 13 PDV- and/or YJV-allergic patients as described previously[38], using the Flow CAST (Bühlmann Laboratories AG, Schönenbuch, Switzerland). Allergen concentrations were 2, 10, 50 and 250 and 1000 ng/mL. A detailed description is given in the Supplementary Methods.

Other methods. SDS-PAGE, Western blotting, DPP IV activity and CD spectroscopy are described in the Supplementary Methods.

Data availability. All data generated or analysed during this study are included in this published article and its Supplementary Information files.

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Author Contributions

M.S. planned and performed the experiments, analysed the data and wrote the manuscript. B.E. coordinated the recruitment of hymenoptera venom-allergic patients and supervised basophil activation tests. M.P. performed basophil activation tests and collected data. C.H., A.K., D.R. and S.P. performed M.S. experiments, analysed the data and revised the manuscript. G.P. recruited HBV- and YJV-allergic patients and collected data. P.S. recruited PDV-allergic patients and collected data. C.M.-A. coordinated the recruitment of PDV-allergic patients and collected and analysed the data. FdlR recruited PDV- and YJV-allergic patients and collected data. T.B. contributed to the interpretation of data and revised the final version of the manuscript. U.D. was responsible for the diagnostic work-up of hymenoptera venom- allergic patients. C.S.-W. supervised the study, contributed to the interpretation of data, and revised the final version of the manuscript. M.O. supervised the study, analysed the data, and wrote the manuscript. S.B. initiated and supervised the study, analysed the data and wrote the manuscript.

Additional Information

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RESEARCH PAPER

OPEN ACCESS

Component-resolved evaluation of the content of major allergens in therapeutic extracts for specific immunotherapy of honeybee venom allergy

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ABSTRACT

Allergen-specific immunotherapy is the only curative treatment of honeybee venom (HBV) allergy, which is able to protect against further anaphylactic sting reactions. Recent analyses on a molecular level have demonstrated that HBV represents a complex allergen source that contains more relevant major allergens than formerly anticipated. Moreover, allergic patients show very diverse sensitization profiles with the different allergens. HBV-specific immunotherapy is conducted with HBV extracts which are derived from pure venom. The allergen content of these therapeutic extracts might differ due to natural variations of the source material or different down-stream processing strategies of the manufacturers. Since variations of the allergen content of therapeutic HBV extracts might be associated with therapeutic failure, we adressed the component-resolved allergen composition of different therapeutic grade HBV extracts which are approved for immunotherapy in numerous countries. The extracts were analyzed for their content of the major allergens Api m 1, Api m 2, Api m 3, Api m 5 and Api m 10. Using allergen-specific antibodies we were able to demonstrate the underrepresentation of relevant major allergens such as Api m 3, Api m 5 and Api m 10 in particular therapeutic extracts. Taken together, standardization of therapeutic extracts by determination of the total allergenic potency might imply the intrinsic pitfall of losing information about particular major allergens. Moreover, the variable allergen composition of different therapeutic HBV extracts might have an impact on therapy outcome and the clinical management of HBV-allergic patients with specific IgE to particular allergens.

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Introduction

Stings of Hymenoptera such as honeybees or vespids can cause severe and even fatal anaphylaxis in allergic individuals. The only curative treatment which is effective in reducing the risk of subsequent systemic reactions is venom-specific immunotherapy (VIT). VIT is effective in 75% to 98% of patients in preventing sting anaphylaxis.[1] However, therapy failures occur more often in honeybee venom (HBV) compared with yellow jacket venom (YJV) allergy.[2]

HBV represents a complex mixture of various substances such as low-molecular weight components (e.g. histamine, noradrenalin, serotonin and dopamine), peptides (e.g., melittin, apamin, kinins and mast cell degranulating peptide) and a plethora of proteins from which several are allergens.[3] VIT is performed with venom extracts which are administered either as aqueous or aluminum hydroxide-adsorbed extracts (depot preparations). The latter are used in the conventional build-up and maintenance phases, while the aqueous extracts are used in ultra-rush, rush, clustered and maintenance phases.[4] Interestingly, in Europe many specialists switch from aqueous extracts to depot preparations after up-dosing.[5,6]

All therapeutic HBV extracts are derived from pure venom, which is usually collected by electrostimulation, a procedure which leads to a relatively pure venom. Another possibility for obtaining venom extract is the dissection of whole venom glands and venom sacs, a method yielding less pure extract since in addition to the venom components, also proteins from the surrounding tissue are contained in the extract. However, only scarce information is available about how the venom is further processed by different manufacturers to produce therapeutic

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grade venom extracts. Although this classification is a little misleading in the literature, aqueous venom extracts are sometimes classified as "purified" and "non-purified" extracts.[4,7,8] This terminology results from the fact that, even though, all manufacturers surely undertake purification steps of the pure venom for injection purposes, some companies claim to offer an ultrapure venom extract for therapy which does not contain vasoactive amines and a reduced content of small peptides.[4] In the commonly used licensed depot preparation, the "purified" extract is adsorbed onto aluminum hydroxide. In comparative trials, the purified aqueous and the purified aluminum hydroxide-adsorbed extracts appear to be better tolerated than non-purified extracts, especially in terms of severe large local reactions.[7,8]

Although the production of therapeutic allergen extracts has to be highly standardized in terms of the production process and of the total allergenic potency,[9] the lack of information about a broader range of clinically relevant allergens and of appropriate molecular tools for their assessment hampers the generation of highly reliable venom extracts with a more favorable overall therapeutic efficacy.

Especially HBV might represent a challenge for the preparation of therapeutic extracts including all relevant allergens in adequate amounts, since over 60% of its dry-weight is made up by the allergens Api m 1 (12%) and Api m 4 (50%).[10] While Api m 1 (phospholipase A2) represents a well-established major allergen, Api m 4 (melittin) is a minor allergen with restricted clinical relevance.[3] Recently it was demonstrated that HBV contains many more additional important major allergens, namely Api m 2 (hyaluronidase), Api m 3 (acid phosphatase), Api m 5 (dipeptidyl peptidase IV) and Api m 10 (icarapin) which exhibit sIgE reactivity with 47.9–52.2%, 49.6–50%, 58.3–61.7% and 61.8–72.2% of allergic patient's sera, respectively.[11,12] Compared to Api m 1 and Api m 4, all these allergens are present in the venom in only minimal amounts.[13,14] This might implicate that especially the amount of these allergens in therapeutic extracts, might be easily affected by natural variations of the source material, different work-up strategies of the manufacturers or even by degradation of particular components.

In a former study we used monoclonal antibodies and demonstrated that, compared with crude HBV, the allergens Api m 3 and Api m 10 are underrepresented or even missing in particular therapeutic HBV extracts which are commonly used for VIT.[13] Very recently, another study correlated treatment failures of HBV VIT with a predominant Api m 10 sensitization and demonstrated the lack or underrepresentation of Api m 10 in different therapeutic HBV extracts.[11]

Such data might be of major importance for the clinical management of HBV-allergic patients with specific IgE to particular allergens. Therefore, in this study we extended former analyses and generated highly specific and sensitive antibodies for the detection of the major allergens Api m 2, Api m 3, Api m 5 and Api m 10 and compared different therapeutic HBV extracts regarding their allergen content. Thereby, we were able to demonstrate the underrepresentation of relevant major allergens in particular therapeutic extracts. Moreover, compared with another study,[11] we found dramatically different results concerning the Api m 3 and Api m 10 content of particular products, a fact that is of major importance for clinical decisions on the selection of licensed immunotherapeutic products in Europe. Additionally, our findings on Api m 10 stability might have an impact on the immunotherapeutic procedure for venom allergy, both in Europe and in the United States.

Results

Generation of allergen-specific antibodies

Polyclonal antibodies with specificity for the major allergens Api m 2, Api m 3 and Api m 10 were generated by the immunization of rabbits with the individual purified and cross-reactive carbohydrate determinat (CCD-)-free recombinant allergens.[12,13,15,16] For the detection of Api m 5 we used a recombinant monoclonal IgE antibody.[17]

All rabbit antisera that resulted from the immunizations with the individual allergens as well as the monoclonal Api m 5-specific antibody showed excellent reactivity with the

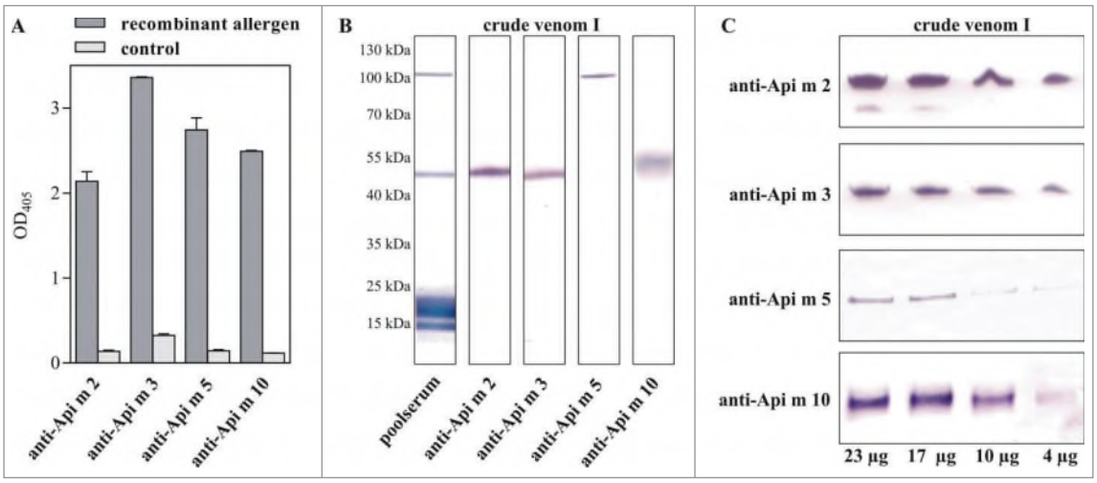

Figure 1. Specificity and sensitivity of allergen-specific antibodies. A, Reactivity of the antibodies with their corresponding recombinant target allergens in ELISA. B, Reactivity of the antibodies with their native target allergens in crude honeybee venom. In comparison, the IgE-reactivity of a poolserum from honeybee venom-allergic patients is shown. Since all investigated allergens represent glycoproteins, the molecular weights do not correspond to that of the calculated weights of the protein portions only, which are stated in some databases. C, Detection of the particular allergens in serial dilutions of crude honeybee venom to assess the sensitivity of the allergen-specific antibodies in immunoblot.

recombinant allergens (Fig. 1A). Moreover, all antibodies proved to be highly specific for the natural allergens in the crude HBV and detected single allergen bands of the expected molecular weight in immunoblots (Fig. 1B). The sensitivity of the detection was assessesd using dilution series of the crude venom and all antibodies showed adequate detection of their target allergens within 4 μg of whole venom (Fig. 1C).

Allergen content of therapeutic HBV extracts

We addressed the major allergen content of 4 different aqueous therapeutic HBV extracts which are approved for immunotherapy in different countries world-wide: Venomil (Allergy Therapeutics, Worthing, UK), Reless (ouside Germany also known as Pharmalgen; ALK-Abelló, Hamburg, Germany), ALK lyophylisiert SQ (ouside Germany also known as Aquagen SQ; ALK-Abelló) and Venomenhal (HAL Allergy, Leiden, Netherlands). Furthermore, the allergen content of these therapeutic HBV extracts was compared with 2 commercially available crude HBV extracts (I: Sigma-Aldrich, Taufkirchen, Germany: II: Latoxan, Portes-lès-Valence, France).

For the analyses, all lyophilized extracts were reconstituted with ddH_2O and the freshly reconstituted extracts were applied for immunoblotting. Staining of the major allergen Api m 1 served as loading control to ensure that all extracts were present on the immunoblot at equal amounts (representative stainings are shown in Fig. 2). Api m 1 was present in all therapeutic extracts as well as in the crude venoms in equal and high amounts. Additionally, Api m 2 was well detectable in all therapeutic extracts (Fig. 2A). Api m 3, Api m 5 and Api m 10 were detectable in comparable amounts in Venomil, Reless (Pharmalgen) and Venomenhal, although, to a slightly lesser extent than in the crude HBV (Fig. 2A). However, all 3 allergens were strongly underrepresented and only barely detectable in ALK lyophylisiert SQ (Aquagen SQ) (Fig. 2A).

Intriguingly, while these results were reproducible for 5 batches of Venomil, 2 batches of Reless and 2 batches of ALK lyophylisiert SQ, in another batch of Venomenhal, Api m 3 and Api m 10 were not detectable. The direct comparison of the 3 batches of this product showed that both allergens were well detectable in 2 batches but undetectable in a third batch (Fig. 2B), while the content of the other investigated allergens was comparable for all batches (data not shown).

In a recent study a comparable Api m 3-specific antiserum was used and a clear reactivity of all therapeutic HBV extracts, comparable to that of crude HBV, was postulated. Due to these divergent results we repeated the analysis using an already published monoclonal Api m 3-specific antibody.[13] Thereby, we were able to completely confirm our previous results using the polyclonal antiserum, namely, a lesser Api m 3 content of all therapeutic extracts compared with the crude venom and an underrepresentation of Api m 3 in ALK lyophylisiert SQ (Aquagen SQ) compared with the other products (Fig. S1).

Stability of Api m 10

To address the variable Api m 10 content of different therapeutic products and the observed discrepant results for particular products compared with the recently published study by Frick et al.,[11] we evaluated the stability of this relevant allergen. Interestingly, applying recombinantly produced and purified Api m 10 in a comparable concentration as found in the crude venom, our analyses demonstrated rapid degradation of the allergen within 3 days, when stored in solution at +4° C (Fig. 3A).

In our analyses we always used freshly reconstituted therapeutic venom extracts. However, the observed instability of Api m 10 could critically influence immunoblot-based analyses in the laboratory. For laboratory purposes the freeze-dried therapeutic venom extracts are routinely solved in ddH_2O or buffers like PBS and not in the supplied albumin-containing saline

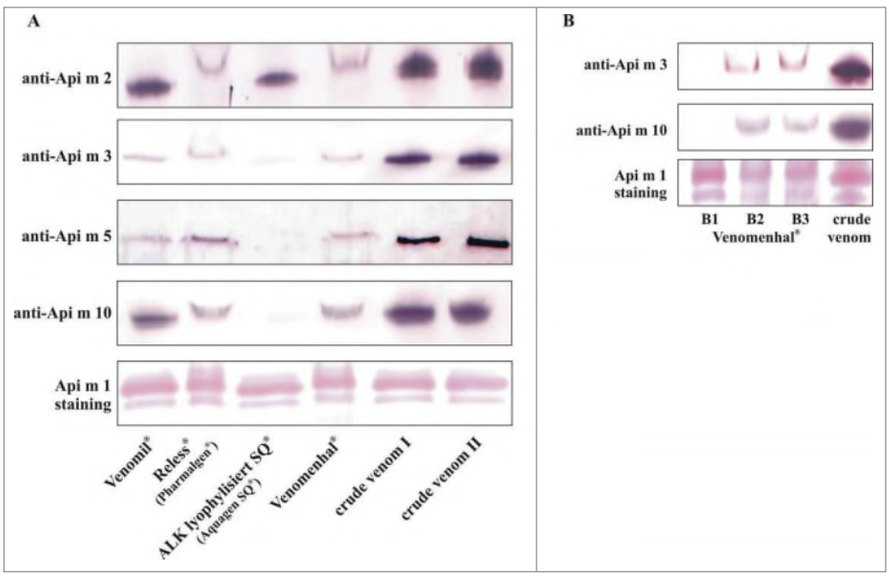

Figure 2. Allergen content of therapeutic honeybee venom extracts. A, Allergen content of therapeutic venom extracts compared with crude venom as assessed by the use of polyclonal (Api m 2, Api m 3 and Api m 10) and monoclonal (Api m 5) antibodies. Representative results of 5 batches of Venomil (Allergy Therapeutics, Worthing, UK), 2 batches Reless (Pharmalgen) (ALK-Abelló, Hamburg, Germany), 2 batches ALK lyophylisiert SQ (Aquagen SQ) (ALK-Abelló) and 2 batches of Venomenhal (HAL Allergy, Leiden, Netherlands) are shown. Ponceau S staining of Api m 1 served as loading control (a representative staining is shown). B, Api m 3 and Api m 10 content of 3 independent batches (B1-B3) of Venomenhal.

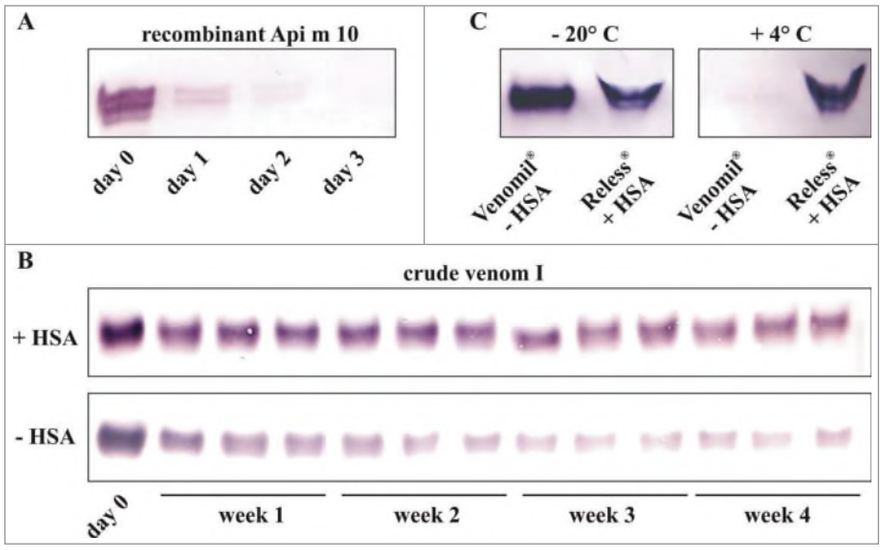

Figure 3. Stability of the major allergen Api m 10. A, Stability of recombinant purified Api m 10 produced in insect cells. Api m 10, in a concentration comparable to that detected in crude venom, was stored for 3 d at + 4° C. B, Stability of Api m 10 in crude honeybee venom reconstituted either with HSA-containing diluent for injection (Allergy Therapeutics) or PBS upon storage at + 4° C for 4 weeks. C, Detection of Api m 10 in water-resolved Venomil (no HSA in the lyophylisate) and Reless (HSA in the lyophylisate) stored for 4 weeks at either - 20° C or + 4° C.

diluent for injection purposes. This implies that some PBS-reconstituted products such as Reless (Pharmalgen) or Venomenhal contain human serum albumin (HSA) since it is already contained in the freeze-dried venom. In contrast, for Venomil the HSA is added together with the diluent in clinical practice, so that ddH_2O- or buffer-reconstituted Venomil contains no HSA. The stabilizing effect of HSA on Api m 10 in crude venom, stored in solution at +4° C, is shown in Fig. 3B. Moreover, these results indicate that laboratory analyses of therapeutic venom extracts might be influenced by the reagents used for their reconstitution and by the subsequent storage of the solubilized extracts, as demonstrated by the different results for ddH_2O-reconstituted Venomil and Reless stored at either -20°C or +4° C (Fig. 3C). However, in clinical practice this fact does not matter since all analyzed products contain HSA after reconstitution with the supplied diluent.

Discussion

In this study we established tools that allow a component-resolved analysis of therapeutic HBV extracts. Four therapeutic HBV extracts commonly used for VIT were analyzed for their content of the major allergens Api m 1, Api m 2, Api m 3, Api m 5 and Api m 10. Intriguingly, numerous differences could be demonstrated for the particular products. The observed varying major allergen content of therapeutic HBV extracts might have a high impact on clinical practice and on the handling of patients with particular sensitization profiles.

Although obtained by methods, yielding relatively pure venom, the analyzed therapeutic HBV extracts showed a diverse content of important major allergens. This degree of variation, as also demonstrated for other allergen extracts,[18-22] indicates that different strategies for down-stream processing of the pure venom for the production of therapeutic grade venom extracts, can substantially affect the representation of major venom allergens, resulting in the potential loss of particular allergens with high clinical relevance. Moreover, the geographical origin or seasonal variations might additionally affect the composition of the source material.[23]

During the last years, studies demonstrated that in addition to Api m 1 also Api m 2, Api m 3, Api m 5 and Api m 10 represent major allergens of HBV.[12,13,15,17,24,25] Additionally, 39 different sensitization profiles were identified in 144 patients with HVB allergy, applying 6 different allergens (Api m 1–5 and 10).[12] Interestingly, in HBV allergy these 6 allergens are necessary to reach a diagnostic sensitivity of approximately 95%, a fact that indicates a more complex allergen composition of HBV compared with YJV. In YJV allergy the 2 major allergens Ves v 1 and 5 are sufficient to reach the same diagnostic sensitivity.[26] Both of the 2 important allergens are present in YJV in substantial and equimolar amounts (Ves v 1 with 6–14% and Ves v 5 with 5–10% of the venom dry weight).[14] In contrast, in HBV Api m 1 is the only major allergen that is present in substantial amounts and all other relevant allergens make up only 0.6–2% of the venom dry weight.[13,14] Therefore, it could be speculated that these differences between the 2 venoms might be a reason for the higher success rate of YJV-specific immunotherapy.

Currently, it is a matter of debate whether particular sensitization profiles are linked to the outcome of VIT with HBV. Very recently, a study correlated treatment failures of HBV VIT with a predominant Api m 10 sensitization.[11] The same study demonstrated the lack or underrepresentation of Api m 10 in different therapeutic HBV extracts commonly used for VIT. As a consequence of the variable Api m 10 content, it was suggested that patients with predominant Api m 10 sensitization should be treated with a HBV extract containing a relevant amount of the allergen. To our opinion this is a major step forward toward a personalized medicine approach in VIT and, surely, will influence the use of VIT products. Likewise, it might be an option to treat patients without Api m 10 sensitization with a product that lacks the allergen.

In this study, the content of 5 relevant major allergens in 4 aqueous HBV extracts which are commonly used for immunotherapy was addressed for the first time. Thereby, we were able to demonstrate comparable allergen contents for Api m 1 and Api m 2. However, substantial differences were demonstrated for the other allergens. While Api m 3, Api m 5 and Api m 10 could be reproducibly detected in different batches of Venomil and Reless (Pharmalgen), all 3 allergens were clearly underrepresented in ALK lyophylisiert SQ (Aquagen SQ). An underrepresentation of Api m 3 and Api m 10 was observed for one but not for 2 other batches of Venomenhal. Only one batch of the product was available on the market at a time and the underrepresentation was observed only for the earliest purchased batch. Therefore, the differences in the Api m 3 and Api m 10 content could either be due to batch to batch variations or to a modified production process.

Whether the underrepresentation of the 3 major allergens, which was observed for ALK lyophylisiert SQ (Aquagen SQ), also holds true for the related depot preparation (ALK-depot SQ or Alutard SQ), can only be speculated at this time point since the analysis of aluminum-adsorbed venom extracts by the here applied methods is very challenging. Recently, the presence of Api m 10-derived peptides was demonstrated for Aquagen SQ (and Alutard SQ) by mass spectrometry (MS) analyses.[27] This is in accordance with our immunoblot analyses which were also able to detect minimal amounts of intact Api m 10 in ALK lyophylisiert SQ (Aquagen SQ). Nevertheless, our results demonstrate obvious differences in the amount of full-length Api m 10 in this particular product compared with other aqueous extracts. Moreover, while our analyses address the content of the full-length protein, the applied MS analyses are not able to discriminate between full-length Api m 10 and Api m 10-derived degradation products. Additionally, the used MS analyses were not quantitative. However, so far it is not known whether the intact allergen and derived degradation products thereof exhibit the same potency in inducing a tolerogenic immune response. Definitely, further studies are needed, which address, if small amounts of Api m 10 or Api m 10-derived peptides in the therapeutic extracts are sufficient to induce tolerance in the majority of patients. Nevertheless, our analyses suggest that intensive purification and processing steps of the crude venom might strongly influence the content of full-length Api m 10 (as well as of Api m 3 and Api m 5), and since underrepresentation was most pronounced in ALK-lyophylisiert SQ (Aquagen SQ), it's processing that removes low molecular weight substances and reduces the amount of bioactive peptides, may be relevant here.[4,7,8]

A recent study demonstrated the lack or underrepresentation of Api m 10 in Venomil, Venomenhal and Aquagen SQ as well as its presence in Pharmalgen.[11] Although in our analyses we applied the same methods, intriguingly, we found dramatically different results in part, a fact that is of major importance for clinical decisions on the selection of licensed immunotherapeutic products in Europe. Our study was able to confirm the underrepresentation of Api m 10 in Aquagen SQ (ALK lyophylisiert SQ) and its presence in Pharmalgen (Reless). However, in strong contrast to that study, we were able to detect comparable amounts of Api m 10 in 5 independent batches of Venomil. For Venomenhal our analyses demonstrated batch to batch variations ranging from Api m 10 content comparable to Venomil and Pharmalgen to undetectable content. These are facts that might be of importance for the handling of patients with Api m 10 sensitization.

Regarding the reasons for these different results, it can only be speculated. The analyses were reproducibly performed with different independent batches in both studies. The sensitivity of detection is not able to give a reasonable explanation for the observed discrepancy, since the evaluated detection limit of our experimental setup is significantly higher than the postulated detection limit by Frick et al.: Whereas 4–10 μg of crude venom was necessary to achieve adequate detection of Api m 10 in our immunoblots, Frick et al. show effective detection when applying 0.5–1.5 μg of crude venom. Most likely, the observed discrepancies might be explained by the rapid degradation of the allergen after solubilizing the lyophilized therapeutic extracts and storage at + 4° C. Unfortunately, Frick et al. do not state how venom extracts were handled in the laboratory after solubilization. Nevertheless, these data clearly demonstrate the need for standardized operation procedures for quality-control of therapeutic extracts in different laboratories.

In clinical practice in Europe, the observed Api m 10 instability might be limited by the fact that all of the licensed products contain potentially stabilizing human serum albumin after reconstitution of the lyophilized extract. However, our results might implicate that the use of small pharmaceutical phials that contain therapeutic extracts for one injection might be superior over larger ones that are stored for several weeks after reconstitution. However, the obtained results may not only influence clinical decisions for the selection of immunotherapeutic products in Europe. Due to the observed instability of Api m 10, they might also have an impact on the immunotherapeutic procedure in the United States, where the clinical routine practice involves the formulation of patient-specific preparations for immunotherapy based on stock solutions of venom extract stored at + 4° C, exactly the conditions that lead to rapid Api m 10 degradation.

In contrast to a former analysis in which we used an Api m 3-specific monoclonal antibody for detection,[13] the study by Frick et al.[11] demonstrated the presence of Api m 3 in all assessed therapeutic products in comparable amounts to that in crude HBV by applying polyclonal antibodies. Our analyses shown here, also used polyclonal rabbit antibodies that were generated against full-length Api m 3 produced in insect cells. Surprisingly, these analyses showed clearly lesser amounts of Api m 3 in all therapeutic extracts, compared with crude HBV as well as an underrepresentation in ALK lyophylisiert SQ (Aquagen SQ) and in 1 out of 3 batches of Venomenhal compared with the other therapeutic extracts. Although, both studies used polyclonal rabbit antibodies, these differences might be explained by slight differences in sensitivity or specificity of detection. In contrast to the antibodies used in our study, which detected a single band of Api m 3, the antibodies used by Frick et al. resulted in multiple bands in some of the analyzed products. Moreover, our results are in full accordance with the data obtained in a former study[13] and with the results obtained using the monoclonal Api m 3-specific antibody in this study.

Considering the complex sensitization profiles of HBV-allergic patients,[12] it might be speculated that some of the

therapeutic extracts could be associated with therapeutic failure in patients with particular sensitization profiles, an issue that should be addressed in future studies. Potential candidates might be patients who are polysensitized to several allergens or who are exclusively sensitized to allergens such as Api m 3 and/or Api m 10 (4.8% of patients[12]), which are not or only barely detectable in particular therapeutic HBV extracts, and against which, only minimal IgG4 is induced during VIT in contrast to other allergens that are present in substantial amounts.[12] Moreover, the immune response to minimal amounts of allergen in particular HBV extracts might differ strongly in individual patients. Notably, HBV in general might represent a particularly challenging allergen source for the preparation of therapeutic extracts, containing all major allergens in sufficient amounts. Studies of other therapeutic vaccines for allergen immunotherapy demonstrated that maintenance doses of 3 to 20 μg of major allergen are associated with clinical improvement after immunotherapy.[28] Taken a maintenance dose of 100 μg, these amounts are only reached for Api m 1 and none of the other major allergens of HBV, even within crude venom. Therefore, the mechanism of tolerance induction against low amounts of major allergens in the majority of HBV-allergic patients clearly should be a focus of future research. The limitation of our study is represented by the fact that at this time no connection between therapeutic outcome, allergen content of products used for therapy and IgE sensitization profiles of patients can be revealed. Therefore, our results clearly demonstrate the need for extended prospective clinical studies focusing on this relationship.

Nevertheless, our results might have implications for i) the clinical management of HBV-allergic patients particular sensitization profiles worldwide, ii) the quality control and regulatory process for patient-named and licensed products used for VIT procedures, and iii) all major stakeholders (doctors, patients, regulators, reimbursement systems/insurance companies) in the affected health markets, helping them to make the right decisions in the emerging era of precision medicine.

Taken together, our data demonstrate obvious differences in the quality of therapeutic HBV extracts in terms of the content of important allergens, a fact that might be of major importance at least for patients with particular sensitization profiles. Moreover, standardization of therapeutic venom extracts by determination of the total allergenic potency might imply the intrinsic pitfall of losing information about particular major allergens. Allergen-specific antibodies represent valuable tools that allow component-resolved analyses of therapeutic extracts on a molecular level that cope with the advanced knowledge of the composition of relevant allergens.

Materials and methods

Allergen-specific antibodies and recombinant allergens

The recombinant CCD-free allergens were produced in *Spodoptera frugiperda* (Sf9) insect cells and purified as described previously.[12,13,15-17] Polyclonal antibodies were generated by immunization of rabbits (Davids Biotechnology, Regensburg, Germany) with either recombinant Api m 2, Api m 3 or Api m 10 according to established protocols. The monoclonal Api m 3- and Api m 5-specific IgE antibodies were generated as described previously.[13,17,29]

Immunoblotting

For immunoblotting, lyophilized HBV extracts were dissolved in ddH_2O to a stock concentration of 1.3 mg/mL. Immediately after dissolving 23 μg/lane (or less for sensitivity testing of the antibodies) were separated by SDS-PAGE under reducing conditions and immobilized onto nitrocellulose membranes (Thermo Scientific, 88018). Blot membranes were blocked with 40 mg/mL nonfat dry milk powder (AppliChem, A0830) in PBS (Life Technologies, 70011051). Polyclonal allergen-specific rabbit antisera were diluted 1:1000 with 20 mg/mL nonfat dry milk powder in PBS. Recombinant monoclonal IgE antibodies were used in form of cell culture supernatants (DMEM (Gibco, 31966–021) supplemented with 10% fetal calf serum (Biochrom, SO115)) of antibody-producing HEK293 cells. All antibodies were applied to the corresponding Western blots and incubated over night at 4° C. After washing for 3 times with PBS, bound allergen-specific antibodies were detected for 1 hour at room temperature via polyclonal goat anti-rabbit IgG (Sigma-Aldrich, SAB3700854) or monoclonal mouse anti-human IgE (BD Biosciences, 555859) antibody, conjugated to alkaline phosphatase, diluted 1:5000 or 1:1000 with 20 mg/mL nonfat dry milk powder in PBS, respectively. After washing for 3 times with PBS bound antibodies were visualized using nitrotetrazolium blue chloride (AppliChem, A1243)/5-bromo-4-chloro-3-indoyl phosphate (AppliChem, A1117) according to the recommendations of the manufacturer. Ponceau S (Sigma-Aldrich, P7170) staining of immobilized Api m 1 served as loading control.

Elisa

F96 maxisorp Nunc-immuno plates (Thermo Scientific, 439454) were coated with recombinant allergens (10 μg/mL) over night at 4° C and blocked with 10 mg/mL BSA (AppliChem, A1391) in PBS (Life Technologies, 70011051). Allergen-specific polyclonal rabbit antisera were diluted 1:5000 and monoclonal recombinant antibody cell culture supernatants 1:2 with 5 mg/mL BSA in PBS, applied to the corresponding wells and incubated for 4 hours at room temperature. After washing 5 times with 0.05% Tween20 (EMD Chemicals, 655204) in PBS, alkaline phosphatase-conjugated polyclonal goat anti-rabbit IgG (Sigma-Aldrich, SAB3700854) diluted 1:5000 or monoclonal mouse anti-human IgE (BD Biosciences, 555859) diluted 1:1000 in 5 mg/mL BSA were added for 1 hour at room temperature. After washing 5 times with 0.05% Tween20 in PBS, detection was performed with 5 mg/mL 4-nitrophenylphosphat disodium salt hexahydrate (AppliChem, A1442) in AP-detection buffer (100 mM Tris, 10 mM $MgCl_2$*6 H_2O, 100 mM NaCl, pH 9,5) and signals were read at 405 nm.

Abbreviations

CCD	cross-reactive carbohydrate determinant
HBV	honeybee venom
HSA	human serum albumin

PBS phosphate-buffered saline
VIT venom immunotherapy
YJV yellow jacket venom

Disclosure of potential conflicts of interest

SB has received speaker's honorarium and/or travel support from ALK-Abelló, Bencard and Thermo Fisher Scientific; has received consultancy fees as an advisory board member and research support from Bencard. UD has been speaker, investigator and / or been a member of advisory boards for Allergopharma, ALK-Abelló, Bencard, GSK, Hermal, MEDA, Novartis Pharma, Stallergenes, Stiefel. MS has received travel support from ALK-Abelló. TB has received research funding, speaker's honorarium and consultancy fees from Thermo Fisher Scientific, has received research support from DFG, Novartis and Thermo Fisher Scientific, has received lecture fees from MSD, Novartis, HIPP GmbH & Co, ALK-Abelló, MedComms Ltd and Astellas Pharma GmbH. CBS-W has received grants from Allergopharma, Leti, PLS-Design and Regeneron; is member of the scientific advisory board of Leti and Bencard; has received consultancy fees from Leti, GLG Consultancy and Allergopharma; is board member of the Norwegian Research Council. MO has received consultancy fees from Siemens Healthcare, Hitachi Chemical Diagnostics and Bencard; has received lecture fees from Thermo Fisher Scientific, Bencard and Siemens Healthcare; is co-founder of PLS-Design GmbH. The other authors declare that they have no conflict of interest.

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Prevalence of Hymenoptera venom allergy and sensitization in the population-representative German KORA cohort

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Abstract
Purpose Allergic reactions to Hymenoptera venoms represent potentially life-threatening conditions. However, studies on their prevalence in Germany and their relation to specific IgE sensitization are rare. The aim of this study was to evaluate the prevalence of Hymenoptera venom allergy as well as the frequency of venom-specific IgE sensitization in a large population-based adult German cohort.
Methods Questionnaire data were collected from the participants of the German population-based KORA (Cooperative Health Research in the Region of Augsburg) S4 baseline study population ($n = 4261$) and the follow-up F4 study population ($n = 3074$), which was conducted seven years later. Moreover, sIgE antibodies to honeybee (HBV) and yellow jacket venom (HJV) as well as to common aeroallergens were measured in the S4 study population.
Results The prevalence of systemic sting reactions ranged between 2.3% and 2.6%. sIgE sensitization ($\geq 0.35 kU_A/L$) to HBV and YJV was demonstrated in 23.1% and 31.7% of the population, respectively (41.6% to HBV and/or YJV). Double-sensitization to both venoms occurred in 13.2% of the individuals. Approximately 53% and 77% of the individuals who reported shock symptoms after honeybee and yellow jacket stings, respectively, exhibited $sIgE \geq 0.35 kU_A/L$ to the culprit venom. In contrast, only 2.8% of the venom-sensitized individuals reported symptoms exceeding local reactions. Local reactions were reported by 4.4 to 4.8% of the population.
Conclusions Self-reported Hymenoptera sting reactions and venom sensitization are frequent in the general German population. In many cases, sensitization and clinically relevant allergy are not observed in the same individual, indicating that comprehensive diagnostic approaches are a prerequisite for the identification of patients at risk for severe reactions.

Keywords Anaphylaxis · Honeybee venom · Insect venom allergy · Specific IgE · Systemic reaction · Venom sensitization · Yellow jacket venom

S. Blank and S. Haemmerle contributed equally to this work.

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Abbreviations

BMBF	German ministry of education and research
CCD	Cross-reactive carbohydrate determinant
CI	Confidence interval
DZL	German Center of Lung Research
HB	Honeybee

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HBV	Honeybee venom
KORA	Cooperative Health Research in the Region of Augsburg
LIH	Luxembourg institute of health
LLRs	Large local reactions
NGFN	German national genome research network
OR	Odds ratio
RAST	Radioallergosorbent test
sIgE	Specific immunoglobulin E
tIgE	Total immunoglobulin E
VIT	Venom-specific immunotherapy
YJ	Yellow jacket
YJV	Yellow jacket venom
ZAUM	Center of allergy and environment

Introduction

Stings by Hymenoptera species are very common; 56.6–94.5% of the general population have been stung at least once in their lifetime [1]. The normal reaction to a Hymenoptera sting consists of pain and inflammation (swelling, redness and itching). Large local reactions (LLRs) at the site of the sting, which are characterized by a swelling with a diameter exceeding 10 cm and last for more than 24 h, occur in 2.4 to 26.4% of the general population [2]. However, in venom-allergic individuals already one sting can induce severe systemic reactions and even fatal anaphylaxis. Systemic reactions can involve cutaneous, respiratory or vascular symptoms or combinations thereof and less commonly might also affect the intestine, uterus and heart [2]. The prevalence of systemic sting reactions in adults ranges between 0.3 and 7.5% [2]. The estimated number of annual mortalities ranges from 0.03–0.45 per one million inhabitants [1]. However, this number could be underestimated as many fatal reactions following insect stings might remain undetected [3]. It was reported that in adults (>18 years) 48.2% and in children 20.2% of cases of severe anaphylaxis are caused by insect stings [4]. Of note, as these data are based on the reporting of physicians, many of whom are not familiar with the existence of such databases, this information might be strongly biased.

The diagnosis of systemic Hymenoptera venom allergy is based on a combination of the clinical history of a systemic sting reaction and the proof of sensitization by skin testing and/or the detection of venom-specific IgE antibodies (sIgE) in the serum of the patients [5, 6]. However, the value of a diagnosed sensitization alone is limited as between 9.3 and 28.7% of the population are sensitized to Hymenoptera venoms without previous clinical history of a sting reaction [5]. For these patients it is likely that the sensitization is of no clinical significance. However, the possibility of a reaction to a future sting cannot be fully excluded. To date, no indications are available on how to effectively manage these cases [7].

In Germany the most common elicitors of Hymenoptera venom allergy are honeybees (HB; *Apis mellifera*) and yellow jackets (YJ; *Vespula spp.*). Patients at risk for a severe reaction following a sting of these insects can be effectively managed by venom-specific immunotherapy (VIT) [6]. VIT is the only disease-modifying and curative treatment of venom allergy which is effective in minimizing the risk for a future severe sting reaction and to increase the patients' quality of life [8, 9]. VIT is reported to be effective in 77–84% of patients treated with honeybee venom and in 91–96% of patients receiving vespid venom [6]. In Germany, approximately one to three million Hymenoptera venom-allergic individuals might require VIT. However, only 20% of these patients receive the necessary therapy [10] despite the fact that insect stings are the most frequent trigger of severe anaphylaxis in adults [11]. Hence, it is of major importance to identify individuals that are at risk to develop severe reactions.

Large population-based studies that assess the prevalence of Hymenoptera-venom allergy in unselected populations are scarce. In this study we addressed sIgE sensitization to honeybee venom (HBV) and yellow jacket venom (YJV) of the participants of the population-based Cooperative Health Research in the Region of Augsburg (KORA) S4 baseline study ($n = 4261$). Moreover, questionnaire-based analyses of the S4 study population and the follow-up F4 study population ($n = 3074$) served to assess the prevalence of local and systemic reactions to insect stings.

Methods

Study population

The KORA study region consists of the city of Augsburg (Germany) and two surrounding districts with about 600,000 inhabitants in 1999 [12]. The KORA S4 baseline study (conducted between 1999 and 2000) involved 4261 participants recruited from a randomized two-stage cluster sample of 6640 individuals aged between 25 and 74 years with equal-sized distribution of sex and age strata (Fig. S1). The KORA F4 study was a follow-up of the S4 study conducted between 2006 and 2008 and involved 3074 participants. All participants gave their informed written consent and the studies were carried out in accordance with the declaration of Helsinki. The study was approved by the ethics committee of the Bavarian chamber of physicians.

IgE measurement

The sIgE levels to HBV (i1) YJV (i3) and aeroallergens (inhalant allergen screen SX1: d1, *Dermatophagoides pteronyssinus*; e1, cat dander; e5, dog dander; g6, timothy grass; m2, *Cladosporium herbarum*; t3, birch; w6, mugwort) as well as the total IgE (tIgE) levels were de-

termined using the Immulite 2000® platform (Siemens Healthcare Diagnostics, Los Angeles, CA, USA) [13].

Questionnaire

To identify insect venom allergy in the S4 study, the following questions were asked: "are you hypersensitive/allergic to insect stings?" (yes, no), "to which kind of insect?" (honeybee, yellow jacket, mosquito, others/don't know) and "what kind of reaction?" (reaction at the site of the sting, signs of shock, others/don't know). Moreover the participants were asked if they react to a second or third insect and, if so, to which insect and with what kind of reaction. Additionally, a history of atopic eczema, hay fever and asthma was recorded.

In the F4 study, the answers to the question "what kind of reaction?" were expanded to: "strong reaction at the site of the sting", "mild to moderate general reaction", "signs of shock" (severe general reaction) and "others/don't know". Moreover, the F4 study participants' age at which the reaction to a sting occurred for the first time was recorded.

Statistical analysis

All data were analyzed using the GraphPad Prism 6 software (San Diego, CA, USA). Gaussian distribution was tested by D'Agostino & Pearson omnibus normality test. The non-Gaussian distributed results were further analyzed by unpaired Mann–Whitney test. *P*-values of >0.05, ≤0.05 and ≤0.0001 are shown as ns, * and ****, respectively. Bivariate analysis was done by the chi-square test with Yates' correction. Odds ratios (OR) are given together with the 95% confidence intervals (CI).

Results

Total IgE levels in the S4 study population

The tIgE levels ranged between 1 and 15,356 kU/L (median 36.1) and were significantly higher in males (median 47.85) than in females (median 28.45; Fig. S2). Levels above 100 kU/L were found in 1023 subjects (616 male, 407 female).

Specific IgE antibody levels in the S4 study population

Using the cut-off of 0.35 kU_A/L, sIgE antibodies to HBV were present in 23.1% (cut-off 0.1 kU_A/L: 34.1%) and to YJV in 31.7% (44.8%) of the subjects (Fig. 1a). In all, 27% (36.6%) of the individuals were sensitized to aeroallergens (SX1). The sensitization rates (especially to HBV) were higher in males than in females (Fig. 1a) and 13.2% (23.8%) of the population were double-sensitized to HBV and YJV (Fig. 1b). The co-sensitization rates to aeroallergens and HBV or YJV were 10.3% (17.9%) and 11.7% (20.6%), respectively. Taken together, 41.6% (55.3%) of the studied subjects were sensitized to HBV and/or YJV.

Most of the positive test results with HBV and YJV were of class 0/1 (>0.1 to <0.35 kU_A/L) and 2 (≥0.71 to 3.5 kU_A/L) and only few of class 4 to 6 (≥17.51 to >100 kU_A/L; Fig. 1c). This distribution was the same for males and females (Fig. S3). For aeroal-

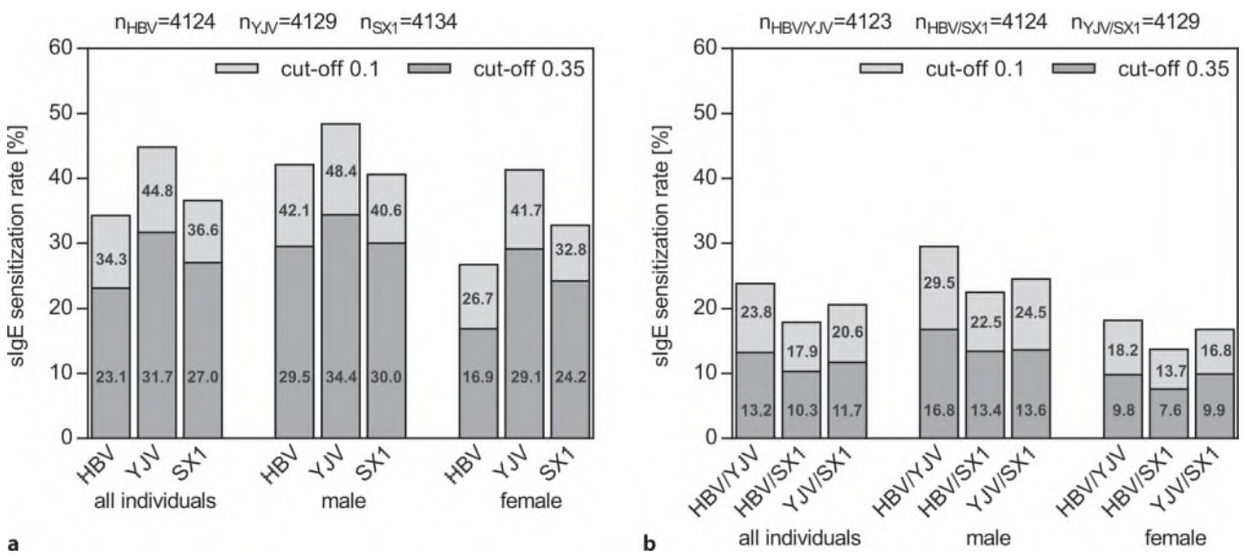

Fig. 1 Prevalence of sIgE sensitization in the KORA S4 baseline study population ($n = 4261$). **a** Prevalence of sIgE sensitization to honeybee venom (HBV), yellow jacket venom (YJV) and aeroallergens (SX1) in the whole study population, males and females. Shown are the thresholds of 0.1 and 0.35 kU_A/L. **b** Prevalence of double sIgE sensitization to HBV and YJV (HBV/YJV), HBV and aeroallergens (HBV/SX1) and YJV and aeroallergens (YJV/SX1)

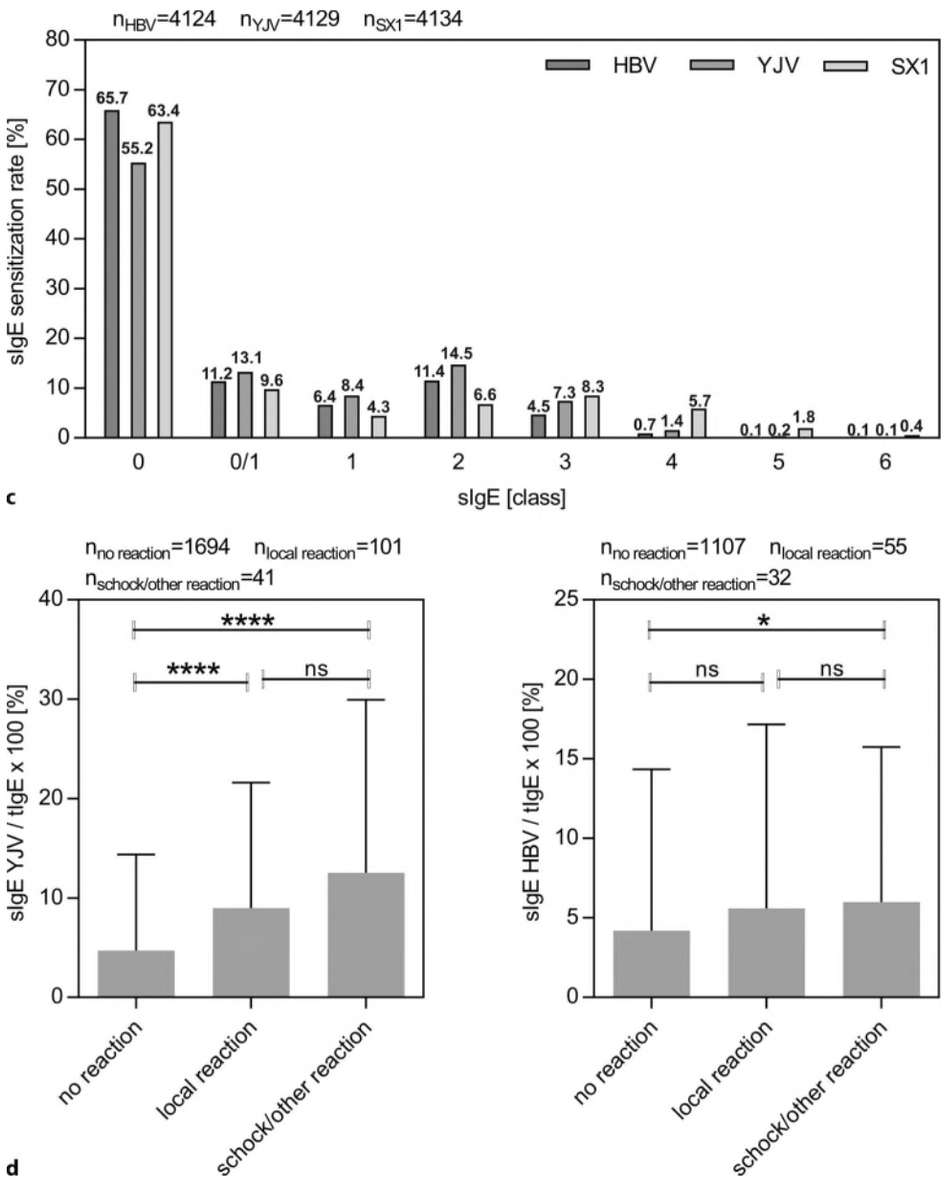

Fig. 1 Prevalence of sIgE sensitization in the KORA S4 baseline study population ($n = 4261$). **c** Distribution of the sIgE reactivity to the different arbitrary sIgE classes used in a clinical context (0: ≤0.1 kU_A/L, 0/1: >0.1–<0.35 kU_A/L, 1: ≥0.35–0.7 kU_A/L, 2: ≥0.71–3.5 kU_A/L, 3: ≥3.51–17.5 kU_A/L, 4: ≥17.51–50 kU_A/L, 5: ≥50.1–100 kU_A/L, 6: >100 kU_A/L). All percentages are rounded to one decimal place. **d** Percentage of sIgE directed against either YJV (i3) or HBV (i1) in relation to the tIgE level in YJV- (left) and HBV- (right) sensitized (>0.1 kU_A/L) individuals with no, local and shock/other symptoms to the respective venoms. Shown is the mean with standard deviation. * $p = \leq 0.05$; **** $p = \leq 0.0001$; *ns* not significant

lergens the most common classes were 0/1 (>0.1 to <0.35 kU_A/L) and 3 (≥3.51 to 17.5 kU_A/L).

Interestingly, in the YJV-sensitized (>0.1 kU_A/L) population, the percentage of sIgE in relation to the tIgE level was significantly lower in individuals without any reaction following a YJ sting compared to those reporting local or shock/other reactions (Fig. 1d, left). These differences were less pronounced in the HBV-sensitized population and only significant between individuals without any reaction and those reporting shock/other reactions following a HB sting (Fig. 1d, right).

Correlation of different parameters with the presence of venom-specific IgE antibodies

The presence of venom sIgE (>0.1 kU/L) was positively correlated with male sex, whereby sIgE to HBV showed a higher odds ratio (OR = 1.99, CI 1.74–2.27) compared to YJV sIgE (OR = 1.33, CI 1.18–1.50; Fig. 2). Elevated tIgE levels (>100 kU/L) were found to be strongly associated with sIgE to HBV (OR = 3.02, CI 2.61–3.49) and YJV (OR = 2.81, CI 2.43–3.24). Additionally, sIgE to aeroallergens (SX1) was correlated to sIgE to HBV (OR = 2.72, CI 2.38–3.11) as well as to YJV (OR = 2.07, CI 1.82–2.36). The presence of sIgE to HBV and YJV was not related to a positive history of asthma or atopic eczema. In contrast, a history of hay fewer was correlated with the presence of sIgE to HBV (OR = 1.264 CI 1.08–1.48), but not to YJV (OR = 1.03 CI 0.89–1.21).

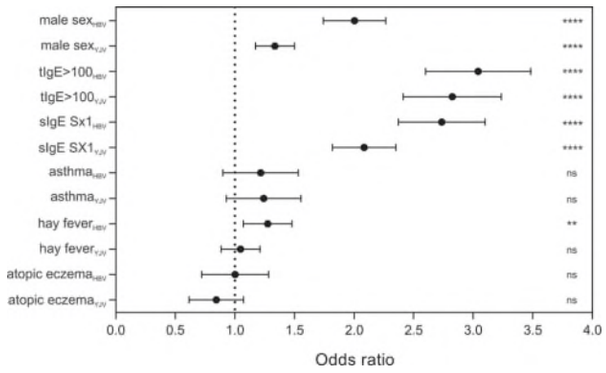

Fig. 2 Correlation of various parameters with the presence of honeybee venom (HBV)- and yellow jacket venom (YJV)-specific IgE. Considered positive were all sIgE levels above 0.1 kU_A/L. Shown are the odds ratios together with the 95% confidence intervals. *P*-values of >0.05, ≤0.05, ≤0.01, ≤0.001 and ≤0.0001 are shown as ns, *, **, ***, and, ****, respectively. *ns* not significant

Prevalence of hypersensitivity reactions to hymenoptera venom in the S4 study population

In the S4 survey, covering 4261 subjects, 6.9% ($n=287$) reported to be hypersensitive/allergic to HBV and/or YJV (Fig. 3a). Reactions to either HBV or YJV were reported by 4.0% ($n=165$) and 4.6% ($n=192$) of the participants, respectively. Participants who showed reactions to mosquito bites or to another/unknown insect (Fig. 3a) were excluded from the further analyses.

Unfortunately, the S4 survey questionnaire was vaguer with regard to the symptoms following the sting compared to the F4 questionnaire. Patients had the possibility to answer with "reaction at the site of the sting" (local), "signs of shock" (shock) and "others/don't know" (other; Fig. 3b). In the S4 study, local, shock and other symptoms were reported by 4.8% ($n=197$), 1.5% ($n=60$) and 0.8% ($n=34$) of the subjects, respectively. Local reactions were more common in females (6.4%; $n=135$) than in males (3.1%; $n=62$). Moreover, 1.0% ($n=21$) of males and 1.9% ($n=39$) of females suffered from shock symptoms after a HB and/or YJ sting. The distribution of shock symptoms to the particular insects was comparable. 0.8% ($n=34$) and 0.9% ($n=36$) of the participants stated HBs and YJs as culprit species, respectively (Fig. 3b).

Interestingly, only 1.8% and 2.8% of the HBV-sensitized (>0.35 kU/L) subjects stated to have had a shock and shock or other reactions following a sting, respectively. Out of the YJV sIgE-sensitized patients 1.6% and 2.8% reported shock and shock or other symptoms, respectively. Intriguingly, 30.3% and 18.8% of the participants who stated shock symptoms following a HB sting had sIgE to HBV of class 0 (≤0.1 kU_A/L) and class 0/1 (>0.1–<0.35 kU_A/L), respectively (Fig. 3c). Of the patient population reporting shock symptoms after YJ stings, 28.1% and 6.3% showed sIgE to YJV of class 0 and class 0/1, respectively.

Prevalence of hypersensitivity reactions to hymenoptera venom in the F4 study population

In the follow-up F4 study ($n=3074$) 6.8% ($n=210$), 3.5% ($n=110$) and 5.0% ($n=153$) of the subjects stated to be hyperreactive/allergic to HBV and/or YJV, HBV and YJV, respectively (Fig. 4a).

In the F4 survey, the answering options with regard to symptoms were more suitable to assess the prevalence of systemic reactions to Hymenoptera venoms since the participants had the possibility to answer "strong reaction at the site of the sting" (local), "mild to moderate general reaction" (general), "signs of shock" (shock) and "others/don't know" (other; Fig. 4b). The frequency of strong local reactions was very comparable to that of local reactions in the S4 study and was 4.4% ($n=134$), 2.7% ($n=40$) and 5.9% ($n=94$) for all, male and female subjects, respectively. Mild to moderate general reactions were reported by 1.4% ($n=44$) of the participants, signs of shock by 1.0% ($n=30$) and other symptoms by 0.2% ($n=6$). The frequency of general and shock symptoms was 1.6% ($n=25$) and 1.1% ($n=17$) in females and 1.3% ($n=19$) and 0.9% ($n=13$) in males. General and shock reactions to HBV occurred both in 0.5% ($n=7/8$) of males and in 0.8% ($n=12$) and 0.4% ($n=6$) of females, respectively. The prevalence of general and shock symptoms to YJV in males and females was 1.1% ($n=16$) and 0.4% ($n=6$) and 1.1% ($n=16$) and 0.8% ($n=13$), respectively (Fig. 4b). The prevalence of systemic reactions (mild to moderate general reactions and shock symptoms) to Hymenoptera (HB and/or YJ) stings was 2.4% ($n=73$) in the F4 study population (2.1% in males and 2.6% in females).

Systemic reactions occurred most frequently for the first time at the age range of 40 to 49 years (22.8%) followed by the age range of 50 to 59 years (20.3%). In the 10-year age ranges between 0 and 39 years the frequencies steadily increased (Fig. 4c).

Discussion

Studies assessing the prevalence of Hymenoptera venom allergy in the general adult population are relatively rare. Those studies that were performed in different European countries between 1992 and 2016 demonstrated a prevalence of self-reported systemic sting reactions ranging from 0.9 to 8.9% [14–19]. The observed divergent results regarding the prevalence might mirror differences in exposure depending on climate and activities [17], in the size of the study population or in the methodology using telephone interviews or different questionnaires. Many other studies were performed on selected patient populations such as factory workers or conscripts [20, 21]. However, in most of these studies the obtained preva-

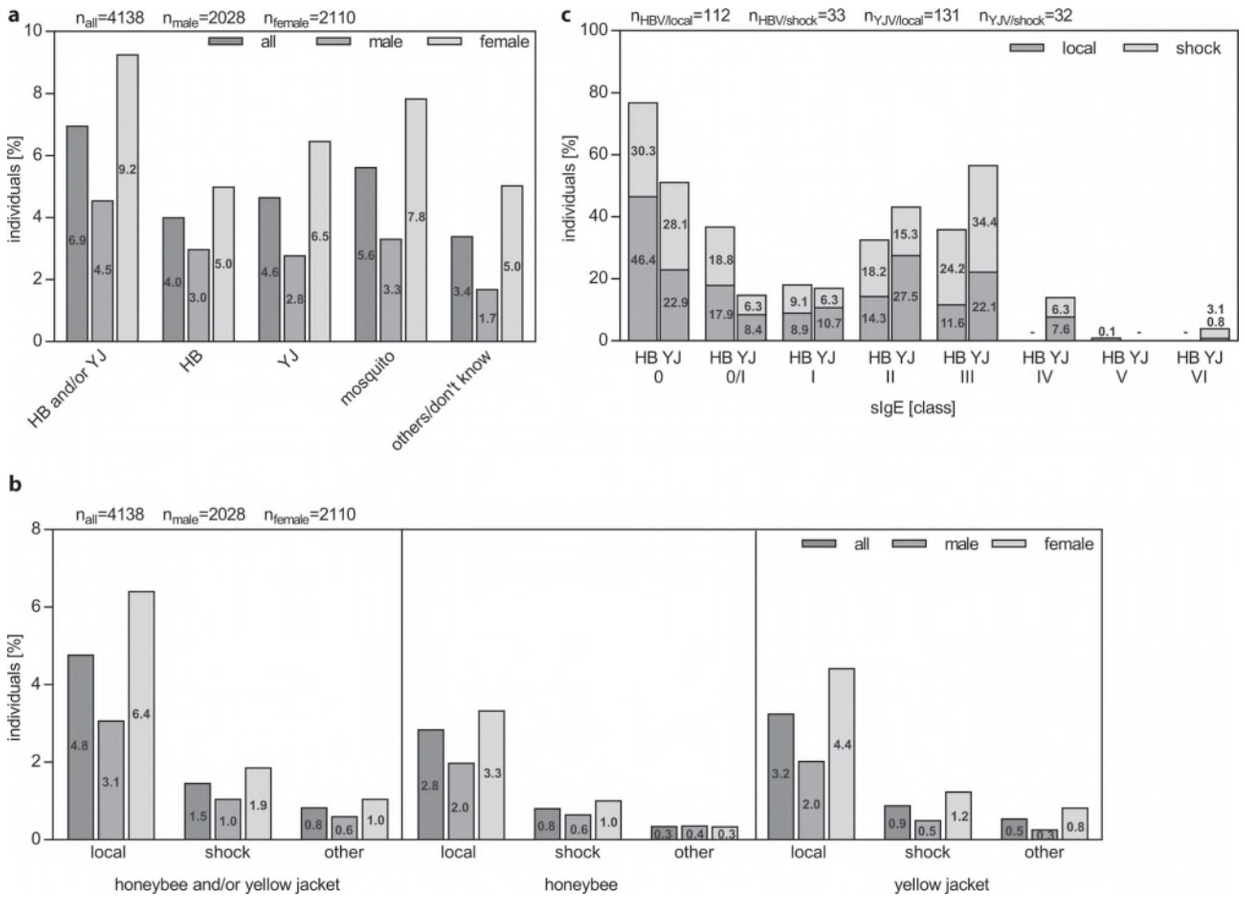

Fig. 3 Prevalence of symptoms to Hymenoptera stings in the KORA S4 baseline study population ($n = 4138$) and its relation to the level of sIgE. **a** Questionnaire-based prevalence of hyperreactivity and/or allergy to honeybee (HB) and yellow jacket (YJ) stings, mosquito bites and to other or unknown species. Reactions to mosquito bites and other/unknown species were excluded from the following analyses. **b** Prevalence of reactions at the site of the sting (local), signs of shock (shock) and other/unknown reactions (other) following a Hymenoptera sting. **c** Frequency of individuals with local reactions and signs of shock to HB and YJ stings in relation to their levels of sIgE to the culprit venom. All percentages are rounded to one decimal place

lence is in a comparable range to the general population. In the past, one study assessed the prevalence of insect venom allergy in the German adult population (German Health Interview and Examination Survey for Adults; $n = 8152$) by addressing reported medical diagnoses of insect venom allergy [22]. Here, a lifetime prevalence of 2.8% (3.6% for females and 2.0% for males) was identified for insect venom allergies. To our knowledge, only one study addressed the prevalence of Hymenoptera venom allergy in relation to venom sensitization in Germany so far [23]. This study covered 232 adults and 45 children from a rural area population out of whom approximately 3.9% of the adults reported a history of a systemic sting reaction. Sensitization to HBV or YJV was present in 24.1% of the adults (16.5% only to HBV, 2.2% only to YJV, 5.4% to both venoms).

In order to address the prevalence of Hymenoptera venom allergy in relation to venom sensitization in a larger German adult cohort, we performed questionnaire-based analyses of the population-based Cooperative Health Research in the Region of Augsburg (KORA) S4 baseline study population ($n = 4261$) as well as of the follow-up F4 study population ($n = 3074$). The questionnaire of the F4 study was clearly more suitable to assess the prevalence of systemic reactions as the participants had the possibility to discriminate between "strong reactions at the site of the sting", "mild to moderate general reactions", "signs of shock" and "other reactions/don't know". Taking mild to moderate and shock reactions to HBV and/or YJV together, covering systemic reactions, their prevalence is 2.4% (2.1% in males and 2.6% in females) whereby reactions to YJV (1.6%) are slightly more common than to HBV (1.1%). Strong reactions at the site of sting were reported by 4.4% of the participants which is a very assimilable range compared to the prevalence of large local reactions of 4.6% found in an Austrian study [15].

In the S4 study the participants only had the possibility to discriminate between "reaction at the site of

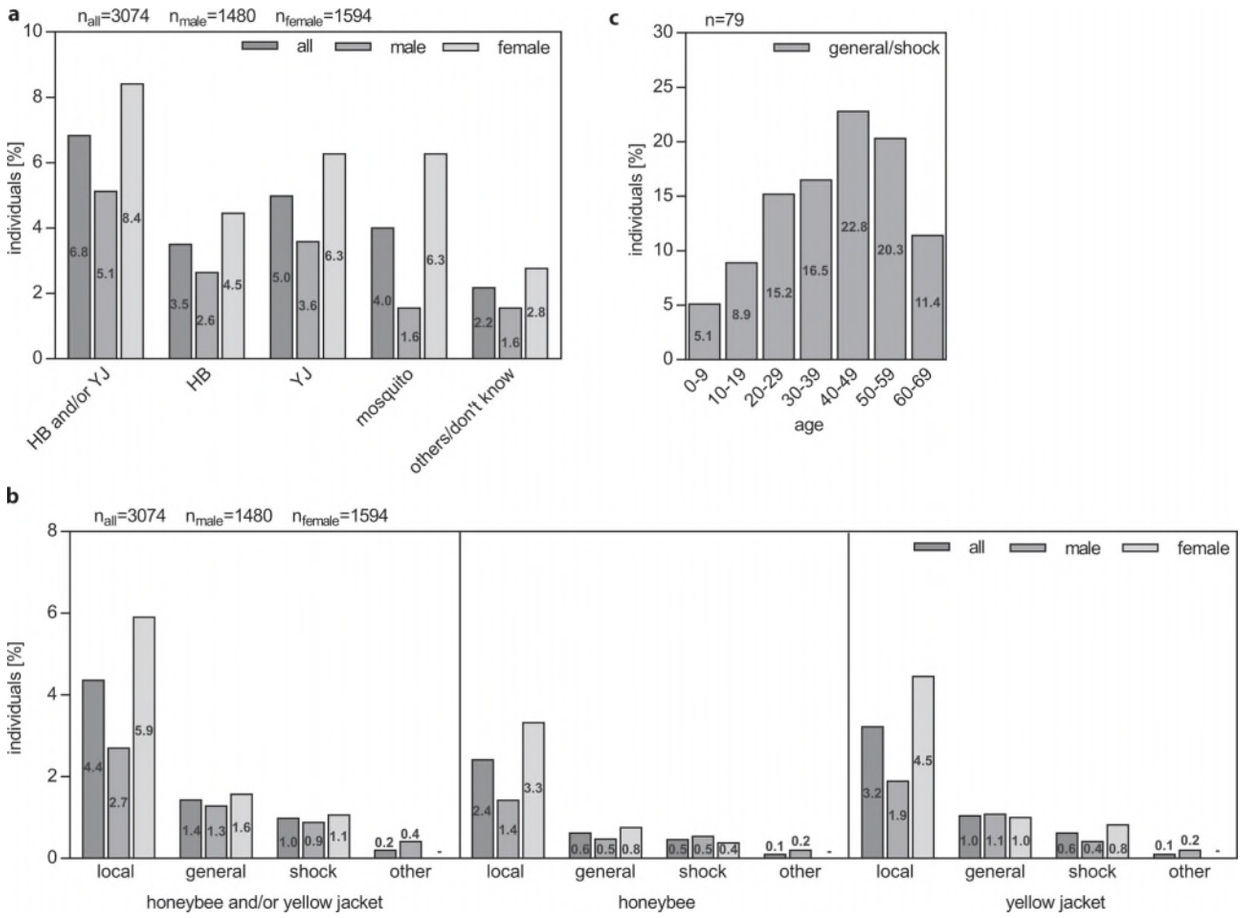

Fig. 4 Prevalence of symptoms to Hymenoptera stings in the KORA F4 follow-up study population ($n = 3074$). **a** Questionnaire-based prevalence of hyperreactivity and/or allergy to honeybee (HB) and yellow jacket (YJ) stings, mosquito bites and to other or unknown species. Reactions to mosquito bites and other/unknown species were excluded from the following analyses. **b** Prevalence of strong reactions at the site of the sting (local), mild to moderate general reactions (general), signs of shock (shock) and other or unknown reactions (other). **c** Proportion of subjects with general or shock reactions in relation to the age range in which the symptoms occurred for the first time. All percentages are rounded to one decimal place

the sting", "signs of shock" and "other reactions/don't know". This selection surely is less suitable to estimate the prevalence of systemic reactions as many participants suffering from mild to moderate systemic reactions most likely would have answered with "other reaction/don't know". This is also reflected by the fact that the percentage of participants giving this answer was much higher in the S4 than in the F4 study. However, estimating that many of the mild to moderate systemic reactions are represented in this group, the prevalence of systemic reactions in the S4 study is in a very comparable range to the F4 study. Local reactions were reported by 4.8% of the participants of the S4 study.

Taken together, the prevalence of self-reported systemic reactions to HBV and/or YJV in the German KORA cohort ranges between 2.3% (S4 study; shock and other reactions) and 2.6% (F4 study; mild to moderate, shock and other reactions). Reactions to YJV are slightly more common compared to HBV.

Applying the threshold level of 0.35 kU_A/L, as done in most other studies, 23.1% and 31.7% of the S4 study population were sensitized to HBV and YJV, respectively; 41.6% were sensitized to HBV and/or YJV and 13.2% to both venoms. Although sIgE to cross-reactive carbohydrate determinants (CCDs) could not be measured in this study, their contribution to the obtained venom sensitization rate seems to be negligible as indicated by the low prevalence of double-positive test results to HBV and YJV (as well as to venoms and aeroallergens). Other studies reported a prevalence of sensitization to Hymenoptera venoms (indicated by positive skin test and/or the detection of sIgE) in the range between 9.3% and 28.7% in adults [2]. A recent Danish study reported a prevalence of sIgE to HBV and YJV of 3.3% and 13%, respectively [17]. Of note, in addition to a varying degree of exposure in different areas, differences in sensitization rates might also result from the use of different assay platforms for sIgE detection. In this study, the Immulite 2000® plat-

form was used which reportedly leads to higher sIgE values compared to the ImmunoCAP® system, due to the different calibration approach that is used [24–28]. Another study that was conducted in Germany found a sensitization rate to Hymenoptera venoms of 27.1% [23]. In this study, the sensitization to HBV (24.8%) was much more common than to YJV (8.5%). However, this fact might be explained by the rural origin of the patient population on the one hand and by the lower sensitivity of the RAST (radioallergosorbent test) that was used in this study for sIgE detection to YJV on the other hand [23].

In general, atopy is not regarded as risk factor for venom allergy [23, 29, 30]. Here, we found no association between asthma or atopic eczema with the presence of venom sIgE and only a weak association between hay fever and sIgE to HBV (OR = 1.264) but not to YJV. In contrast, as shown previously by others [23], the association between the presence of venom sIgE and sIgE to common aeroallergens as well as to the level of total IgE could be clearly demonstrated. Moreover, male sex represented a stronger risk factor for the presence of sIgE to HBV compared to YJV. This might be explained by the fact that more males are working in professions associated with outdoor work such as foresters or gardeners and, hence, with a higher degree of exposure to HB stings.

Intriguingly, only 2.8% of the HBV- and YJV-sensitized patients reported shock or other reactions following a sting. In the study mentioned before, 7.1% of the positively tested patients stated a history of a systemic sting reaction [23]. However, it is not known if a certain fraction of the sensitized individuals would react to a future sting. Nevertheless, these results underline that venom sIgE-testing should not be a part of general health screenings [17]. Although venom-sensitized individuals suffering from systemic reactions exhibit a significantly higher percentage of venom sIgE in relation to their tIgE level, this parameter is likely not suitable to discriminate between sensitized individuals and those with clinically relevant allergy due to high variability within the population.

Of note, 30.3% and 18.8% of the participants who had shock symptoms following a HB sting had sIgE to HBV of class 0 (≤0.1 kU_A/L) and class 0/1 (>0.1–<0.35 kU_A/L), respectively. Of the patient population reporting shock symptoms after YJ stings, 28.1% and 6.3% showed sIgE to YJV of class 0 and class 0/1, respectively. This is in concordance with a former study reporting that only 38% of individuals with abnormal sting symptoms to any insect had sIgE ≥0.35 kU_A/L to HBV and/or YJV [17] and again illustrates the importance of a comprehensive approach for accurate diagnostics of Hymenoptera venom allergy including clinical history, sIgE measurement, skin tests and cellular tests. Moreover, future studies are needed to address the impact of the recently available component-resolved diagnostics [7, 31, 32] on this diagnostic gap on a population level.

In conclusion, the frequency of sensitization to Hymenoptera venoms in the general German population is high. However, a considerable gap exists between the presence of sIgE and a clinical history of venom allergy on the one hand and between systemic reactions and detectable sIgE on the other hand.

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Conflict of interest S. Blank reports non-financial support from ALK-Abelló, grants, personal fees and non-financial support from Bencard Allergie GmbH, personal fees from Teomed AG, personal fees from Thermo Fisher Scientific, grants from Allergy Therapeutics, outside the submitted work. In addition, S. Blank has a patent "Cloning of honey bee allergen C" licensed to Thermo Fisher Scientific. C.B. Schmidt-Weber reports grants and personal fees from Bencard, grants from Leti Pharma, grants and personal fees from Allergopharma, grants and personal fees from PLS-Design, outside the submitted work. In addition, C.B. Schmidt-Weber has a patent on diagnostic success prediction in AIT, which is pending. M. Ollert reports non-financial support from Siemens Healthcare Diagnostics, during the conduct of the study; personal fees from Thermo Fisher Phadia, personal fees from Siemens Healthcare Diagnostics, personal fees from Hitachi Chemical Diagnostics, personal fees from Hycor, outside the submitted work; and Scientific co-founder of the university biotech spin-off PLS-Design GmbH, Hamburg, Germany. S. Haemmerle, T. Jaeger, D. Russkamp and J. Ring declare that they have no competing interests.

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Mastocytosis as a risk factor for insect venom allergy

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The prevalence of anaphylaxis in mastocytosis patients (20-30%) is much higher than the estimated frequency of anaphylaxis in the general population (0.05–2%) The triggers that can induce massive degranulation of mast cells (MC) and cause anaphylaxis in adult subjects with mastocytosis are numerous, but hymenoptera stings are the most frequent (19-60% of cases of anaphylaxis), followed by foods (3-16% of cases) and drugs (5-9%) .
The literature confirmed that there is a preferential association between HVA and mastocytosis and that the prevalence of mastocytosis in patients with HVA is significantly higher than in the general population. Allergic/anaphylactic symptoms after hymenoptera sting are mostly present in patients with an indolent variant of systemic mastocytosis (SM) without skin lesions where they represented the initial clinical manifestation and the reason for bone marrow (BM) biopsy in the majority of cases.
Therefore, patients with both diseases represent a population requiring specific management.

During the last few years it has increasingly been seen that there is a preferential association between HVA and mastocytosis for several reasons:

- The prevalence of HVA in SM patients (20-30%) is higher than in the general population (0.3%-8.9% in the European adults population)
- The hymenoptera venom sting represents the most common trigger of anaphylaxis in adult mastocytosis patients (22 - 60 % of cases)
- The association between HVA and mastocytosis is also confirmed by the higher prevalence of CMD in patients with systemic HVA (1-7.9%) (see Table 1) than in the general population (1-1.3 cases per 10,000) .The lower prevalence rate of CMD in patients with HVA reported in some studies could be explained by the low sensitivity of the screening test used , by the lack of a BM evaluation or some sensitive BM diagnostic tests

Clinical features of patients with HVA and Clonal Mast Cell disorders of patients with HVA and CMD

- In the past, diagnostic work-ups for SM in patients with HVA have usually been limited to evaluating the presence of Maculo-papular Cutaneous Mastocytosis (MPCM) or Urticaria. Instead, in later years, it has been shown that HVA is more frequently reported in SM patients without skin involvement .This is a very crucial point because if we focus attention on skin lesions only, there is a risk of not diagnosing a high percentage of SM.
- The CMDs associated with HVA are represented other than SM, by monoclonal MC activation syndromes (MMAS), characterized by the absence of skin lesions and the demonstration of BM MC clonality by detection of KIT D816V Kit mutation and/or abnormal expression of Cd25 and/or CD2 on MC, but lacking sufficient criteria for SM
- An increased SBT appears to be a useful criterion for selecting patients with HVA eligible for BM evaluation when SM is suspected ; nevertheless a CMD cannot be excluded in subjects with systemic severe HVA but with normal SBT.The REMA Score, proposed by the Spanish group, identified four clinical elements (male sex, presyncopal and/or syncopal episodes, absence of urticarial/angioedema, and serum tryptase >25 ng/mL) as independent predictive factors of CMD in patients suffering from severe mediator symptoms without mastocytosis in the skin .The application of this score, which shows high sensitivity (91%)

and specificity (75%), provides a good tool for screening patients with suspected mastocytosis with HVA but without typical skin lesions.

- The characteristics of patients with HVA and ISMs(-) are the prevalence of male sex, a significantly lower MC burden, lower levels of serum tryptase and lower frequency of dense compact MC aggregates in BM sections than in ISM skin positive [ISMs(+)] patients. They also frequently show coexisting populations of phenotypically normal and aberrant MC in BM and a lower frequency of multilineage KIT mutation
- The anaphylactic reactions of patients with CMD and HVA are characterized in most of cases by the absence of angioedema and erythema and the predominance of cardiovascular symptoms, such as hypotension leading to loss of consciousness The majority of patients do not report MC activation symptoms between acute episodes; therefore most of these patients may have HVA severe reactions as the unique clinical manifestations of mastocytosis.
- Progression to aggressive mastocytosis has not been yet reported in SM patients with HVA and, on the contrary, HVA seems to be very rare in patients with the aggressive subtypes of SM, who harbor the highest mast cell load .
- In order to minimize the risk of failure in identifying a CMD in patients with normal or very slightly increased SBT and very low MC burden the technical approach used is very important. In these cases very sensitive techniques for BM MC immunophenotyping and detection of the KIT-D816V mutation (as RT-qPCR) are needed .

Immunotherapy

There is no preventive pharmacological treatment available for HVA. Venom Immuno-Therapy (VIT) represents a safe and effective treatment, that decreases the risk of subsequent systemic reactions and reduces morbidity and mortality ..

The only curative treatment which is effective in reducing the risk of subsequent systemic reactions and improving patients' quality of life is VIT. VIT in the general population is reported to be effective in 77-84% of patients treated with honeybee venom and in 91-96% of patients receiving vespid venom .

After some debate, which were mainly due to safety concerns, it is now generally accepted that VIT is clinically justified in those patients with severe HVA and documented mastocytosis .In fact it is now generally accepted that VIT should be given always. Based on the data of literature available up to now VIT conferred full protection in the majority (86%) of re-stung mastocytosis patients, although this percentage is slightly smaller than that reported in patients without SM .

According to the published case series, conventional, cluster and rush protocolsare well- tolerated and effective in patients with SM associated with anaphylaxis to hymenoptera venom- induced anaphylaxis.

In patients with HVA and SM not fully protected at field re-stings, an increase of the maintenance dose to 200 mcg venom can be recommended. Before increasing the dose, it is mandatory to ensure that the diagnosis is correct and to exclude a new sensitization. (Bonadonna 2016).

Furthermore, in mastocytosis patients a pretreatment with an H1 antihistamine can be used in order to reduce the number and severity of LLRs and mild SRs to VIT, such as urticaria and angioedema. More recently, several case reports have shown that pretreatment with anti-IgE monoclonal antibodies may permit more rapid and higher doses of allergen immunotherapy: ISM patients who experienced SRs to VIT were able to tolerate immunotherapy following pretreatment with omalizumab .

In the normal HVA population, the literature confirmed that a minimum of a five-year treatment is better for long-term effectiveness and life-long therapy should be considered in patients with severe initial SSR, systemic adverse events during VIT, and honeybee venom allergic patients with high risk of future honeybee stings. Patients with mastocytosis and HVA, who were protected during VIT, may have very severe reactions after VIT discontinuation

Moreover, the probability of having mastocytosis (in any form) is quite high when VIT protection is

lost after treatment. This would suggest that patients with HVA-induced anaphylaxis who lose protection after a proper course of VIT should be investigated for mastocytosis. When a diagnosis of mastocytosis is established these patients should continue life-long VIT;[therefore from a practical point of view, regardless of the tryptase value, it has been suggested that an accurate haematological work-up be performed before stopping immunotherapy in those patients with very severe reactions with hypotension and without urticaria and angioedema in order to exclude CMD. In general, in order to improve the compliance of patients in the HVA population who have to continue life–long injections, a 3-4 month extended interval can be proposed, and this schedule, adopted after 5 years of immunotherapy, seems to be safe and effective . We can hypothesize that mastocytosis patients can also adopt this schedule even if up to now there have been no studies about the efficacy and protection in case of re-sting.

All VIT-treated mastocytosis patients, even in the maintenance phase, should carry epinephrine self-injectors with them, because of the persistent risk of SSR and the possibility that SSR may also occur after a sting of an insect whose venom was not used for VIT [.

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The additional value of cellular tests in insect venom allergy

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Keywords: Basophils, mast cells, CD63, CD203c, basophil activation test (BAT), mast cell activation test (MAT), anaphylaxis, insect venom allergy, honeybee, wasp, diagnosis, immunotherapy monitoring, systemic adverse events, severity of sting reaction,

INTRODUCTION

Cellular tests have become a pervasive test for allergic response through the development of flow cytometry, discovery of activation markers such as CD63 and unique markers for identifying basophils (CCR3 and CD123+/HLA-DR-), and a recent development of mast cell activation test (MAT) (1-3). The impact of cellular tests is due to the unique ability of effector cells (basophils and mast cells) to degranulate upon cross-linking of the specific IgE (sIgE) bound on membrane-bound high affinity IgE-receptor (FcεRI) by allergen exposure. CD63 is a membrane protein localized to the same secretory granule that contains histamine. Translocation of CD63 to the cell membrane during degranulation can be measured by flow cytometry and this surface up regulation correlated with the anaphylactic degranulation. Other activation markers, like CD203c correlated with the basophil or mast cell piecemeal degranulation.

At the moment basophil activation test (BAT) with CD63 is clinically the best validated test (1). It is preferably performed on peripheral whole blood samples, and for stimulation we mainly use allergen extracts, although recombinants also working very well. Recent BAT developments are focused on automation, expansion and validation, preferably for allergies to foods and drugs. However, the feasibility of BAT is challenging because it requires fresh blood and from 5% to 15% of individuals have uninterpretable BAT results caused by nonresponding basophils (basophils do not respond to IgE-mediated, but only to non–

IgE-mediated stimulants). To possibly resolve some of this obstacles, MAT was recently developed in which primary human blood-derived mast cells or LAD2 mast cells are passively IgE sensitized with patients sera, stimulated with allergen and then analysed for CD63 response (2-3). MAT was especially useful in subjects with nonresponding basophils, but the overall sensitivity was lower than for BAT, particularly because of lower sensitivity in patients with lower sIgE levels (classes 1 and 2; <3.5 KU_A/L) (3).

Presentation and interpretation of cellular test results

Overall, cellular tests measure the quality i.e. the allergenic activity of the specific IgE antibodies for cross-linking and thus activation of basophils or mast cells at different allergen concentrations. In case that the test is performed on whole blood (and thus with patients basophils) different cofactors might moderate the basophil response including blocking and/or inhibitory IgG antibodies and patients cellular specific factors like the effectiveness of FcεRI signaling pathway. For some normalization and better comparison between individuals we recently start to use CD63 ratio, which represents the ratio of the percentage of CD63 basophils at allergen stimulation to the percentage of CD63 basophils after stimulation with anti-IgE or anti-FcεRI (4).

There are two common measures of BAT; basophil reactivity, the number of basophils that respond to a given stimulus, and basophil sensitivity, which is depending on allergen concentration at which basophils started to respond. Basophil sensitivity requires measurement at least 4, preferably log allergen concentrations. The graded response to allergen is fitted to a curve of reactivity versus allergen concentration, and the eliciting concentration at which 50% of basophils respond (EC50) is determined. EC50 can be expressed as ‘CD-sens’ by inversion and multiplication by 100 (1). More recently, the area under the dose curve (AUC) combine reactivity and sensitivity into one and can be calculated even in cases where responses do not fit well to a typical dose–response curve (1).

CLINICAL APPLICATIONS

Patients with negative standard venom diagnostic tests

A subset of patients (4–6%) with a history of systemic reactions after insect stings have negative venom-specific IgE and skin test results. These patients can subsequently experience another severe or even fatal reaction to an insect sting. In fact, in cases of fatal sting anaphylaxis, venom-specific IgE are very low or even undetectable in more than 30% of patients. Diagnostic sting provocation tests are considered as unethical for such cases. BAT allows the identification of approximately two-thirds of those patients and thus the introduction of the VIT (5-7). Some of those patients are also positive for venom recombinants (8). In patients with systemic mastocytosis and negative standard venom diagnostic tests, diagnosis of venom allergy with BAT should be performed with care (9).

Patients sensitized to bee and wasp venom ‘double positivity’

Up to 60% of the patients with insect venom allergy have sIgE to both bee and wasp venom. It is important to identify the relevant venom for VIT, especially if the patient has had an anaphylactic reaction to only one insect. Furthermore, the majority of double positive patients have a clinically apparent allergy to only one venom. The double positivity might be due to a true double sensitization to both venoms, irrelevant recognition of cross-reacting carbohydrate determinants or cross reactivity between homologous venom proteins. BAT repeatedly shows a positive result to only one venom in about one-quarter to one-third of patients with double positivity (10). In such cases a positive BAT can identify the primary sensitizing allergen. In the case of patients with double-positive BAT, the venom to which the patient is markedly more sensitive might represent the primary sensitizing allergen (11). Unfortunately, this breakthrough concept was not further evaluated, and currently there is no cut-off values which could distinguish between high and low BAT venom responses. The positioning of BAT in the of double positivity issue is currently emerging because recent publication clearly demonstrated that more than 70% of double positive patients are also double positive with recombinant venom components (12, 13). Since the rate of asymptomatic sensitization

in insect venom allergy, a currently unexplained aspect of the disease, is high and clinical double reactivity to apidae and vespidae is rare, it seems that the measuring of the recombinant IgE sensitization profile is not a sufficient tool to infer the clinical relevancy of double positivity. Further research of cellular tests in this field are thus obviously needed.

RESEARCH APPLICATIONS

Monitoring the efficiency and safety of venom immunotherapy

A clear decrease in basophil sensitivity is found up to 4 years after initiation of VIT, without a change in basophil reactivity (1). A previous report about an 8-year follow-up of patients submitted to VIT showed that the decrease in basophil sensitivity seemed to be also associated with the induction of tolerance (14). Some studies suggest that side-effects during the build-up phase of VIT are predicted by a high basophil sensitivity (15). The utility of BAT as the tool of choice to monitor the effect of VIT should be further developed and explored.

Prediction of severity of sting reaction

A major future perspective of cellular tests is the potential to distinguish the severity and threshold of allergic reactions and thus significantly improved personalized allergy management. This potential was recently demonstrated in peanut allergy, both for BAT (4) and MAT (2, 3). Moreover, first clinical trial of BAT as predictor of of severity of peanut allergy was recently started. Similar studies are urgently needed also in the field of insect venom allergy. Notably, there is currently no reliable laboratory test or biomarker which can predict the severity of initial, previous, or future allergic sting reactions and thus the clinical management decision on which patients should be treated relies only on clinical data.

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Invited review article

Mast cells and IgE in defense against venoms: Possible "good side" of allergy?☆

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a r t i c l e i n f o

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Abbreviations:
ABS, rabbit anti-basophil serum; AES, rabbit anti-eosinophil serum; BMCMCs, bone marrow-derived cultured mast cells; BV, honeybee venom; bvPLA$_2$, honeybee venom phospholipase A$_2$; CPA3, carboxypeptidase A3; CBH, cutaneous basophil hypersensitivity; DNP, dinitrophenol; DNP-HSA, dinitrophenol-conjugated human serum albumin; ESCMCs, embryonic stem cell-derived cultured mast cells; ET-1, endothelin-1; F(ab), antigen-binding fragment of an immunoglobulin molecule; FcεRI, the high affinity receptor for IgE; IgE, immunoglobulin E (antibody); IgG, immunoglobulin G (antibody); IL, interleukin; ILC2, innate lymphoid cells, type 2; i.d., intradermal; i.p., intraperitoneal; i.v., intravenous; LPS, lipopolysaccharide; MC(s), mast cell(s); Mcl-1, myeloid cell leukemia 1; MCP4, mast cell protease 4; NRS, normal rabbit serum; PAMPs, pathogen-associated molecular patterns; RVV, Russell's viper venom;

a b s t r a c t

Physicians think of mast cells and IgE primarily in the context of allergic disorders, including fatal anaphylaxis. This 'bad side' of mast cells and IgE is so well accepted that it can be difficult to think of them in other contexts, particularly those in which they may have beneficial functions. However, there is evidence that mast cells and IgE, as well as basophils (circulating granulocytes whose functions partially overlap with those of mast cells), can contribute to host defense as components of adaptive type 2 immune responses to helminths, ticks and certain other parasites. Accordingly, allergies often are conceptualized as "misdirected" type 2 immune responses, in which IgE antibodies are produced against any of a diverse group of apparently harmless antigens, as well as against components of animal venoms. Indeed, certain unfortunate patients who have become sensitized to venoms develop severe IgE-associated allergic reactions, including fatal anaphylaxis, upon subsequent venom exposure. In this review, we will describe evidence that mast cells can enhance innate resistance to reptile or arthropod venoms during a first exposure to such venoms. We also will discuss findings indicating that, in mice which survive an initial encounter with venom, acquired type 2 immune responses, IgE antibodies, the high affinity IgE receptor (FcεRI), and mast cells can contribute to acquired resistance to the lethal effects of both honeybee venom and Russell's viper venom. These findings support the hypothesis that mast cells and IgE can help protect the host against venoms and perhaps other noxious substances.

☆ This review is a modified and updated version of a similar invited review that will appear in the American Journal of Pathology: Galli SJ. The 2014 Rous-Whipple Award Lecture. The mast cell-IgE paradox: From homeostasis to anaphylaxis. Am J Pathol. In press.

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s.c., subcutaneous; shRNA, small hairpin RNA; Th2, T helper cell type 2; VIP, vasoactive intestinal polypeptide; WT, wild type

Mast cells, basophils and IgE in the pathology of allergic disorders

Allergies, that currently afflict 20–30% of people worldwide, are detrimental immune responses against any of a large variety of environmental antigens.[1] Such antigens (called allergens) share the ability to elicit acquired type 2 immune responses which are orchestrated by $CD4^+$ T helper type (Th)2 cells and include the production of allergen-specific IgE antibodies.[2–4] In such Th2 cell-associated "type 2" immune responses, IgE enables antigen-specific function of effector cells by binding to high affinity receptors for IgE (FcεRI) on the cells' surface.[5,6] FcεRI are expressed on mast cells, that reside in most vascularized tissues in mammals and other vertebrates, and on basophilic granulocytes ("basophils"), that ordinarily circulate in very low numbers in the blood but which can be recruited to sites of inflammation.[3,5–10]

When mast cell- or basophil-bound IgE antibodies recognize antigens that are at least bivalent, rapid aggregation of the FcεRI initiates complex intra-cellular signaling pathways. This ultimately results in the release, by such activated effector cells, of a wide variety of mediators with diverse biological effects.[5,6,8–11] Some of these mediators are stored in the cells' cytoplasmic granules, ready for immediate release, including, in mast cells, histamine, heparin and other proteoglycans, proteases such as carboxypeptidase A3 (CPA3), tryptases and chymases, and some cytokines; in addition, products of arachidonic acid metabolism (via the cyclo-oxidase or lipoxygenase pathways; e.g., prostaglandins and cysteinyl leukotrienes) and a diverse group of cytokines, chemokines and growth factors are secreted after upregulation of their transcription as a result of FcεRI–dependent cell activation.[3,5–7,12,13] Basophils activated via FcεRI aggregation can release a panel of mediators partially overlapping with those of mast cells, but, as compared to mast cells, they contain much lower amounts of proteases and appear to produce fewer cytokines and chemokines.[8–10]

Innate activation of mast cells

In addition to IgE and specific antigen, many stimuli can activate at least some mast cell populations via innate mechansims, including products of complement activation (e.g., C3a, C5a), products of pathogens (e.g., LPS and other pathogen-associated molecular patterns [PAMPs]), certain cyokines or growth factors (including IL-33 and the Kit ligand, stem cell factor), products of other hematopoietic cells, certain endogenous peptides (including endothelin-1 [ET-1] and vasoactive intestinal polypeptice [VIP]), and components of the venoms of many different vertebrates and invertebrates.[10,14–18] Within or among different mammalian species, individual mast cell subpopulations can vary in their susceptibility to activation via these innate mechanisms, likely reflecting such factors as microenvironmentally regulated differences in levels of expression of the cognate receptors.[14,19] Also, various stimuli can differ in their ability to elicit the release of granule-stored, lipid, or cytokine mediators. For example, certain peptides such as substance P can activate some mast cell populations to robustly release the granule-stored mediators; however, compared to the same cells activated via the FcεRI, such stimuli may less potently elicit release of lipid mediators or cytokines.[14,20,21] In contrast, for at least some mast cell populations, PAMPs are more effective in eliciting release of cytokines and chemokines than granule-stored mediators.[16,17] Because mast cells or basophils particpating in innate or adative immune responses may encounter simultaneously or sequentially several different stimuli of activation, it may be difficult to predict which mast cell- or basophil-derived mediators will be released and in what amounts in these settings, and even more challenging to guess what the net effects of all such mediators might be during that particular biological response.

Possible beneficial functions of mast cells, basophils and IgE

It is now generally accepted that mast cells and basophils can contribute importantly to the pathology associated with allergic disorders, including potentially fatal anaphylaxis.[3,22,23] Yet the evolutionary advantages which might be conferred by IgE, mast cells and basophils have been more difficult to define. A major hypothesis about the potential "beneficial functions" of such allergic effector mechanisms is that IgE-associated type 2 immune responses contribute to host defense against helminths and certain other parasites.[4,24–26]

It should be noted, however, that it has been challenging to prove that IgE, mast cells or basophils dramatically influence the survival of parasite-infected animals. Abnormalities in host responses to certain parasites have been observed in mice that genetically lack IgE,[27,28] mast cells,[29–33] or basophils[28,33] but such studies generally have not included an analysis of the effects of those deficiencies on the overall survival or reproductive success of the infected hosts. And some findings even suggest that, in certain settings, IgE or mast cells may have effects during host responses to parasites (e.g., effects which direclty or indirectly result in increased parasite egg production) that may favor the parasite rather than the host.[34–36]

The complexity of the relationships between parasites and their hosts is not surprising, given that vertebrates have been co-evolving with such parasites for millions of years. It therefore also is not surprising that, depending on the parasites and the particular setting, immune effector mechanisms such as IgE, mast cells and basophils might be exploited by the parasites to their own advantage. For example, one can speculate that by eliciting a type 2 immune response that results in IgE-dependent mast cell activation and release of vasoactive mediators in response to parasite antigens at sites of parasite infection, the parasite could influence local blood flow and vascular permeability in ways that enhance the parasite's nutrition.

In contrast to parasites, most allergens do not represent a direct threat to the non-sensitized host. This is why such type 2 immune responses are widely considered to be "misdirected" or "maladaptive" immune responses.[37,38] However, Margie Profet proposed a radically different notion in 1991, based in part on the observation that the common feature of most allergens is their origin from sources such as seafood, nuts, or venoms which either might contain toxins (e.g., foods) or always do (e.g., venoms).[39] Profet proposed that acute allergic reactions, manifested as immediately occurring symptoms in response to allergen exposure, such as sneezing, coughing, vomiting and diarrhea, evolved as defense mechanisms allowing the sensitized host to respond immediately to, and to expel, neutralize and/or avoid, noxious substances which

might be indicative of potentially life-threatening situations.[39] Even before Profet's 1991 paper, James Stebbings, Jr. hypothesized that "a major function of the immediate hypersensitivity reactions has been the protection of terrestrial vertebrates from the bites of, or invasion by, arthropods".[40] However, until recently,[41] Profet's "toxin hypothesis" was largely ignored by the scientific community; Stebbings' paper was even more neglected.[42]

Evidence for a beneficial role of basophils in acquired immunity to the feeding of ixodid ticks

Galli et al. generated a rabbit anti-guinea pig basophil antiserum (ABS) and showed that intravenous (i.v.) administration of ABS markedly reduced numbers of blood basophils in vivo, without reducing numbers of blood eosinophils or the total white blood cell count, or changing numbers of intradermal mast cells.[43] ABS did cause a reduction in skin mast cell numbers if injected intradermally, raising the possibility that the antiserum targeted an antigen shared by basophils and mast cells. Administration of this ABS i.v. to guinea pigs sensitized to express "cutaneous basophil hypersensitivity" (CBH), an antigen-induced, delayed onset, erythematous skin reaction that contains large numbers of infiltrating basophils,[44] revealed that ABS could be used to reduce markedly the numbers of basophils infiltrating the skin at such sites.[43]

Brown et al. then used this rabbit ABS, and a rabbit anti-eosinophil serum (AES), to investigate whether basophils or eosinophils contributed to adaptive immune response that diminish the feeding success of larval Amblyomma americanum ticks.[45] A. americanum (the Lone star tick) is an ixodid tick that is a vector for a variety of diseases including Rocky Mountain spotted fever (Rickettsia rickettsia), Q fever (Coxiella burnetii), tularemia (Francisella tularensis), granulocytic ehrlichiosis (Ehrlichia ewingii), monocytotropic ehrlichiosis (Ehrlichia chaffeensis), and others.[46] Moreover, Platts-Mills and colleagues recently demonstrated that the bites of this tick can induce the development of IgE antibodies to a tick carbohydrate, galactose-α-1,3-galactose, that is also present in non-primate meat and meat products, sensitizing such individual to develop a delayed form of urticaria or anaphylaxis after they consume such meats or meat products[47] or in response to treatment with a therapeutic antibody containing this carbohydrate.[48]

It was known that when guinea pigs subjected to a single round of exposure to the feeding of larval A. americanum ticks are challenged with a second exposure to the larval ticks about a month later, tick feeding success is markedly diminished, and such acquired resistance can be transferred by administering serum from sensitized to naïve guinea pigs.[49] It also was known that tick feeding sites in sensitized guinea pigs contained large numbers of basophils and eosinophils.[50] Brown et al. found that treatment of tick-sensitized guinea pigs with rabbit ABS markedly diminished basophils in the bone marrow, blood, and skin at sites of tick feeding of the sensitized guinea pigs, and also resulted in diminished numbers of eosinophils at such feeding sites.[45] Notably, ABS treatment also essentially ablated the acquired resistance to tick feeding conferred by sensitization (Fig. 1)[45]. Treatment with rabbit AES had a lesser, but still statistically significant, effect on tick feeding success, whereas normal rabbit serum was without effect (Fig. 1).[45]

To our knowledge, this was the first in vivo experimental evidence that went beyond correlative and observational studies (i.e., demonstrating the presence of basophils at tick feeding sites) indicating that basophils might represent one important component of acquired, antibody-dependent resistance to the feeding of an arthropod. Years later, it was possible to pursue similar experiments in mice genetically deficient in either mast cells or basophils. In convincing studies, Hiroshi Matsuda and Yukihiko Kitamura and colleagues (using mast cell-engrafted genetically mast cell deficient (WB/Re-W/+ X C57BL/6-W^v/+)F_1-"W/W^v" mice),[51] and Hajime Karasuyama and colleagues (using mice genetically deficient in basophils[52]), provided evidence that both mast cells and basophils, as well as IgE, can contribute to acquired immunity to the feeding of Haemaphysalis longicornis ticks in mice. By contrast, it appears that basophils may have a more important role than mast cells in acquired resistance to the feeding of larval Dermacentor variabilis ticks in mice.[53]

"Mast cell knock-in mice"

Yukihiko Kitamura and colleagues discovered that (WB/Re-W/+ X C57BL/6-W^v/+)F_1-"W/W^v" mice (now known as WBB6F_1-Kit^W/Kit^{W-v} mice, since "W" later was shown to encode c-kit[54,55]) not only

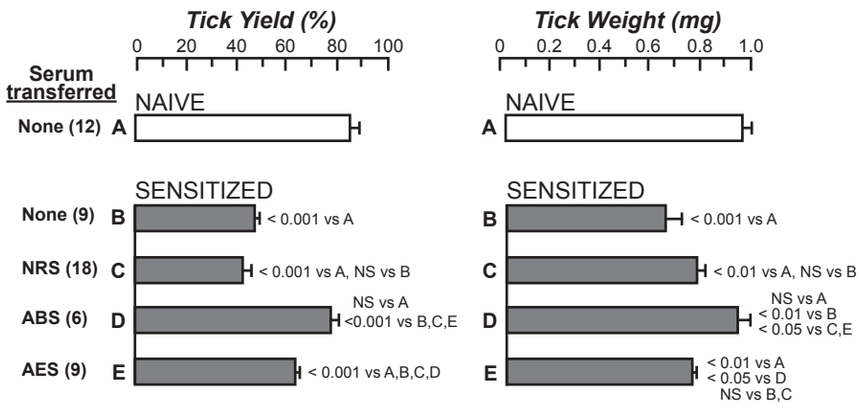


Fig. 1. Effects of treatment with anti-basophil serum (ABS), anti-eosinophil serum (AES) or normal rabbit serum (NRS) on feeding success of larval Amblyomma americanum ticks in a second infestation of guinea pigs. One hundred larval Amblyomma americanum ticks were placed on the flanks of "nonsensitized" (naïve) guinea pigs or guinea pigs which had been "sensitized" 26 days earlier by a primary infestation of A. americanum. The naïve guinea pigs (A) and one group of sensitized hosts (B) received no serum, other sensitized animals were treated with NRS (C), ABS (D), or AES (E) as described in[45]. The number (Tick Yield, left) and weight (Tick Weight, right) of engorged ticks was determined at 90 h of infestation. ABS completely ablated immunity; AES partially impaired resistance; NRS had no effect. Data (mean + SEM) were pooled from three separate experiments, with the total number of animals in each group shown in parentheses. NS = not significant ($P > 0.05$). Differences among the experimental groups were analyzed by the Newman–Keuls multiple sample comparison test. [This is a modified version of Fig. 2 in Brown SJ, Galli SJ, Gleich GJ, Askenase PW. Ablation of immunity to Amblyomma americanum by anti-basophil serum: cooperation between basophils and eosinophils in expression of immunity to ectoparasites (ticks) in guinea pigs. J Immunol 1982;129:790–6 (Ref. 45) reprinted with the permission of the publisher. Copyright 1982. The American Association of Immunologists, Inc.].

had a moderate macrocytic anemia, a phenotype which had been reported decades earlier, but were profoundly deficient in tissue mast cells.[56] They also showed that mast cells developed in WBB6F$_1$-W/W^v mice which had been engrafted with bone marrow cells from the wild type (WT) littermate WBB6F$_1$-+/+ mice.[56] However, the recipient W/W^v mice also were cured of their anemia,[56] as was initially shown by Elizabeth Russell.[57] Moreover, eventually, non-irradiated WBB6F$_1$-W/W^v mice engrafted with sufficient (e.g., 1×10^7) WBB6F$_1$-+/+ or other genetically-compatible WT whole bone marrow cells also undergo virtually complete replacement of multiple other hematopoietic lineages (including granulocytes and lymphocytes) with cells of donor origin.[58–60]

Because transfer of WT bone marrow cells into W/W^v mice did not result in the selective engraftment of mast cells, due to the presence in bone marrow cells of hematopoietic stem and progenitor cell populations, an effort was undertaken to attempt to achieve a more selective "repair" of the mast cell deficiency of W/W^v mice by transferring in vitro-derived, "lineage-committed", mast cells to the mice instead of whole bone marrow cells. This approach appeared plausible because it was clear that large populations of cells with features of "immature" mast cells could be generated in vitro from mouse hematopoietic progenitor cells[61] and that such cells exhibited features of additional mast cell maturation when exposed to sodium butyrate in vitro.[62] Nakano et al. showed that the transfer of WBB6F$_1$-+/+ mouse bone marrow-derived cultured mast cells (BMCMCs) i.v., i.p. or i.d. into WBB6F$_1$-W/W^v mice had no effect on the anemia of the recipient mice but resulted in the appearance of mast cells in their tissues, and that, over time, these mast cell populations came to exhibit certain phenotypic features similar to those present in the corresponding anatomical sites in WT mice.[63]

Since that first study, many groups have used such mast cell-engrafted or, as we refer to them in the Galli lab, "mast cell knock-in mice" (Fig. 2), to analyze mast cell development, phenotype, heterogeneity and function in vivo.[14,71,72] An attractive aspect of this approach is that one can transfer into different genetically mast cell-deficient recipients either WT mast cells or mast cells that have been genetically manipulated or that are derived from various mutant or transgenic mice, so that one can compare the function in vivo of mast cells that are normal or that lack (or have altered function of) various receptors, signaling molecules or mediators. Moreover, Tsai et al. showed that one also can generate such mast cells from embryonic stem cells, permitting the analysis in vivo of mast cells which lack products which, if absent in the germ line, would result in embryonic or perinatal lethality.[64]

In addition to using the original mast cell-deficient WBB6F$_1$-Kit^W/Kit$^{W\text{-}v}$ mice to prepare "mast cell knock-in mice", this approach also can be employed using C57BL/6-Kit$^{W\text{-}sh}$/Kit$^{W\text{-}sh}$ mice, which have the advantage of being inbred, fertile and not anemic.[68,69,73] We recently showed that this approach also can be pursued using C57BL/6-Cpa3-Cre$^+$-Mcl-1$^{fl/fl}$ mice, which are mast cell-deficient (and also have substantially diminished numbers and function of basophils) due to the lineage-restricted ablation of the anti-apoptotic factor, myeloid cell leukemia 1 (Mcl-1), in lineages with sufficiently high expression of the Cpa3 gene.[70] Because the latter mice are mast cell deficient but, unlike WBB6F$_1$-Kit^W/Kit$^{W\text{-}v}$ or C57BL/6-Kit$^{W\text{-}sh}$/Kit$^{W\text{-}sh}$ mice, have normal c-kit, we informally call them "Hello Kitty" mice[70] (Fig. 2).

New models for analyzing the functions of basophils and mast cells

In addition to the models described above, those interested in the biology of mast cells, basophils or their mediators are now fortunate to have a large number of additional models to choose from, including other lines of mice that exhibit constitutive or inducible deficiencies in populations of mast cells or in basophils, or which constitutively or inducibly lack various mast cell mediators or other molecules.[72] As reviewed in detail elsewhere,[72,74,75] each of the various models currently available has features that must be kept in mind when interpreting the results of studies using such mice, and the importance of particular mast cell (or basophil) roles in individual biological responses may vary both according to the details of the model used to study that biological response (e.g., whether one is studying a "weak" or "strong" model of that response) and based on the strain background of the mice. Accordingly, we have recommended that investigators consider using more than one type of genetic model to investigate the functions and importance of mast cells (or basophils) and/or their individual products in biological responses in vivo.[72]

Identifying a beneficial role for mast cells in enhancing innate resistance to venoms

Early work by Higginbotham and colleagues suggested that mast cells might be able to diminish the toxicity of certain venoms by degranulating and releasing heparin in response to venom exposure.[76,77] However, this work was conducted before the description of mice deficient in mast cells or their individual mediators, so the importance of the roles of mast cell and their products in innate resistance to venoms could not be investigated more definitively in vivo. Even after Kitamura's description of genetically mast cell-deficient mice, some time elapsed before any attention was paid to this question. One step in that direction was the finding that ET-1 can initiate a homeostatic mechanism whereby proteases released by mast cells activated by ET-1 can degrade that vasoactive peptide and thereby diminish its potential toxicity in vivo.[65] Using mast cell knock-in C57BL/6-Kit$^{W\text{-}sh}$/Kit$^{W\text{-}sh}$ mice engrafted with ET$_A$ receptor-deficient or WT mast cells derived in vitro from ET$_A^{-/-}$ or ET$_A^{-/+}$ embryonic stem cells, Maurer et al. found that mast cell activation by ET-1 via the ET$_A$ receptor contributed to this effect.[65]

A homology search conducted by Martin Metz revealed that ET-1 was structurally similar to sarafotoxin 6b, one of the major toxins in the venom of the Israeli mole viper (Atractaspis engaddensis).[78] Sarafotoxin 6b can induce activation of cells in the envenomated animals by binding to endothelin receptors.[79] Metz et al. showed that mast cells not only diminished the toxicity of sarafotoxin 6b and enhanced the survival of mice injected with that peptide, but also did so in mice injected with the whole venom of A. engaddensis.[15] Testing the ability of mast cells to influence responses to whole venoms is important, since snakes (and arthropods) don't envenomate their prey with a single toxin but with a complex mixture of toxins that can induce pathology by different mechanisms.[80] Initial pharmacological studies suggested that chymase was the critical mast cell protease in this setting.[65] However, later work by our group, employing both pharmacological approaches and shRNA knock down of CPA3 in adoptively-transferred mast cells,[15] as well as elegant studies by Schneider et al., who exploited a mouse they created which expressed only a catalytically inactive CPA3[81], indicated that CPA3 is the key mast cell-derived protease that detoxifies both ET-1 and sarafotoxin 6b. Using mast cell knock-in mice, Metz et al. also provided evidence that mast cells were important in substantially enhancing the innate resistance of mice to honeybee (Apis mellifera) venom and to the venoms of two North American pit vipers, the western diamondback rattlesnake (Crotalus atrox) and the southern copperhead (Agkistrodon contortrix contortrix).[15]

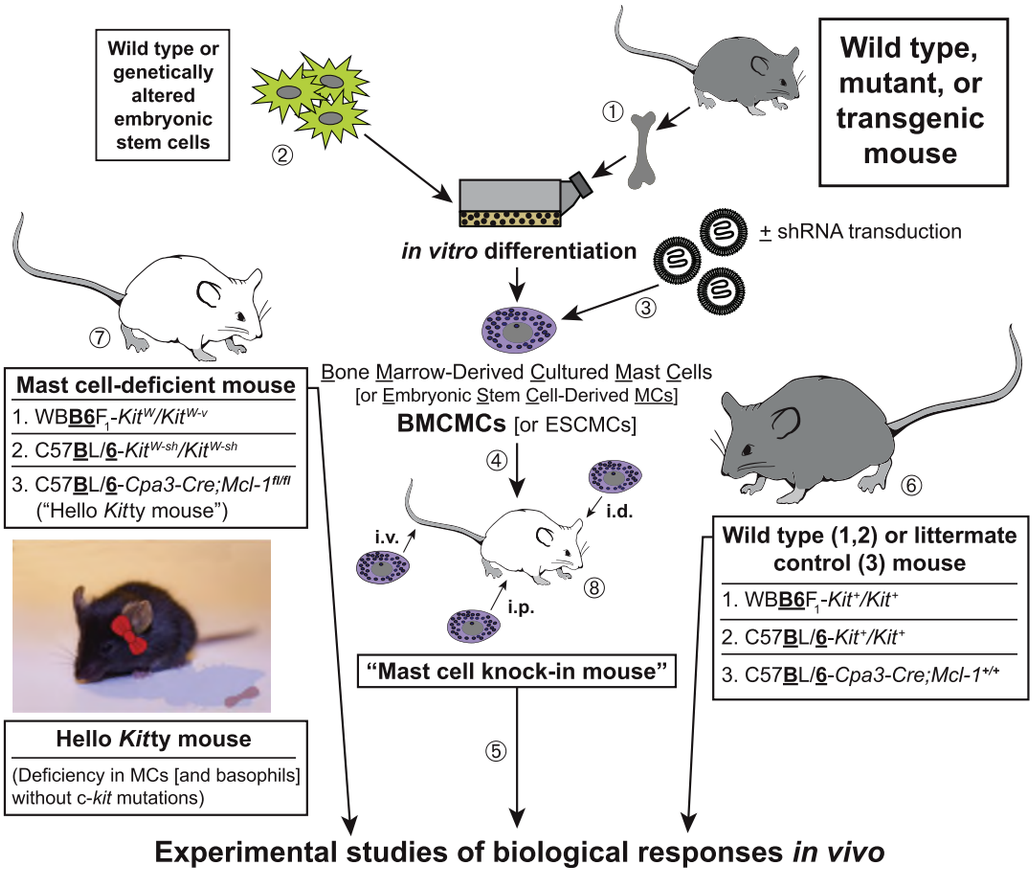


Fig. 2. Making "mast cell knock-in mice". (1) Mast cells can be generated from bone marrow cells (or other hematopoietic cells; e.g., those in the fetal liver) from wild type mice or from mutant or transgenic mice with specific genetic alterations of interest.[61–63] Alternatively, (2) embryonic stem (ES) cell-derived cultured mast cells (ESCMCs) can be generated from wild type or genetically altered ES cells[64,65], or (3) various mast cell populations can be transduced in vitro with shRNA to diminish expression of specific genes of interest.[15,66] (4) Such bone marrow-, ES cell-, [or fetal liver-] derived cultured mast cells, or shRNA-transduced mast cells, can then be transplanted into mast cell-deficient c-kit mutant mice, such as WBB6F$_1$-Kit^W/Kit$^{W\text{-}v}$ mice[63,67] or C57BL/6-Kit$^{W\text{-}sh}$/Kit$^{W\text{-}sh}$ mice,[68,69] or into C57BL/6-Cpa3-Cre;Mcl-1$^{fl/fl}$ mice[70] (which we informally refer to as "Hello Kitty mice", which have wild type c-kit), to produce mast cell knock-in mice. Note: BMCMCs can be injected into genetically mast cell-deficient mice intravenously (i.v.), intraperitoneally (i.p.), or intradermally (i.d.), or into the joints or meninges, etc., but there is a more limited experience with the engraftment of other types of MCs, such as EMCMCs, than with BMCMCs. (5) A suitable interval is then allowed for engraftment and phenotypic "maturation" of the adoptively-transferred mast cells (the length of this interval can be varied based on the route of mast cell transfer, the anatomical site of interest, the particular biological response being analyzed, etc.). The importance of mast cell function(s) in biological responses can be analyzed by comparison of the responses in the appropriate wild type or littermate control mice (6), the corresponding mutant mast cell-deficient mice (7), and selectively mast cell-engrafted mutant mice (mast cell knock-in mice) (8). The contributions of specific mast cell products (surface structures, signaling molecules, secreted products, and so on) to such biological responses can be analyzed by comparing the features of the responses of interest in mast cell knock-in mice engrafted with wild type mast cells versus mast cells derived from mice or ES cells that lack or express genetically altered forms of such products or that have been transduced with shRNA to silence the specific genes that encode these products. An important part of the analysis of the mast cell knock-in mice used in particular experiments is to assess the numbers and anatomic distribution, and, for certain experiments, aspects of the phenotype, of the adoptively-transferred mast cells, as, depending on the type of in vitro-derived mast cells used, the route of administration, and other factors, these may differ from those of the corresponding native populations of mast cells in the corresponding wild type mice.[14,71,72]. [This is a modified version of Fig. 2 in Metz M, Grimbaldeston MA, Nakae S, Piliponsky AM, Tsai M, Galli SJ. Mast cells in the promotion and limitation of chronic inflammation. Immunol Rev 2007; 217:304–28 (Ref. 71), reprinted with the permission of the publisher, John Wiley and Sons.].

A project led by Mitsuteru Akahoshi and Chang Ho Song then analyzed whether mast cells might enhance innate resistance to another pair of biologically active peptides, the endogenous mammalian peptide VIP and the structurally similar peptide helodermin (also known as exendin-2), which is one of the toxins present in the venom of the Gila monster (Heloderma suspectum).[18] Testing of both mast cell knock-in mice (including C57BL/6-Kit$^{W\text{-}sh}$/Kit$^{W\text{-}sh}$ mice engrafted with WT versus chymase [mMCP4]-deficient mast cells) (Fig. 3A, B) and mice which had mast cells but were genetically deficient in mMCP4[82] or CPA3[81] or produced a catalytically inactive CPA3[81] (Fig. 3C), showed that mast cells could diminish the toxicity of VIP, helodermin, and the whole venom of H. suspectum, and that this was largely or wholly dependent on mast cell-derived mMCP4 rather than CPA3[18]. Similar approaches were used to provide evidence that mast cells and mMCP4 can contribute to enhanced innate resistance of mice to the venoms of two scorpions, one from the old world, the Deathstalker (Leiurus quiquestriatus hebraeus), and one from the new world, the Arizona bark scorpion (Centruroides exilicauda).[18]

It is possible that future work will reveal that mast cell activation can increase, rather than decrease, the toxicity of some venoms. However, our initial evidence indicated that mast cells can increase the innate resistance of mice upon their first exposure to the venoms of 3 species of poisonous snakes, the Gila monster, the honeybee, or two especially dangerous scorpions. Moreover, mast cells contain at least two different proteases, CPA3 and chymase (mMCP4), which permit mast cells to respond, after their activation via cognate receptors that can bind either the endogenous or the structurally similar reptile-derived peptides, to high and potentially toxic levels of ET-1 and VIP, respectively, as well as to high levels of the similar peptides contained in the reptile venoms (sarafotoxin 6b in Israeli mole

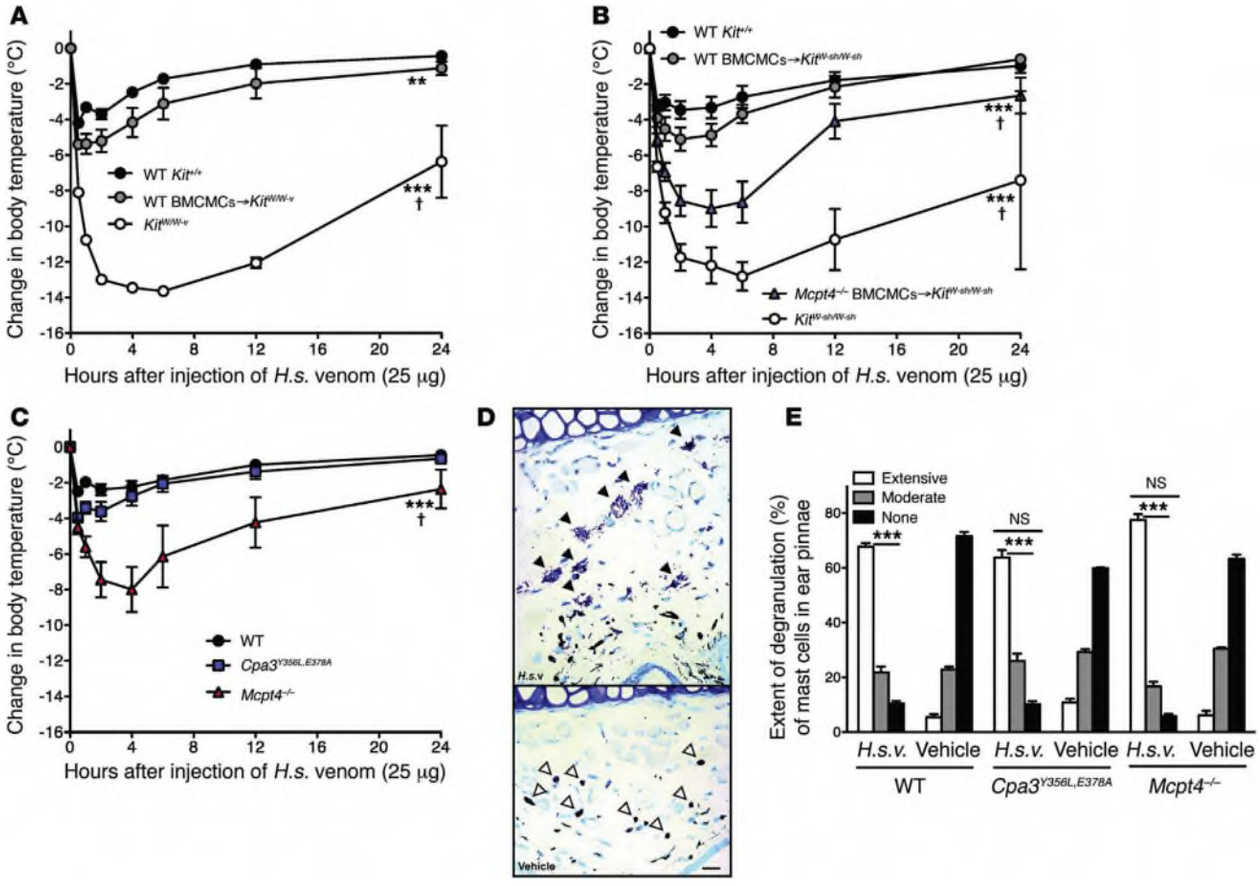


Fig. 3. Mast cells can diminish Heloderma suspectum venom (H.s.v.)-induced hypothermia and mortality through MCP4-dependent mechanisms. Changes in rectal temperatures after i.d. injection of H.s.v. (25 μg in 20 μl DMEM solution) into the ear pinnae (one ear pinna of each mouse) of: (A) WT WBB6F$_1$-Kit$^{+/+}$, mast cell-deficient WBB6F$_1$-Kit$^{W/W\text{-}v}$, and WT BMCMCs→Kit$^{W/W\text{-}v}$ mice (i.e., WBB6F$_1$-Kit$^{W/W\text{-}v}$ mice which had been engrafted, 6–8 weeks before venom challenge, in one ear pinna with 2 million BMCMCs derived from WT WBB6F$_1$-Kit$^{+/+}$ mice) (the death rates of Kit$^{+/+}$, WT BMCMCs→Kit$^{W/W\text{-}v}$, and Kit$^{W/W\text{-}v}$ mice within 24 h after H.s.v. injection were 0% [0/21], 7% [1/15, P = 0.42 vs. Kit$^{+/+}$ mice], and 65% [13/20, P < 0.0001 vs. Kit$^{+/+}$ mice], respectively); (B) WT C57BL/6-Kit$^{+/+}$, mast cell-deficient C57BL/6-Kit$^{W\text{-}sh/W\text{-}sh}$, WT BMCMCs→Kit$^{W\text{-}sh/W\text{-}sh}$, and Mcpt4$^{-/-}$ BMCMCs→Kit$^{W\text{-}sh/W\text{-}sh}$ mice (the death rates of Kit$^{+/+}$, WT BMCMCs→Kit$^{W\text{-}sh/W\text{-}sh}$, Mcpt4$^{-/-}$ BMCMCs→Kit$^{W\text{-}sh/W\text{-}sh}$, and Kit$^{W\text{-}sh/W\text{-}sh}$ mice within 24 h after H.s.v. injection were 5% [1/19], 11% [2/18, P = 0.48 vs. Kit$^{+/+}$ mice], 43% [6/14, P = 0.01 vs. Kit$^{+/+}$ mice], and 50% [10/20, P = 0.006 vs. Kit$^{+/+}$ mice], respectively); or (C) WT C57BL/6-Kit$^{+/+}$ mice, C57BL/6-Cpa3^{Y356L,E378A} mice (which have a catalytically inactive CPA3) and C57BL/6-Mcpt4$^{-/-}$ mice (which are deficient in mMCP4) (the death rates of Kit$^{+/+}$, Cpa3^{Y356L,E378A}, and Mcpt4$^{-/-}$ mice within 24 h after H.s.v. injection were 7% [1/15], 0% [0/14, P = 0.52 vs. Kit$^{+/+}$ mice], 40% [6/15, P = 0.007 vs. Kit$^{+/+}$ mice], respectively). Each figure shows data pooled from at least three independent experiments with each group of mice (n = 2–5 mice per group per each individual experiment). **P < 0.01, ***P < 0.001 versus WT WBB6F$_1$-Kit$^{+/+}$ or WT C57BL/6-Kit$^{+/+}$ mice; †P < 0.01–0.001 versus each other group (A–C). (D) Extensive degranulation of mast cells (some indicated by closed arrowheads) 1 h after i.d. injection of H.s.v. (25 μg in 20 μl DMEM), but not vehicle (DMEM) alone (mast cells without evidence of degranulation are indicated by open arrowheads) in WT C57BL/6 mice (Toluidine blue stain; scale bar: 50 μm). (E) Degranulation of mast cells 60 min after i.d. injection of H.s.v. (25 μg in 20 μl DMEM) or vehicle (DMEM) alone in WT C57BL/6, Mcpt4$^{-/-}$, or Cpa3^{Y356L,E378A} mice (injection was into one ear pinna of each mouse). ***P < 0.001 versus corresponding vehicle-injected groups; NS = not significant (P > 0.05) versus values for WT mice. [This is a reproduction of Fig. 1 from Akahoshi M, Song CH, Piliponsky AM, Metz M, Guzzetta A, Abrink M, Schlenner SM, Feyerabend TB, Rodewald HR, Pejler G, Tsai M, Galli SJ. Mast cell chymase reduces the toxicity of Gila monster venom, scorpion venom, and vasoactive intestinal polypeptide in mice. J Clin Invest 2011;121:4180–91 (Ref. 18), reprinted with the permission of the publisher, the American Society for Clinical Investigation.].

viper venom and helodermin in Gila monster venom, respectively) (Fig. 4). By undergoing degranulation and releasing proteases that can inactivate potentially toxic endogenous peptides or peptides in venoms, mast cells can help to restore homeostasis, albeit while also enhancing features of the ensuing local and perhaps systemic inflammatory responses.

Depending on the mammalian species, mast cells can contain several tryptases and chymases of distinct substrate specificity, as well as CPA3[13,72,83]. This raises the possibility that one of the reasons that the mast cells of various species contain several different proteases in their cytoplasmic granules is so that these cells, which are positioned in large numbers in the skin, the most common site of envenomation, are equipped to release a panel of proteases with the potential to degrade a variety of structurally distinct toxic compounds contained in animal venoms. Mast cells might also contribute to innate resistance to venoms in other ways, such as by increasing local vascular permeability and thereby favoring the interstitial access of circulating molecules that can antagonize the effects of venom proteases[84] and other toxins.

IgE can contribute to host defense against arthropod and reptile venoms

Many animals and some humans experience multiple episodes of envenomation by arthropods such as bees, wasps, and scorpions, or by various reptiles. Such envenomation not only provokes an innate inflammatory response and pathology related to the biological activities of the venoms' toxins,[85–87] but also can induce allergic sensitization associated with the development of specific IgE antibodies.[88–93] In some unfortunate people, such IgE responses to venoms put these individuals at risk to develop potentially fatal episodes of anaphylaxis.[3,7,23,93] But recent findings suggest, in accord with Profet's “toxin hypothesis of allergy”, that

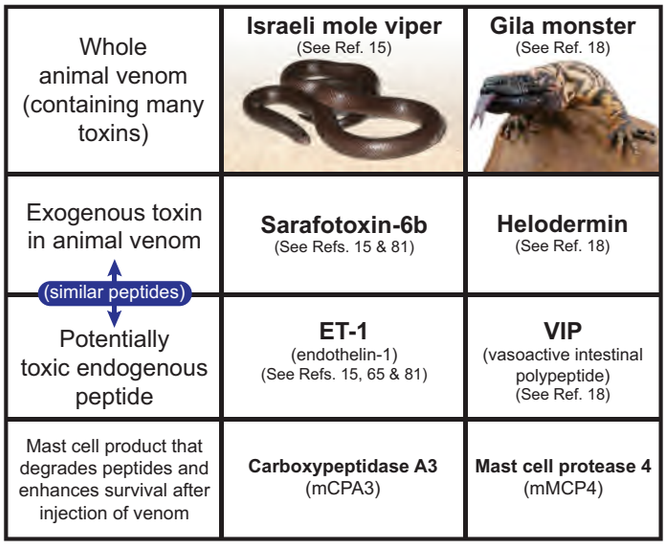


Fig. 4. Mast cells can enhance innate resistance to high levels of endogenous peptides and structurally similar peptides in reptile venoms. Mast cell cytoplasmic granules contain proteases such as carboxypeptidase A3 (CPA3 [mCPA3 = mouse CPA3]) and mast cell protease 4 (MCP4 [mMCP4 = mouse MCP4]) that, upon secretion by activated mast cells, can degrade certain endogenous peptides, such as endothelin-1 (ET-1) and vasoactive intestinal polypeptide (VIP), respectively, as well as structurally similar peptides contained in the venoms of poisonous reptiles, such as sarafotoxin 6b in the venom of the Israeli mole viper (Atractaspis engaddensis) and helodermin in the venom of the Gila monster (Heloderma suspectum). The ability of mast cells to be activated to degranulate by components of venoms such as these, which can act at the same receptors which recognize the corresponding structurally similar endogenous peptides, permits mast cells to release proteases that can reduce the toxicity of these peptides and which help to enhance the survival of mice injected with the whole venoms of these reptiles, that contain many toxins in addition to sarafotoxin 6b and helodermin. This mechanism may also permit mast cells to restore homeostasis in settings associated with markedly increased levels of the endogenous peptides. [This is a reproduction of Fig. 4 from Galli SJ. The 2014 Rous-Whipple Award Lecture. The mast cell-IgE paradox: From homeostasis to anaphylaxis. Am J Pathol, in press (ref. [42]), reprinted with the permission of the publisher, Elsevier for the American Society for Investigative Pathology. The photograph of the Israeli mole viper is by Amikam Shoob, courtesy of Elazar Kochva, Tel Aviv University, and the photograph of the Gila monster is from reptiles4all and is used under license from Shutterstock.com.].

this same "allergic" mechanism — involving IgE and mast cells — also can enhance host resistance to venoms.

Honeybee (A. mellifera) venom consists of a mixture of cytolytic peptides (e.g., melittin), enzymes (e.g., phospholipase A_2 [PLA_2; considered the main allergen in bee venom]), hyaluronidase, neurotoxins and bioactive amines,[85] and accounts for a large fraction of venom allergies in humans.[93] The venom of the Russell's viper (Daboia russelii), one of the most dangerous snakes in the Indian subcontinent,[94] is a complex mixture of growth factors and enzymes with pro-coagulant and neurotoxic activities.[87] We found that, in mice, IgE-associated type 2 immune responses against honeybee venom or Russell's viper venom were able to increase significantly host resistance to challenge with potentially lethal doses of those venoms.[95]

This was unexpected because both IgE and IgG_1 antibodies produced during type 2 immune responses can orchestrate anaphylaxis and other allergic reactions in mice[7,23,96,97] and because type 2 immune responses against venoms (that include the development of anti-venom IgG_1 [in mice] and IgE antibodies) are classically thought to exacerbate the outcome of subsequent venom exposure.[89–93,98] By contrast, IgG class antibodies raised against animal venoms (or their $F(ab')_2$ fragments), are used to treat envenomated humans or animals.[99]

So it was important to identify which antibodies contributed to the enhanced resistance to honeybee venom observed in mice with type 2 immune responses to that venom. Our evidence showed that IgE antibodies were the critical elements of the acquired host resistance to honeybee or Russell's viper venom. We found: 1) that most or all of the acquired resistance induced in mice by a single exposure to honeybee venom was transferable to naïve mice with only 250 μL of serum from honeybee venom-immunized mice; 2) that when such "immune serum" was depleted of IgE either by adding a neutralizing antibody to IgE[35,100] or by heating (56 °C, 1 h, which eliminates the ability of IgE to bind to FcεRI and induce passive cutaneous anaphylaxis[101] while the function of other antibody classes, including IgG_1, is not affected[102]), the immune serum's ability to transfer enhanced resistance to naïve mice was essentially lost; and 3) that such "immune serum" failed to transfer enhanced venom resistance to mice lacking either the IgE antibody-binding α chain of the FcεRI or the γ chain of FcεRI that is necessary for signaling initiated by aggregation of the receptor.[5,6]

We also found: 1) that genetically IgE-deficient mice[96] could not develop acquired immunity to honeybee venom, even though they developed a robust IgG_1 antibody response to the venom; 2) that "immune serum" from WT mice could passively transfer enhanced resistance to honeybee venom to naïve IgE-deficient mice, unless such "immune serum" was first treated to neutralize IgE or impair its ability to bind to FcεRI, and 3) that naïve genetically mast cell-deficient C57BL/6-$Kit^{W\text{-}sh}$/$Kit^{W\text{-}sh}$ or C57BL/6-Cpa3-Cre^{+}-Mcl-$1^{fl/fl}$ mice which received immune serum from honeybee venom-immunized C57BL/6 WT mice actually exhibited worse survival after challenge with a high dose of honeybee venom than did mast cell-deficient mice which had received serum from PBS mock-immunized C57BL/6 WT mice.[95] The latter finding suggested that mast cells can contribute to IgE-mediated acquired resistance to honeybee venom, as well as enhance the innate resistance of mice to a first exposure to that venom.[15] Independently of our work, Palm et al. showed that mice immunized with the major allergen contained in honeybee venom, bee venom phospholipase A_2 ($bvPLA_2$), exhibited enhanced resistance to the ability of $bvPLA_2$ to induce hypothermia upon its injection into mice, and provided evidence that this enhanced immunity required B cells and was diminished significantly in mice which lacked the IgE-binding α chain of the FcεRI[103]. Taken together, these two initial studies[96,103] support the hypothesis that one physiological function of IgE is to protect the host against noxious substances.

Subsequently, we found that the acquired enhanced resistance to Russell's viper venom (RVV) which we observed in mice that had developed type 2 immune responses to that venom[95] also was highly dependent on IgE (Fig. 5) and FcεRI (see Fig. 4, A–E in Ref. 104), and could be effectively transferred by immune serum into normal mice (see Fig. 3, F–J in Ref. 104) but not into C57BL/6-Cpa3-Cre;Mcl-$1^{fl/fl}$ mice which were genetically markedly deficient in mast cells and which also had diminished numbers of basophils (see Fig. 4, G and H in Ref. 104). Notably, two different types of genetically mast cell-deficient mice also exhibited significantly diminished innate resistance to the toxicity and lethality of RVV (Fig. 6A–C, E, F), supporting Higginbotham's hypothesis that mast cells can contribute to enhanced innate resistance to this venom.[76] Compared to the corresponding mast cell-sufficient mice, such naïve mast cell-deficient mice also exhibited many fewer attempts to scratch sites of RVV injection (Fig. 6D, G). The latter finding supports the idea proposed both by Stebbings[40] and Profet[39] that elements of allergic responses, in this case, mast cells, can confer benefit to hosts experiencing attacks by arthropods[40] or other sources of toxins[39] by altering the host's behavior in ways that would help to eliminate, or at least permit the host to become aware of, the threat.

As noted above, Palm et al. reported that immunization of mice with honeybee venom-derived $bvPLA_2$, which represents

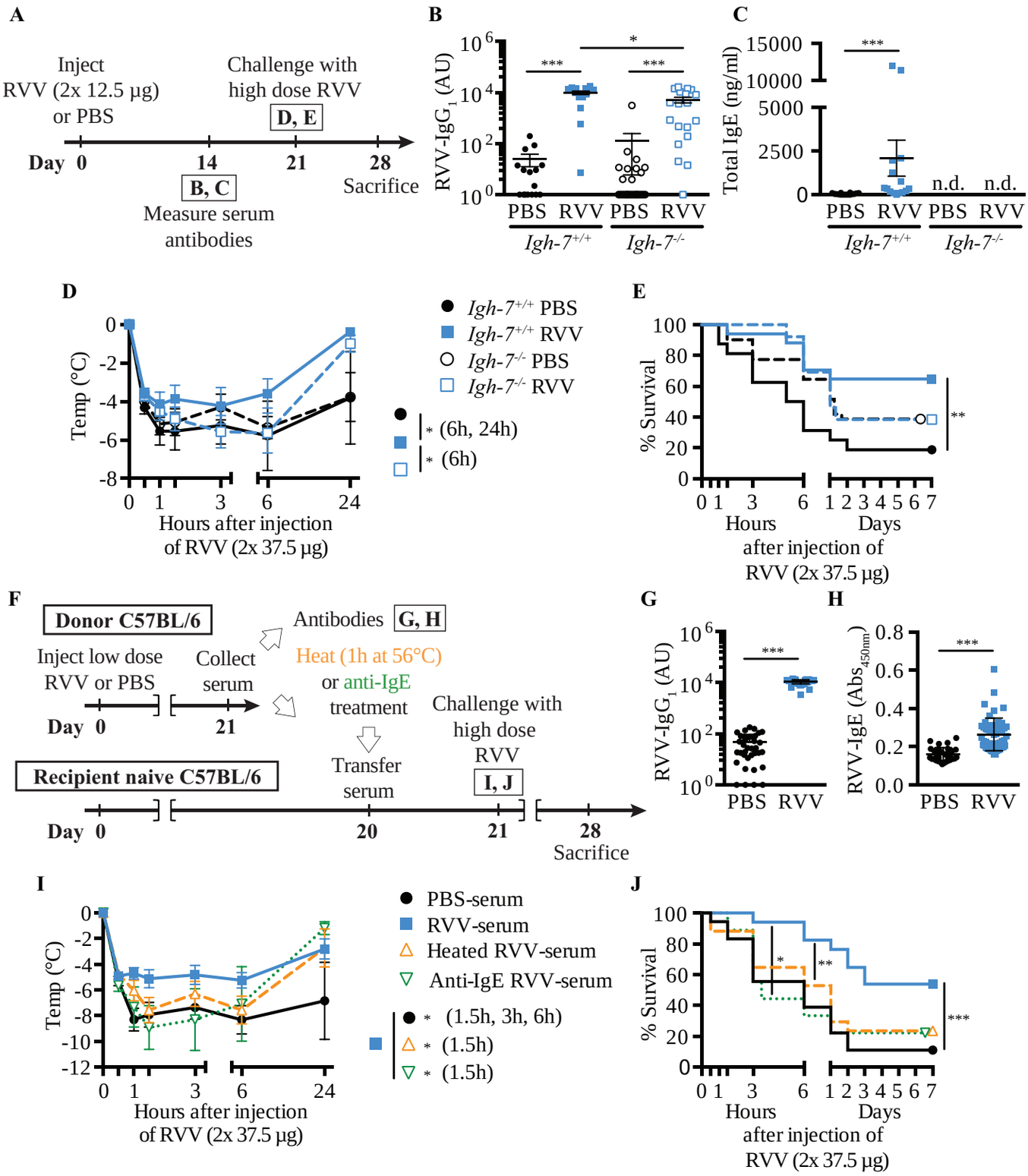


Fig. 5. Evidence that IgE antibodies contribute to acquired enhanced resistance to the toxicity and lethality of Russell's viper venom. A. Outline of experiments with IgE-deficient (Igh-$7^{-/-}$) and control (Igh-$7^{+/+}$) C57BL/6 mice (B–E). B, C. Serum RVV-specific IgG_1 (B) and total IgE (C). D, E. Body temperature (D) and survival (E). F. Outline of serum transfer experiments in C57BL/6 mice (G–J). G, H. Serum RVV-specific IgG_1 (G) and RVV-specific IgE (H). I, J. Body temperature (I) and survival (J). Data were pooled from 3 to 4 experiments (n = 9–25/group). P values: Mann–Whitney test (B, C, G, H), Student's t test (D, I) and Mantel–Cox test (E, J). [This is a reproduction of Fig. 3 from Starkl P, Marichal T, Gaudenzio N, Reber LL, Sibilano R, Tsai M, Galli SJ. IgE antibodies, FcεRIα and IgE-mediated local anaphylaxis can limit snake venom toxicity. J Allergy Clin Immunol. In press (Ref. 104), reprinted with the permission of the publisher, Elsevier.].

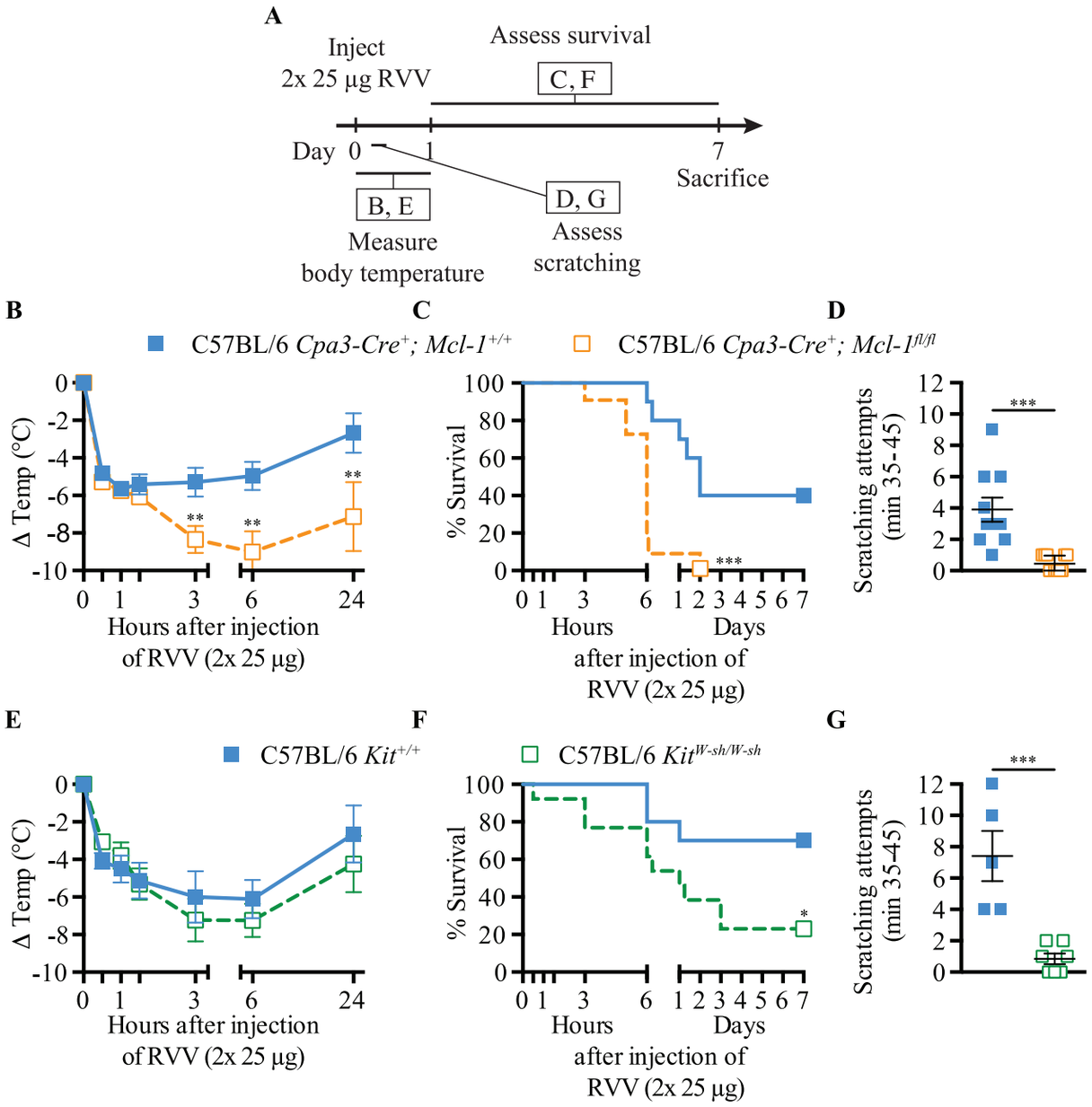
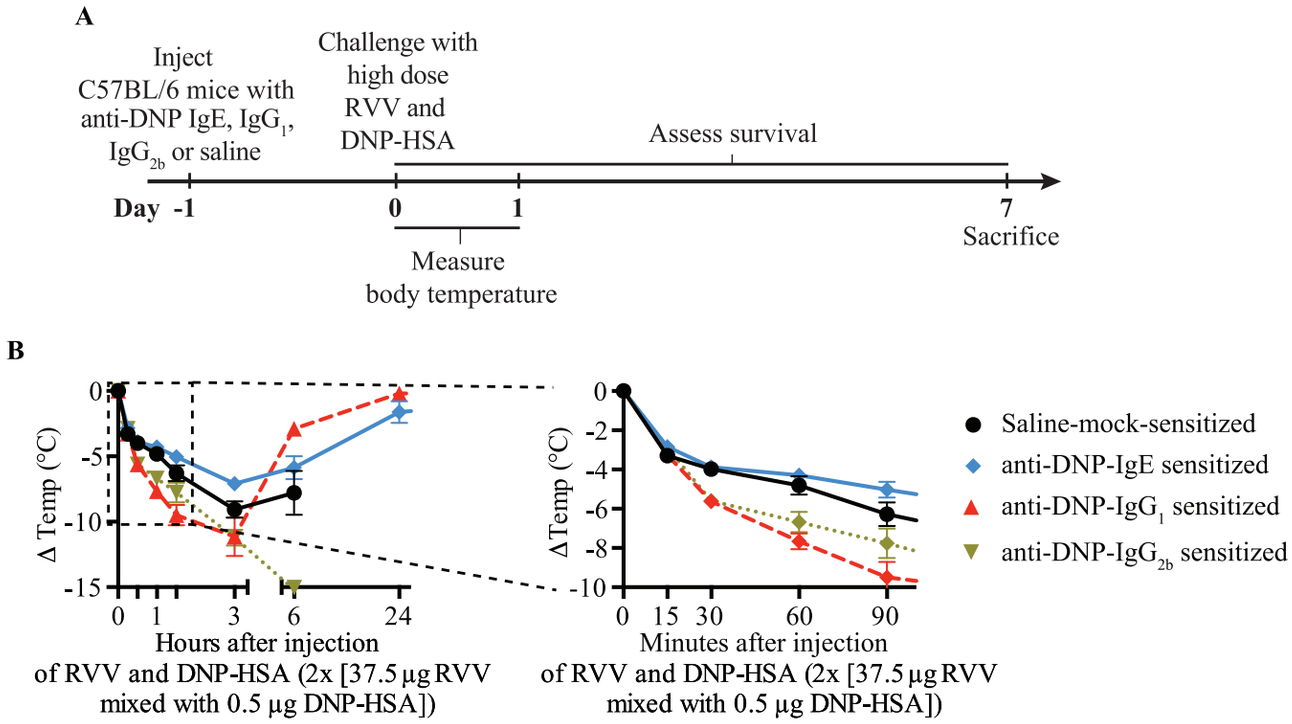


Fig. 6. Evidence that mast cells contribute to innate resistance to the toxicity and lethality of Russell's viper venom, as well as to behavioral responses to envenomation. A. Experimental outline. B and E, body temperature; C and F, survival; D and G, scratching attempts, of mast cell-deficient Cpa3-Cre^{+}; Mcl-1$^{fl/fl}$ (B–D) and Kit$^{W\text{-}sh/W\text{-}sh}$ (E–G) mice and corresponding control mice after RVV injection. P values: (B, D, E, G) Student's t test; (C, F) Mantel–Cox test. Data were pooled from 2 to 4 experiments (n = 5–21/group). [This is a reproduction of Fig. 2 of Starkl P, Marichal T, Gaudenzio N, Reber LL, Sibilano R, Tsai M, Galli SJ. IgE antibodies, FcεRIα and IgE-mediated local anaphylaxis can limit snake venom toxicity. J Allergy Clin Immunol. In press (Ref. 104), reprinted with the permission of the publisher, Elsevier.].

approximately 10% of the dry weight of whole BV[105] can reduce the toxicity-related hypothermia induced by subsequent challenge with a high dose of the same allergen in an antibody- and FcεRIα-dependent manner.[103] However, it was not clear whether an IgE response to a single constituent of an animal venom would be able to enhance resistance to the entire group of toxins contained in that venom. To investigate this, we passively sensitized WT mice locally against DNP-HSA by s.c. injections of anti-DNP IgE[106] (or with anti-DNP IgG$_1$ or IgG$_{2b}$ as controls), or mock-sensitized the mice with saline, then challenged the animals s.c. at the same site 24 h later by injecting a mixture of RVV and DNP-HSA (Fig. 7A). We used amounts of anti-DNP IgE and DNP-HSA which were able to induce a local increase in vascular permeability at the DNP-HSA injection site without resulting in systemic hypothermia, and showed that the amount of DNP-HSA used did not by itself influence the toxicity of RVV (see Fig. E5 in the Online Repository of Ref. 104).

We found that pre-sensitization with anti-DNP IgE significantly increased the resistance of C57BL/6 (Fig. 7B, C) or BALB/c (see Fig. E5, H–I in the online repository of Ref. 104) mice to challenge with a potentially lethal amount of RVV admixed with DNP-HSA.[104] However, pre-sensitization of C57BL/6 mice with anti-DNP IgG$_1$ or IgG$_{2b}$, DNP-specific IgG isotypes with the capacity to activate effector cells via Fcγ receptors[107], not only failed to increase protection but also resulted in increased hypothermia at early time points compared to vehicle-treated or IgE-sensitized mice (Fig. 7B).[104] These findings show that local tissue responses mediated by IgE and antigen can enhance host resistance against RVV even when that antigen is not a native constituent of the venom,



Comparisons of body temperature after injection of RVV and DNP-HSA in various groups of mice.

	Vehicle *vs.*			IgE *vs.*	
Min	IgE	IgG_1	IgG_{2b}	IgG_1	IgG_{2b}
15	*	.91	.18	*	.82
30	.76	**	**	***	***
60	.39	**	*	***	***
90	.09	**	.19	***	**
180	*	.14	.08	**	***

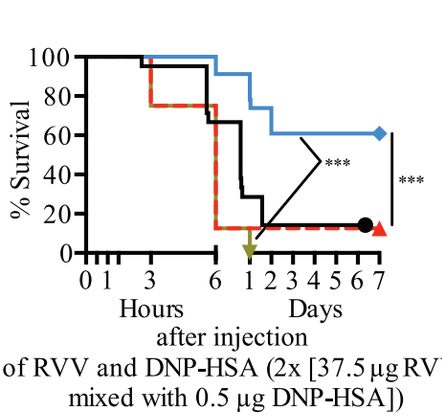


Fig. 7. IgE-dependent local mast cell activation induced by activation with a single antigen can enhance resistance to the lethality of Russell's viper venom. A. Experimental outline. B, C. Body temperature (B) and survival (C) of C57BL/6 mice treated with 3 s.c. injections of saline alone or containing 50 ng anti-DNP IgE, IgG_1 or IgG_{2b} antibody and challenged 18 h later with 2 s.c. injections, each containing 37.5 µg RVV and 0.5 µg DNP-HSA. Data were pooled from 2 to 5 independent experiments (n = 10–25/group). P values: Student's t test (B); Mantel–Cox test (C). [This is a reproduction of Fig. 5 from Starkl P, Marichal T, Gaudenzio N, Reber LL, Sibilano R, Tsai M, Galli SJ. IgE antibodies, FcεRIα and IgE-mediated local anaphylaxis can limit snake venom toxicity. J Allergy Clin Immunol. In press (Ref. 104), reprinted with the permission of the publisher, Elsevier.].

and are consistent with the general idea that the host needs only to generate an IgE response against a limited number of the components of a complex venom (perhaps as few as one component) in order to manifest enhanced acquired resistance to that venom.

Conclusions

Tissue resident cells with morphological, biochemical, and functional properties of mammalian mast cells, and which can produce histamine, heparin and serine proteases, are present in tunicates, whose ancestors appeared in evolution before the development of adaptive immunity.[108,109] Such tunicates also have been reported to have cells resembling basophils.[110] After the appearance of acquired immunity and the development of antibodies, these ancient hematopoietic lineages acquired the ability to bind immunoglobulins such as IgE (in mammals) to their surface. This allowed such tissue-resident cells to become "immunologically primed" or "sensitized" to undergo activation for mediator release upon encountering relatively small amounts of the antigen identified by their surface-bound IgE antibodies. The most extreme example of an IgE-associated immune response resulting in the activation of mast cells (and basophils) is fatal anaphylaxis, in which the rapid, systemic and extensive release of mediators stored in these FcεRI-bearing effector cells results in a catastrophic and quickly lethal outcome.

Observational and epidemiological studies in humans, as well as studies in experimental animals (including those employing mice genetically deficient in mast cells, basophils or IgE), strongly suggest that one beneficial role of IgE, mast cells and basophils is to help to defend the host against ectoparasites such as ticks, and to diminish

the numbers of parasites and burden of disease in mammals infected with certain helminths. However, in addition to parasites, vertebrates also have been subjected to evolutionary pressure through millions of years of co-evolution with venomous arthropods, reptiles, and other species. Evidence in mice indicates that mast cells can enhance innate resistance of mice to 4 species of poisonous snakes, the Gila monster, 2 species of scorpions, and the honeybee, and that mast cell proteases (specifically, CPA3 and the chymase MCP4) can contribute to such mast cell-dependent innate defenses by degrading toxins present in some of these venoms. Moreover, type 2 immune responses induced by a single exposure to honeybee venom or Russell's viper venom, which "arm" mast cells with IgE antibodies that bear specificity for components of those venoms, can significantly increase the survival of such mice to challenge with doses of the venoms which would be lethal in naïve mice.[95]

This evidence supports the notion[39–41] that key elements of "allergic reactivity", including mast cells and IgE, indeed can importantly enhance innate and acquired host resistance to venoms. Yet much work remains to be done to answer several related, but unresolved, questions. These include: 1) in addition to releasing proteases, are there other mechanisms by which mast cells can contribute to enhanced resistance to venoms during innate or acquired immune responses [e.g., ex vivo studies indicate that mast cell-derived heparin, that is highly anionic, can bind and thereby reduce the toxicity of cationic toxins in Russell's viper venom[76]]; 2) in what ways do venoms induce Th2 cell and IgE responses (for honeybee venom, this appears to involve a pathway by which products of bvPLA$_2$ acting on host lipid membranes induce IL-33 production, which in turn can activate ILC2 cells to release cytokines that drive IgE production[103]); 3) during vertebrate evolution, what has been the relative importance of exposure to ectoparasites (and the pathogens for which they serve as vectors), infection with helminths and other parasites, and interactions with venomous animals in shaping the features, function and immunological roles of mast cells, basophils, and IgE?; 4) Given that mast cells and basophils cooperate to enhance acquired resistance to the feeding of certain ticks, and that the hematophagous fluids produced by tick salivary glands can contain peptides similar to those in venoms,[111] is there a role for basophils in enhancing resistance to some venoms?; and 5) why do some subjects develop such severe IgE-dependent reactivity to venom that they are at risk for fatal anaphylaxis (an outcome far from a "protective" immune response).

Our initial findings indicate that the propensity to develop protective vs. potentially harmful type 2 immune responses to venoms, at least in mice, can depend on the genetic background of the animal, the type and amount of venom to which the animal is exposed, and/or the frequency of such venom exposures.[104] But this is only the beginning of addressing this important issue. In considering this question, it should be noted that many people who develop type 2 immune responses to BV do not exhibit anaphylactic reactivity despite having venom-specific IgE antibodies.[112] Also, there is abundant evidence that Th2 cell-mediated responses are subject to immune regulation which can diminish pathology related to IgE-dependent reactivity to the inducing antigen, including honeybee venom.[113–115] One might speculate that such immune regulation of type 2 immune responses ideally would reduce the pathology associated with these responses while preserving their ability to confer enhanced protection when the eliciting antigen is a toxin.

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Conflict of interest

The authors have no conflict of interest to declare.

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REVIEW

OPEN

Component resolved diagnostics for hymenoptera venom allergy

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Purpose of review
Component-resolved diagnostics makes use of defined allergen molecules to analyse IgE-mediated sensitizations at a molecular level. Here, we review recent studies on the use of component-resolved diagnostics in the field of Hymenoptera venom allergy (HVA) and discuss its benefits and limitations.

Recent findings
Component resolution in HVA has moved from single molecules to panels of allergens. Detection of specific immunoglobulin E (sIgE) to marker and cross-reactive venom allergens has been reported to facilitate the discrimination between primary sensitization and cross-reactivity and thus, to provide a better rationale for prescribing venom immunotherapy (VIT), particularly in patients sensitized to both honeybee and vespid venom. Characterization of IgE reactivity to a broad panel of venom allergens has allowed the identification of different sensitization profiles that in honeybee venom allergy were associated with increased risks for side effects or treatment failure of VIT. In contrast, component resolution so far has failed to provide reliable markers for the discrimination of sensitizations to venoms of different members of Vespidae.

Summary
Component-resolved diagnostics allows a better understanding of the complexity of sensitization and cross-reactivities in HVA. In addition, the enhanced resolution and precision may allow identification of biomarkers, which can be used for risk stratification in VIT. Knowledge about the molecular composition of different therapeutic preparations may enable the selection of appropriate preparations for VIT according to individual sensitization profiles, an approach consistent with the goals of personalized medicine.

Keywords
allergy, anaphylaxis, insect venom, risk stratification, specific immunoglobulin E diagnostics

INTRODUCTION

The diagnosis of Hymenoptera venom allergy (HVA) is based on the clinical history of a systemic/anaphylactic sting reaction and the detection of sensitization to relevant insect venoms by skin testing and/or detection of specific IgE antibodies in a serum sample [1▪]. In addition, cellular tests such as the basophil activation test (BAT) can be used not only in cases with a clear history but also with negative or unclear results of skin or in-vitro IgE tests. Depending on the geographical region, different insect species are more or less likely to be involved. The most frequent Hymenoptera sting reactions in central and northern Europe are caused by yellow jacket (Vespula spp.) and honeybee (Apis mellifera), whereas in southern Europe and the Americas, other wasps (e.g. Polistinae) are relevant. In addition, systemic sting reactions can be caused by ants, such as the jumper ant (Myrmecia) in Australia, the Asian needle ant (Pachycondyla) in Asia and the fire ant (Solenopsis) in the Americas.

HYMENOPTERA VENOM ALLERGENS

Whole venom preparations used for the detection of IgE-mediated sensitization contain a plethora of different components (such as proteins,

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KEY POINTS

- Currently, 75 venom allergens from 31 Hymenoptera species have been identified and officially accepted as allergens (www.allergen.org).
- Component resolution in routine diagnostics of HVA allows improved discrimination between primary sensitization and cross-reactivity, particularly in yellow jacket and honeybee venom allergy.
- Component-resolved diagnostics provides additional information on the complexity of the IgE and IgG4 immune response to Hymenoptera venom and allows characterization of individual sensitization profiles.
- Molecular sensitization profiles can be used for risk stratification that may lead to improved patient-related outcomes in VIT.

lipoproteins, glycoproteins, lipids etc.). The progress of molecular biology over the last decades has allowed a detailed characterization of relevant Hymenoptera venom allergens from different culprit insects. The currently known Hymenoptera venom allergens are summarized in Table 1. The list contains 75 allergens from 31 species and for some of these allergens, additional isoforms have been described. In the last decades, mainly the prototypical venom proteins (phospholipases, hyaluronidases and antigen 5) of several species were identified and accepted by the WHO/International Union of Immunology Societies' allergen nomenclature subcommittee as novel allergens (www.allergen.org).

The latest additions to the official list of hymenoptera venom allergens are Poly p 2, a hyaluronidase of Polybia paulista; Pol d 3, a dipeptidylpeptidase IV (DPPIV) of Polistes dominula and Pac c 3, an antigen 5 from Pachycondyla chinensis, the Asian needle ant. Poly p 2 from Polybia paulista, a clinically relevant social wasp that frequently causes stinging accidents in southeast Brazil, seems to have more pronounced IgE reactivity than the yellow jacket hyaluronidases Ves v 2 [3]. Pol d 3 from Polistes dominula is a member of the cross-reactive DPPIV protein family found in the majority of species and initially identified in honeybee venom (HBV) and yellow jacket venom (YJV). Pac c 3 was recently produced in recombinant form and was shown to exhibit significant IgE reactivity in patients with anaphylaxis [4▪]. ImmunoCAP inhibition studies further showed the high degree of cross-reactivity to Ves v 5.

Additional potential allergens not yet included in the official allergen list have recently been described or in some cases evaluated as recombinant proteins. Poly p 1, a phospholipase A1 from Polybia paulista was cloned and produced in bacteria and assessed with regard to IgE reactivity for diagnostic purposes [5▪]. The main venom components of the ectoparasitic ant-like bethylid wasp were recently described [6]. Notably, the most abundant components were acid phosphatase and antigen 5. Identifying an acid phosphatase – a marker allergen found in HBV – in wasp venom might open novel questions about composition of venoms from even highly diverse species.

SPECIFIC IgE TO WHOLE VENOM PREPARATIONS

Skin and/or sIgE tests with whole venom preparations are regarded as the gold standard in the diagnostics of HVA. Today, a number of companies offer test systems for the detection of sIgE to insect venoms. Results are usually expressed as kU_A/l of allergen-specific IgE based on calibration against a heterologous IgE standard curve [7]. The international cut-off for sIgE detection historically has been set to $0.35\,kU_A/l$. However, the lower limit of quantification (LoQ), that is the analytical sensitivity, of the most widely used modern autoanalyser-based singleplex IgE assays has been accepted by the regulatory authorities as $0.1\,kU_A/l$ [8]. Thus, IgE antibody levels between 0.1 and $0.35\,kU_A/l$ should be reported by the laboratory and must be interpreted by the clinician within the context of the patient's history, clinical symptoms and total serum IgE concentrations.

By using the cut-off value of $0.35\,kU_A/l$, positive IgE test results to HBV have been reported in 90–100% of patients with HVA [9,10▪]. With the same cut-off level, 83–97% of YJV-allergic patients have been reported to test positive for IgE to YJV [9,10▪,11]. Interestingly, sIgE against Ves v 5 could be detected in patients with a clear history of YJV anaphylaxis who were negative for sIgE to YJV, suggesting that the whole venom preparations had a shortage of Ves v 5 immunoreactivity [12–15]. Spiking of YJV with recombinant Ves v 5 increased sensitivity from 83 to 97% [11,15,16]. This improved YJV reagent has been available for routine diagnostics on the ImmunoCAP platform since 2012. In patients with low total IgE and a clear history of anaphylaxis, careful evaluation is needed as sIgE can sometimes be hard to detect [17,18]. Here, IgE antibody levels between 0.1 and $0.35\,kU_A/l$ should be considered and interpreted by the clinician as indicated above.

MULTIPLE SENSITIZATIONS, CROSS-REACTIVITY AND COMPONENT-RESOLVED DIAGNOSTICS

Testing sIgE to whole venom preparations of different Hymenoptera has one major limitation: In cases

Table 1. Current list of Hymenoptera venom allergens as of July 2017 (www.allergen.org)

Allergen	Name/function	MW (kDa)	% DW	Potential ***N***-glycosylation	Diagnostic availability
Honeybees (*Apis mellifera, Apis cerana, Apis dorsata*)					
Api m 1, Api c 1, Api d 1	Phospholipase A_2	17	12	1	+ (rApi m 1)
Api m 2	Hyaluronidase	45	2	3	+ (rApi m 2)
Api m 3	Saure phosphatase	49	1–2	2	+ (rApi m 3)
Api m 4	Melittin	3	50	–	+ (rApi m 4)
Api m 5	Dipeptidylpeptidase IV	100	<1	6	+ (rApi m 5)
Api m 6	Protease inhibitor	8	1–2	–	–
Api m 7	Protease	39		3	–
Api m 8	Carboxylesterase	70		4	–
Api m 9	Carboxypeptidase	60		4	–
Api m 10	CRP/Icarapin	55	<1	2	+ (rApi m 10)
Api m 11	MRJP 8,9	60,65		3,6	–
Api m 12	Vitellogenin	200		1	–
Bumble bees (*Bombus terrestris, Bombus pennsylvanicus*)					
Bom t 1, Bom p 1	Phospholipase A_2	16		1	–
Bom t 4, Bom p 4	Protease	27		–	–
Yellow Jackets (*Vespula vulgaris, *Vespula flavopilosa, Vespula germanica, Vespula maculifrons, Vespula pensylvanica, Vespula squamosa, Vespula vidua*)**					
Ves v 1, Ves m 1, Ves s 1	Phospholipase A_1	34	6–14	–	+ (rVes v 1)
Ves v 2, Ves m 2	Hyaluronidase	45	1–3	2	–
Ves v 3	Dipeptidylpeptidase IV	100		6	–
Ves v 5, Ves f 5, Ves g 5, Ves m 5, Ves p 5, Ves s 5, Ves vi 5	Antigen 5	23	5–10	–	+ (rVes v 5)
Ves v 6	Vitellogenin	200		4	–
Hornets (*Vespa crabro, Vespa magnifica, Vespa mandarina*)					
Vesp c 1, Vesp ma 1, Vesp m 1	Phospholipase A1	34		–	–
Vesp ma 2	Hyaluronidase	35			–
Vesp c 5, Vesp ma 5, Vesp m 5	Antigen 5	23		1	–
Bald-faced hornet (e.g. *Dolichovespula maculata, Dolichovespula arenaria*)					
Dol m 1	Phospholipase A_1	34		2	–
Dol m 2	Hyaluronidase	42		2	–
Dol m 5, Dol a 5	Antigen 5	23		–	–
European paper wasps (*Polistes dominula, Polistes gallicus*)					
Pol d 1, Pol g 1	Phospholipase A_1	34		1	+ (rPol d 1)
Pol d 3	Dipeptidylpeptidase IV	100		4	–
Pol d 4	Protease	33		6	–
Pol d 5, Pol g 5	Antigen 5	23		–	+ (rPol d 5)
American paper wasps (*Polistes annularis, Polistes exclamans, Polistes fuscatus, Polistes metricus*)					
Pol a 1, Pol e 1	Phospholipase A_1	34		–	–
Pol a 2	Hyaluronidase	38		2	–
Pol e 4	Protease	?			–
Pol a 5, Pol e 5, Pol f 5, Pol m 5	Antigen 5	23		–	–
South American paper wasps (*Polybia paulista, Polybia scutellaris*)					
Poly p 1	Phospholipase A_1	34		–	–
Poly p 2	Hyaluronidase	33		2	–
Poly p 5, Poly s 5	Antigen 5	21		–	–

Table 1 (*Continued*)

Allergen	Name/function	MW (kDa)	% DW	Potential *N*-glycosylation	Diagnostic availability
Ants					
Fire ants (*Solenopsis invicta, Solenopsis richteri, Solenopsis saevissima*)					
Sol i 1	Phospholipase A_1	18		3	–
Sol i 2, Sol r 2, Sol s 2		14		–	–
Sol i 3, Sol r 3, Sol s 3	Antigen 5	24–26		2	–
Sol i 4		12		–	–
Jumper ant (*Myrmecia pilosula*)					
Myr p 1		7.5, 5.5		–	–
Myr p 2	Pilosulin-3	8.5, 2.4		–	–
Myr p 3	Pilosulin-4.1			–	–
Asian needle ant (*Pachycondyla chinensis*)					
Pac c 3	Antigen 5	23		–	–

Modified with permission from [2].
*Of note, the Vespula vulgaris antigen Ves v 4, a protease, has been cloned and recombinantly expressed, but has so far not officially been confirmed as an allergen.

of double- or triple-positive test results against different venoms, the assays do not allow discrimination between cross-reactivity and primary sensitization to multiple venoms. According to the assessment of the current American Stinging Insect Hypersensitivity Practice Parameter [19▪], immunological cross-reactivity is extensive between hornet and YJV, somewhat less extensive between yellow jacket and hornet with wasp venoms and less common between honeybee and Vespidea venoms. Data from European studies, however, suggest that there is quite a substantial cross-reactivity also between honeybee and yellow jacket venom [10▪,20–22]. In our own patient cohort (n = 815) from the south/west region of Germany, 45% of patients with anaphylactic sting reactions display positive sIgE to both HBV and YJV [1▪,20].

Double positivity may either reflect true double sensitization to both venoms or may be caused by IgE antibodies to cross-reactive carbohydrate determinants (CCD), which are present in the majority of hymenoptera venom allergens [21,23,24] or to homologous peptide sequences in proteins present in both venoms.

Analysis of single allergens in HBV and YJV allowed the identification of allergens that are present only in one or the other venom and may therefore, serve as so-called marker allergens to unequivocally identify a primary sensitization to a given venom. In the discrimination between primary HBV and YJV sensitization, Ves v 1 and Ves v 5 serve as marker allergens for YJV sensitization whereas Api m 1, Api m 3, Api m 4 and Api m 10 for HBV sensitization.

Other allergens are likely to be cross-reactive based on sequence homology, such as the hyaluronidases Api m 2 and Ves v 2, the dipeptidylpeptidases Api m 5 and Ves v 3 and the vitellogenins Api m 12 and Ves v 6 [25–28]. Significantly, the cross-reactivity of the hyaluronidases appears to be mostly based on IgE reactivity to CCD epitopes as demonstrated with YJV hyaluronidases (Ves v 2a, Ves v 2b) with and without CCDs [28]. In contrast, IgE reactivity to CCD-free HBV hyaluronidase Api m 2 is quite prevalent in HBV-allergic patients ranging from 43 to 52% [29,30▪▪,31▪▪]. Structural data demonstrate that, despite high sequence identity, Api m 2 and Ves v 2 display very little homology when it comes to allergen surface epitopes [32]. Thus, IgE reactivity to CCD-free Api m 2 may also with a grain of salt be interpreted as indicator for a primary HBV sensitization.

RECOMBINANT VENOM ALLERGENS FOR ROUTINE DIAGNOSTICS OF HYMENOPTERA VENOM ALLERGY

Recently, recombinant venom allergens that are devoid of CCD reactivity have been introduced for routine sIgE diagnostics to improve the discrimination between primary sensitization and cross-reactivity [10▪,13,22]. The first study on the use of rApi m 1 and rVes v 5 in IgE diagnostics reported a frequency of sensitization of 97% to Api m 1 among HBV and 87% to Ves v 5 among YJV sensitized patients, using a then available liquid-phase detection system [10▪]. Subsequent studies, using the currently available test systems and marker allergens for YJV, reported sensitization frequencies for rVes v 5 as 84.5–90% and for rVes v 1 as 33–54%. The combination of sIgE measurements in rVes v 5 and

rVes v 1 allowed for the detection of 92–98% of YJV-allergic patients [11,15,20,33,34]. Additional measurements of sIgE to Ves v 2 and Ves v 3 were suggested to further increase the ability to detect YJV sensitization [12], which, however, could not be confirmed by a follow-up study [35].

In contrast to the initial report [10▪], the frequency of sensitization to rApi m 1 in HBV-allergic patients was found to be lower in subsequent studies that used currently available test systems. Reported frequencies range from 58 to 80% [13,31▪▪,36,37], leaving a considerable gap in the ability to detect HBV sensitization by using component-based diagnostic tools. Thus, lack of sensitization to Api m 1 in patients suspected of having HBV allergy has been regarded as insufficient to rule out primary HBV sensitization.

The difference in Api m 1 sensitization rates (ranging from 58 to 80%) has been suggested to reflect regional differences [14] or differences in the definition of the patient population [13]. In addition, the sensitivity of Api m 1 may partly depend on the test system used. Recently, direct comparison of sIgE levels with Api m 1 measured on the Immulite fluid phase test system and the ImmunoCAP solid phase test system suggested a higher sensitivity for the Immulite system [38▪,39▪]. It was speculated that IgE binding capacity of the recombinant Api m 1 used in the ImmunoCAP system may be diminished because of altered protein folding [38▪,39▪]. However, this notion was contradicted by a direct comparison of IgE binding with purified natural Api m 1, which was found to be identical in CCD-negative sera [40]. A more likely cause is an apparent difference in the heterologous calibration system between the two assays, as suggested by one study [39▪]. Indeed, two comparative studies using chimeric IgE antibodies to different allergens provided convincing evidence that the Immulite system tends to overestimate the actual levels of sIgE to a given allergen as approximately three to five-fold [41,42], hence explaining the higher frequency of test results exceeding the cut-off level chosen.

Aside from Api m 1, additional major allergens have been reported in HBV allergy. Analysis of sIgE reactivity to a panel of HBV allergens (Api m 1–5, Api m 10) allowed the detection of 94% of patients with HBV allergy and demonstrated that patients with HBV allergy display a broad spectrum of sensitization profiles [31▪▪]. Particularly interesting was the finding that HBV-specific marker allergens rApi m 3 and rApi m 10 allowed the detection of primary HBV sensitizations in about two thirds of Api m 1 negative sera [43], demonstrating that these components help to reduce the diagnostic gap in detecting HBV sensitization.

HBV allergens currently available for routine sIgE diagnostics include rApi m 1, rApi m 2, rApi m 3, Api m 4, rApi m 5 and rApi m 10 (Table 2). By using this panel of allergens, a recent study [29] reported a combined sensitization frequency of only 79% among the HBV-allergic patients studied and concluded that the currently available allergens are still not sufficient to reliably identify HBV sensitization. The lower frequency of sensitization to at least one of the HBV components observed in this study, as compared with the study by Kohler et al. [31▪▪] may have been due to differences in the patient populations, in particular the number of HBV monosensitized and HBV and YJV double-sensitized patients. We previously demonstrated that in patients with HBV allergy, concomitant sensitization to YJV was associated with higher levels of both total and HBV-specific IgE, as well as higher levels of sIgE to all HBV allergens tested [31▪▪], suggesting effects that were independent of serological cross-reactivity. Accordingly, HBV monosensitized patients mostly displayed lower sIgE levels and recognition of fewer allergens, whereas double-sensitized patients often recognized multiple HBV allergens and with higher sIgE levels [31▪▪]. The same trend was observed in a separate population of patients with YJV allergy, suggesting that this might reflect a more advanced state of atopic immune deviation in the double-sensitized population [31▪▪].

Even more difficult than resolution between HBV and YJV sensitization is the discrimination between sensitization to yellow jacket and Polistes venoms, which is of particular relevance in Mediterranean countries and the Americas. Due to a high degree of IgE cross-reactivity, unequivocal discrimination is rarely achieved. Significantly, Polistes venom proteins are devoid of CCD reactivity [44], so that cross-reactivity is mostly caused by homologous proteins as described for the hyaluronidases (Ves v 2 and the homologous protein in Polistes venom), for the dipeptidylpeptidases (Ves v 3, Pol d 3), the group 5 antigens (Ves v 5, Pol d 5) and to a lesser extent for group 1 antigens (Ves v 1, Pol d 1). Among these venoms, which is the most likely primary sensitizer may however be indicated by the relative level of sensitization to them. To this end, the combination of group 1 and group 5 allergens were reported to identify the most probable sensitizing insect in two thirds of the patients studied, whereas the hyaluronidases (Ves v 2 and the homologous protein in Polistes venom) did not provide any additional value [45]. Currently available allergens for routine sIgE diagnostics to yellow jacket and Polistes venom include rVes v 1, rVes v 5, rPol d 1 and rPol d 5 (Table 2).

Table 2. Selected test systems for sIgE detection to Hymenoptera venom allergens as of July 2017

Allergen	Manufacturer	Test system	Significance
Honeybee			
Api m 1	Thermo Fisher Siemens Euroimmun Dr Fooke Laboratories Macro Array Diagnostics (ALEX and Faber test)	Singleplex, multiplex Singleplex Multiplex Singleplex Multiplex	Marker allergen for HBV sensitization, allows discrimination between HB and YJ/*Polistes* venom sensitization
Api m 2	Thermo Fisher Siemens Euroimmun Dr Fooke Laboratories Macro Array Diagnostics (ALEX)	Singleplex Singleplex Multiplex Singleplex Multiplex	Major HBV allergen. Due to limited cross-reactivity with hyaluronidases of YJV or *Polistes* venom in the absence of CCDs, IgE to Api m 2 can be used as indicator for HBV sensitization
Api m 3	Thermo Fisher	Singleplex	Marker allergen for HBV sensitization, allows discrimination between HB and YJ/*Polistes* venom sensitization
Api m 4	Macro Array Diagnostics (Faber test)	Multiplex	Marker allergen for HBV sensitization, allows discrimination between HB and YJ/*Polistes* venom sensitization
Api m 5	Thermo Fisher	Singleplex	Major HBV allergen. High cross-reactivity with dipeptidylpeptidase of YJV and *Polistes* venom prevents its use as marker allergen
Api m 10	Thermo Fisher Euroimmun Dr Fooke Laboratories Macro Array Diagnostics (ALEX)	Singleplex Multiplex Singleplex Multiplex	Marker allergen for HBV sensitization, allows discrimination between HB and YJ/*Polistes* venom sensitization
Yellow jacket			
Ves v 1	Thermo Fisher Euroimmun	Singleplex Multiplex	Marker allergen for YJV sensitization, allows discrimination between YJV and HBV sensitization; n.b. cross-reactivity with phospholipase of *Polistes* venom
Ves v 5	Thermo Fisher Siemens Euroimmun Dr Fooke Laboratories Macro Array Diagnostics (ALEX)	Singleplex, multiplex Singleplex Multiplex Singleplex Multiplex	Marker allergen for YJV sensitization, allows discrimination between YJV and HBV sensitization; n.b. high cross-reactivity with antigen 5 of *Polistes* venom
European paper wasp			
Pol d 1	Euroimmun	Multiplex	Marker allergen for *Polistes* venom sensitization, allows discrimination between *Polistes* and HBV sensitization; n.b. cross-reactivity with phospholipase of YJV
Pol d 5	Thermo Fisher Euroimmun Macro Array Diagnostics (ALEX)	Singleplex Multiplex Multiplex	Marker allergen for *Polistes* venom sensitization, allows discrimination between *Polistes* and HBV sensitization; n.b, high cross-reactivity with phospholipase of YJV

CCD, cross-reactive carbohydrate determinant; HBV, honeybee venom; YJV, yellow jacket venom.

Additional members of the Vespidae family play a prominent role in other areas of the world, such as the genus Polybia in South America. Polybia belong to the subfamily of Polistinae and a number of allergens have recently been cloned and characterized. These Polybia allergens display a high degree of sequence homology to their counterparts in yellow jacket and Polistes venoms. Again, no unique marker allergen has been identified, which would likely allow reliable discrimination between Polistinae and Vespinae venom sensitization.

Finally, cross-reactivity is also observed between ant venom and YJV. In particular, the antigen 5 allergens exhibit significant degree of sequence identity, rendering differentiation of sensitization to venom of Formicoidea and Vespoidea superfamily members difficult.

In conclusion, the currently available recombinant Hymenoptera venom allergens are useful for the identification of sensitizations to YJV and HBV allergens, not confounded by CCD reactivity, even if a minority (5–20%) of HBV-allergic patients is not sensitized to any of the available HBV allergens. Although Ves v 1 and Ves v 5 negative results exclude YJV sensitization with a high likelihood, negative results to the HBV-specific allergens Api m 1, Api m 2, Api m 3, Api m 4 and Api m 10 do not necessarily exclude HBV sensitization. Here,

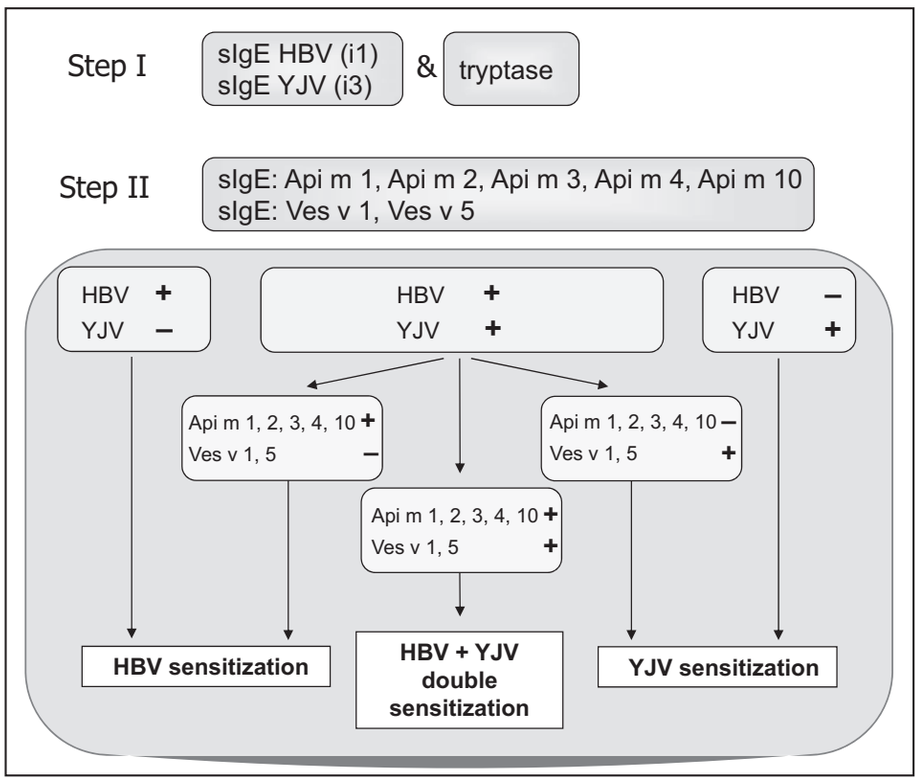

FIGURE 1. Two-step diagnostic algorithm for an improved discrimination between yellow jacket venom and honeybee venom sensitization. Step I: Baseline in-vitro diagnostics; Step II: Component-resolved diagnostics in cases of honeybee venom and yellow jacket venom double-positive cases, or in cases of discrepancies between history, skin test and serology. Please refer to Table 2 for availability of single allergens for routine diagnostics by different manufacturers. HBV, honeybee venom; YJV, yellow jacket venom. Modified with permission from [2].

consideration of low (0.1–0.35 kU_A/l) IgE levels to HBV marker allergens, comparison of IgE levels with cross-reactive allergens or the identification of additional HBV marker allergens would be helpful towards optimizing the diagnostic precision. No major marker allergens that would support definitive discrimination of sensitizations to venoms of different Vespidae subfamilies have so far been identified.

Based on the available allergens, a diagnostic algorithm has been suggested for an improved discrimination between YJV and HBV sensitization (Fig. 1, modified from [2]).

COMPONENT-RESOLVED DIAGNOSTICS FOR PERSONALIZED RISK STRATIFICATION IN VENOM IMMUNOTHERAPY

Component-resolved diagnostics not only supports improved diagnostic precision in HVA but also enables detailed characterization of sensitization profiles of individual patients. Particularly in HBV allergy, patients display a wide spectrum of sensitization profiles [31▪▪], which might be associated with different risks in venom immunotherapy (VIT).

In this context, it was of interest that some of the newly identified major allergens in HBV, namely Api m 3 and Api m 10, were reported to be underrepresented or absent in a number of therapeutic preparations [46▪▪]. This observation prompted us to ask whether treatment failure in honeybee VIT may be associated with certain sensitization profiles. In a retrospective study of VIT-treated HBV-allergic patients, comparison of sIgE levels with HBV and individual allergens identified predominant sensitization to Api m 10 (>50% of sIgE to HBV) as the best predictor of treatment failure with an odds ratio 8.44. No such signal was obtained for dominant sensitization to any of the other allergens [30▪▪]. In this study, again some of the therapeutic HBV preparations analysed displayed lack of Api m 10, whereas their Api m 1 content was comparable with that of crude HBV [30▪▪]. A follow-up analysis of the allergen composition of four different therapeutic venom preparations confirmed the previously reported lack or underrepresentation of Api m 10 [47▪]. In addition, shortage of Api m 3 and Api m 5

was observed in some preparations. Of note, although some allergens are present in large quantities (Api m 1, Api m 2 and Api m 4 with 10%, 3% and >40% of venom dry weight, respectively), Api m 3, Api m 5 and Api m 10 belong to the low abundance allergens with less than 1% of the venom dry weight. Currently, we do not fully understand the role of Api m 10 in HBV allergy and tolerance induction during VIT. However, the high prevalence of Api m 10 sensitization (>50%), the shortage of Api m 10 in widely used therapeutic HBV preparations and the significant association of dominant Api 10 sensitization and treatment failure strongly suggest that Api m 10 is a relevant allergen and that this kind of component-resolved diagnostics may be useful for the risk stratification in honeybee VIT. Even though prospective studies are still lacking, the clinical implication would be that HBV-allergic patients with dominant sensitization to Api m 10 are at increased risk for treatment failure in honeybee VIT and should preferably be treated with HBV preparations demonstrated to contain an adequate amount of Api m 10, sufficient to induce a robust IgG4 response in treated patients.

In a recent study, component-resolved diagnostics using nApi m 1, rApi m 2 and Api m 4 demonstrated a high prevalence of Api m 4 sensitization among HBV-allergic patients who experienced systemic reactions during the induction of honeybee VIT [48]. A subsequent prospective study stratified HBV-allergic patients according to their sIgE to Api m 4 into two groups (<0.98 or >0.98 kU_A/l) and confirmed higher rates of systemic reactions during the VIT induction phase in the latter group. In addition, this group was characterized by increased baseline skin reactivity, increased base line HBV sIgE and more persistent responses in intradermal testing during VIT [49▪▪].

This data supports the concept that component-resolved diagnostics will enable us to define different endotypes of HBV allergy and based on the individual's sensitization profile, allow a personalized risk stratification as well as an optimization of treatment protocols.

UNRESOLVED/OPEN ISSUES

For the use in clinical routine, test reagents ideally should allow a definite discrimination between sensitization to one or the other Hymenoptera venom. Marker allergens such as Ves v 5, Ves v 1 and Api m 1, Api m 3, Api m 4 and Api m 10 allow such discrimination between YJV and HBV sensitization in the majority of cases. However, the limited sensitization prevalence to HBV marker allergens only allows detection of 80–90% of HBV-allergic patients [29,30▪▪,31▪▪]. To further reduce this diagnostic gap, it should be helpful to take advantage of the low end of the assays' measuring range and consider IgE levels down to the LoQ of 0.1 kU_A/l, particularly in patients with low total IgE. In addition, direct quantitative comparison of IgE levels with corresponding cross-reactive HBV and YJV allergens such as Api m 2 and Ves v 2, Api m 5 and Ves v3 or Api 12 and Ves v 6 might prove useful towards identifying the primary sensitizer. The best example are the hyaluronidases Api m 2 and Ves v 2. Api m 2 is a major allergen in HBV allergy, in contrast, IgE reactivity to Ves v 2 is mostly CCD-related and only few patients with YJV allergy display CCD-independent reactivity to Ves v 2 [27,28]. The same approach could be useful in the discrimination between sensitizations to Vespid and Polistinae venoms [45]. However, so far no manufacturer offers test reagents that allow this kind of direct comparison of IgE reactivity with cross-reactive Hymenoptera venom allergens.

The BAT by using whole venom preparations has been demonstrated to be helpful in the investigation of double-sensitized patients or in patients with a clear history of sting reactions but negative sIgE and skin tests [17,50]. The BAT may be of similar diagnostic advantage by using single CCD-free allergens, as recently demonstrated in YJV-allergic patients [12]. So far, component resolution in BAT has only been performed in academic research settings. For routine testing, however, a stringent standardization of allergen preparations, stability and test procedures would be required. Provided this can be achieved, BAT may be helpful to close diagnostic gaps that sIgE determinations leave open.

Finally, data obtained so far on the potential of component-resolved diagnostics for improved risk stratification in VIT is still limited. Prospective randomized studies are needed to put current hypotheses to the test. This is especially true for the retrospective data on Api m 10 regarding the VIT response in patients with HBV allergy.

CONCLUSION

Component resolution provides for a better understanding of the complexity of sensitization and cross-reactivities in HVA. In addition, it has opened up new avenues for identification of biomarkers that may allow risk stratification for VIT responses. The continuously expanding field of venom allergens will permit enhanced resolution and precision in the diagnostic testing of patients with suspected HVA. In addition, improved methods of monitoring therapeutic outcomes and detailed knowledge about the molecular composition of different

therapeutic preparations will enable the selection of appropriate venom preparations for VIT according to the individual sensitization profile. This will help move VIT from a generalized towards a precision-targeted immunotherapy approach, consistent with all other efforts to achieve the goals of personalized medicine.

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Diagnostics in Hymenoptera venom allergy: current concepts and developments with special focus on molecular allergy diagnostics

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Abstract
Background The high rate of asymptomatic sensitization to Hymenoptera venom, difficulty in correctly identifying Hymenoptera and loss of sensitization over time make an accurate diagnosis of Hymenoptera venom allergy challenging. Although routine diagnostic tests encompassing skin tests and the detection of venom-specific IgE antibodies with whole venom preparations are reliable, they offer insufficient precision in the case of double sensitized patients or in those with a history of sting anaphylaxis, in whom sensitization cannot be proven or only to the presumably wrong venom.
Methods Systematic literature research and review of current concepts of diagnostic testing in Hymenoptera venom allergy.
Results and discussion Improvements in diagnostic accuracy over recent years have mainly been due to the increasing use of molecular allergy diagnostics. Detection of specific IgE antibodies to marker and cross-reactive venom allergens improves the discrimination between genuine sensitization and cross-reactivity, and this provides a better rationale for prescribing venom immunotherapy. The basophil activation test has also increased diagnostic accuracy by reducing the number of Hymenoptera venom sensitizations overlooked with routine tests. This paper reviews current concepts of diagnostic testing in Hymenoptera venom allergy and suggests fields for further development.

Keywords Skin test · Recombinant allergens · cross-reactive carbohydrate determinants · Basophil activation test · Diagnostic algorithm

Abbreviations

BAT	Basophil activation test
CAST	Cellular antigen stimulation test
CCD	Cross-reactive carbohydrate determinant
HBV	Honeybee venom
HRP	Horseradish peroxidase
HVA	Hymenoptera venom allergy
sIgE	Specific IgE antibodies
SPT	Skin prick test
VIT	Venom immunotherapy
YJV	Yellow jacket venom

Introduction

Hymenoptera venom allergy (HVA) is one of the most common causes of anaphylaxis in adults and is frequently associated with severe anaphylaxis [1, 2]. It results in significant morbidity and impairment in quality of life [3]. A prevalence of up to 3.5% is reported in Europe [4]. Causal treatment in the form of venom immunotherapy (VIT) is effective and well tolerated.

In Germany the main perpetrators of HVA are yellow jackets (*Vespula*) and honeybees (*Apis*). Bumblebees (*Bombus*) and hornets (*Vespa*) are rarely involved in sting reactions and allergy is usually due to cross-reactivity to honeybee venom (HBV) and yellow jacket venom (YJV), respectively. In America and Mediterranean countries paper wasps (*Polistes*) or white-faced

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Table 1 Conditions that can cause symptoms mimicking anaphylaxis, modified from the guideline for acute therapy and management of anaphylaxis, adapted from [15]

Cardiac arrhythmias
Hypertensive crisis
Pulmonary embolism
Status asthmaticus
Tracheobronchial obstruction
Carcinoid syndrome
Pheochromocytoma
Hypoglycemia
Dissociative disorders and conversion (e. g., Globus hystericus)
Somatoform disorders (e. g., psychogenic dyspnea, "vocal cord dysfunction")
Seizure disorders
Hereditary/acquired angioedema
Intoxication (alcohol, opioids, histaminosis)

hornets (*Dolichovespula*) and some ants (*Formicidae*) are implicated in sting anaphylaxis but these are currently of little relevance in Germany.

According to the current guideline for diagnosis and therapy of bee and wasp venom allergy, only patients with a clinical history of anaphylactic sting reactions should undergo diagnostic testing, and only those with evidence of IgE-mediated sensitization to Hymenoptera venom should be offered VIT [5]. Making a confident diagnosis of HVA is complicated by several factors: The high rate of asymptomatic Hymenoptera venom sensitization in the general population, failure to identify or test for the culprit insect, loss of sensitization profiles over time and conditions mimicking anaphylaxis can all lead to an incorrect diagnosis. Up to 50% of those allergic to Hymenoptera venom are double sensitized to HBV and YJV but usually only one of these sensitizations is clinically relevant [6]. Often the insect responsible for the systemic reaction goes unidentified. When the insect was identified, it should be noted that the ability of the general population to correctly identify Hymenoptera is limited [7]. In order to minimize the risks of not detecting clinically relevant sensitizations, reliable diagnostic tests that accurately identify venom sensitizations are essential.

The diagnosis of HVA is based on a clinical history of Hymenoptera sting-related anaphylaxis and detection of IgE-mediated sensitization. Over recent years, the sensitivity of diagnostic tests has improved, largely due to increasing implementation of molecular allergy diagnostics and to some extent the use of the basophil activation test (BAT). Despite these improvements, current diagnostic tests are not without shortcomings. In particular, in the case of patients double sensitized to HBV and YJV and in those in whom no sensitization is detected, an accurate diagnosis of HVA remains challenging. In this paper we review the diagnostic tests currently available for the investigation of HVA, their benefits and limitations, and suggest areas for further improvement.

Clinical history

Clinically irrelevant sensitizations to Hymenoptera venom occur in 27–40% of the general adult population and up to 50% of children [8–10]. It is important, therefore, to clarify if symptoms consistent with anaphylaxis occurred following a Hymenoptera sting. The risk of a systemic reaction in sensitized subjects with no previous history of HVA lies between 3.3 and 5% [10, 11]. Large local sting reactions occur in up to 26% of the general population and are defined as swellings of >10 cm in diameter lasting for >24 h [12]. In patients with previous large local reactions, the risk of a systemic reaction following a subsequent sting is reported to be less than 10% [13]. As this risk is low no diagnostic work-up is recommended. Similarly, unusual Hymenoptera sting reactions such as serum sickness like reactions or toxic reactions resulting from a large number of stings require no diagnostic work-up [5].

Symptoms of venom anaphylaxis usually occur within thirty minutes of the sting and are frequently associated with skin signs such as pruritus, flushing, urticarial, and angioedema [14]. Common gastrointestinal symptoms in Hymenoptera venom anaphylaxis are nausea and vomiting. Patients suffering anaphylaxis may report prodromal tingling of the palms and soles, restlessness, and a sense of impending doom. Severe anaphylaxis involves the respiratory and cardiovascular systems. Characteristic features are bronchoconstriction and dyspnea, tachycardia, hypotension, diaphoresis, and loss of consciousness. Urinary and fecal incontinence occur with profound circulatory dysregulation and the most severe systemic reactions result in cardiorespiratory arrest. When initial skin signs such as urticaria are followed by cardiovascular or respiratory symptoms, the clinical diagnosis of HVA is straightforward [14]. When this characteristic symptom evolution is absent, the diagnosis can be difficult. A number of conditions can simulate HVA, including chronic spontaneous urticaria, vasovagal syncope, anxiety disorders, cardiogenic shock, and arrhythmias. An incomplete list of differential diagnoses to be considered is shown in Table 1, adapted from [15]. In addition, the clinician should be alert to anaphylaxis featuring predominant circulatory dysregulation without skin signs. This pattern is often observed in patients with underlying clonal mast cell disorders that may have a normal baseline serum tryptase level [16]. The Spanish Network on Mastocytosis (Red Española de Mastocitosis) has developed a scoring system that may help to identify such patients [17, 18].

The identity of culprit insect should be clarified. A recent study assessing the accuracy of the general population in identifying four different Hymenoptera

Table 2 Skin tests with HBV and YJV may be carried out in a stepwise manner or simultaneously depending on severity of anaphylaxis and individual patient risk factors [5, 21]

1)	Skin prick test: 1, 10, 100 µg/ml and intradermal test 1 µg/ml
2)	Skin prick test: 1, 10, 100, 300 µg/ml
3)	Intradermal tests: 0.001; 0.01; 0.1; and 1 µg/ml

species showed almost one third failed to correctly identify yellow jackets, half failed to identify *Polistes* and approximately 10% did not recognize honeybees [7]. Therefore, it is important to remain skeptical regarding the patients' account of the culprit insect. It is often assumed the culprit insect can be identified based on the whether or not the stinger remains in the skin following injection. Due to structural differences, the sting apparatus of a honeybee is more likely than that of a yellow jacket to lodge in the skin. However, whether or not a stinger remains in the skin is influenced by skin characteristics at the sting site. Information on the remaining of a stinger is indicative but not reliable for identifying the stinging insect.

Skin testing

In some countries skin testing is considered the gold standard [19, 20]. In Europe standardized, dialyzed whole venom preparations are available for honey- and bumblebee, yellow jacket, hornet, *Polistes*, and *Dolichovespula*. The process of dialysis removes low molecular weight substances such as biogenic amines that cause nonspecific test reactions. In Germany, bumblebees, hornets, *Polistes*, and *Dolichovespula* are rarely the primary sensitizer in HVA. It is usually sufficient to test with HBV and YJV preparations. Immigrants from Mediterranean or American countries, however, may be primarily sensitized to *Polistes* and/or *Dolichovespula*. In this case testing with further venoms should be considered.

The skin prick test (SPT) is quick, simple to perform, and inexpensive. As severe systemic reactions have occurred following intradermal tests, it has been recommended that intradermal tests should be preceded by a SPT. In 2013 the safety and efficacy of simultaneous intradermal testing in 478 Hymenoptera venom allergic patients with 0.02 ml of 0.001, 0.01, 0.1, and 1.0 µg/ml of HBV and YJV was assessed. A systemic reaction incidence of 0.6% was reported [21] but no severe reactions occurred and none of the reactions could have been prevented by stepwise testing. A recent study of 300 patients with suspected HVA in which skin testing consisted of simultaneous intradermal tests with 0.02 ml of 1.0 µg/ml of five different commercially available venom preparations reported one delayed adverse reaction [22]. Several different protocols for skin testing with Hymenoptera venom exist. Currently used approaches are summarized in Table 2 [5, 21]. Despite the low risk of systemic reactions, the current German guideline for diagnosis and therapy of bee and wasp venom allergy recommends stepwise skin testing in patients with a history of severe anaphylaxis [5].

When interpreting skin tests it is important to know the temporal relationship to the anaphylactic sting event. Skin testing directly after the event should be avoided, since tachyphylaxis may result in false negative results. Most reliable results are obtained 1–6 weeks after the sting event, most likely due to boostering of the relevant venom-specific IgE antibodies through the sting. The rate of loss of sensitization to Hymenoptera venom in skin tests is reported to be 12% per year, with 33% of skin tests becoming negative after 2.5 years [23]. While sensitization remains detectable for many years in a number of patients, negative results may merely reflect a long latency between sting event and diagnostic testing. The use of medications such as corticosteroids, antihistamines, and antihistaminergic antipsychotics can

Table 3 Frequently used medicaments that suppress skin tests together with the duration for which they should be discontinued prior to testing are listed, adapted from [24, 25]

Drug group	Suppression	Period of discontinuation
H1 Antihistamine 1st generation	+++	>3 days
H1 Antihistamine new generation	+++	>7 days
H2 Antihistamine	–/+	2 days
Ketotifen (mast cell stabilizer)	+++	>5 days
Topical glucocorticosteroid (GCS) in the test area >4 weeks	+	>1 week
Systemic short-term GCS		
<50 mg prednisolone	–	3 days
>50 mg prednisolone	–/(+)	>1 week
Systemic long-term GCS		
<10 mg prednisolone	–	0
>10 mg prednisolone	–/+	>3 weeks
Benzodiazepines	+++	>7 days
Omalizumab	+++	4–8 weeks
Tricyclic antidepressants	+++	>14 days
Promethazine (neuroleptic)	++	>5 days

suppress skin test responsiveness and give rise to false negative results. Table 3 lists medications that should be discontinued prior to skin testing, adapted from reviews [24, 25].

Specific IgE antibodies to whole venom preparations

Detecting specific IgE antibodies (sIgE) to whole insect venoms is one of the main diagnostic methods in HVA. At the same time, detection of sIgE to insect venoms is an analytical measurement allowing only the presence or absence of IgE-mediated sensitization to be detected. A diagnosis of HVA can only be made in conjunction with the patient's clinical history.

For the detection of specific IgE to whole venom, various test systems encompassing liquid or solid phase systems and single or multiplex tests are available. The sensitivity of sIgE to HBV in HBV allergic patients is reported to be high (98–100%) [26, 27]. The reported sensitivity of sIgE to YJV is lower (83–93%) [26–28]. It is of limited use to calculate diagnostic specificity and positive predictive values for detecting HVA when evaluating the performance of sIgE testing, since the test system only enables the presence or absence of IgE-mediated sensitizations to be detected and cannot assess clinical relevance.

As with skin tests, in order to make use of the booster effect, venom sIgE should be measured 1–6 weeks after a sting event [5, 12, 29]. Failure to detect venom sIgE in patients with a convincing history of Hymenoptera venom anaphylaxis may be due to a long time interval between the sting reaction and diagnostic work-up. sIgE has been found to decrease between 1 and 4 years after Hymenoptera venom anaphylaxis and may fall below the level of detection with very long latency periods. Earlier assumptions that venom sIgE is consumed by an anaphylactic sting reaction have not been verified [12]. Boostering of venom sIgE following stings from Hymenoptera, to which the patient is not allergic, may give rise to false-positive results.

The internationally accepted cut-off level for detecting sIgE is 0.35 kU/l, however, the analytical sensitivity of modern assays is 0.10 kU/l [30]. As the level of venom sIgE is related to total IgE, venom sIgE between 0.10 and 0.35 kU/l may be clinically relevant in patients with low total IgE and this must be evaluated in the context of the patient history.

An introduction to Hymenoptera venom allergens

Currently 12 honeybee and 5 yellow jacket venom allergens have been characterized in detail and are listed in the official allergen data bank of WHO/IUIS subcommittee on allergen nomenclature [31]. Some allergens present in HBV are specific to honeybee and are not present in the venom of yellow jacket or other

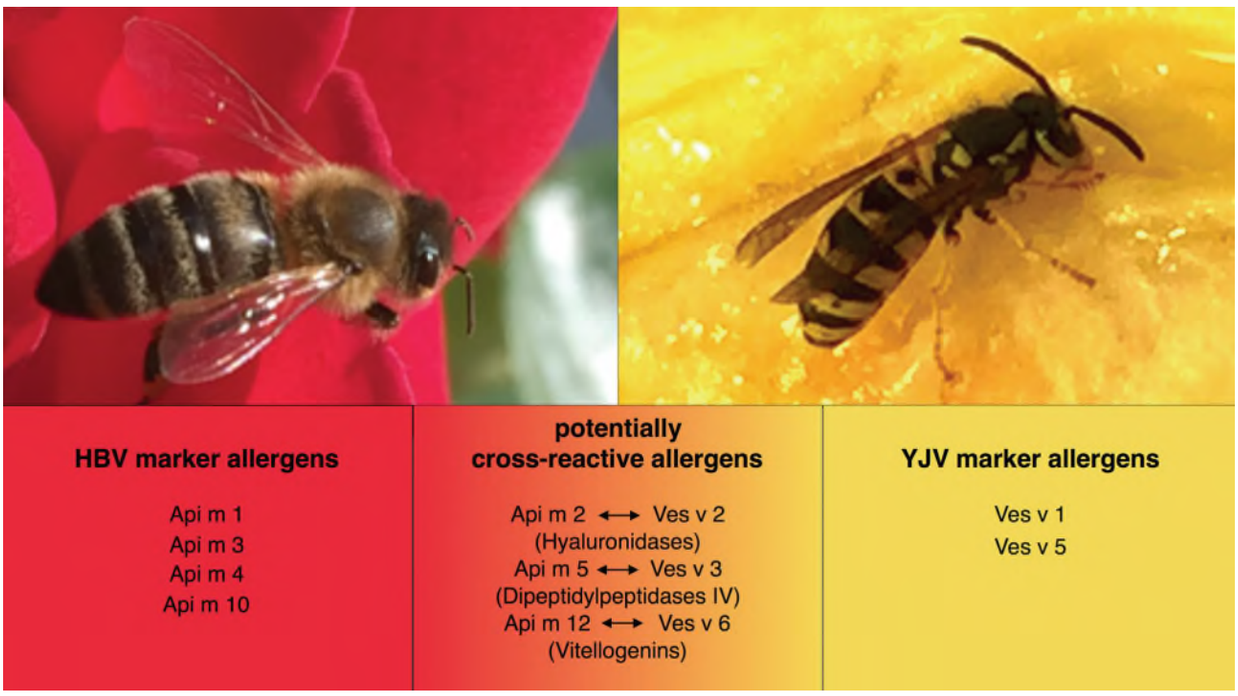

Fig. 1 Honeybee and yellow jacket venom and their respective marker and cross-reactive allergens. *Apis mellifera* marker allergens: *Api m 1, 3, 4* and *10*; *Apis mellifera* potentially cross-reactive allergens: *Api m 2, 5* and *12*. *Vespula vulgaris* marker allergens: *Ves v 1* and *5*; *Vespula vulgaris* potentially cross-reactive allergens: *Ves v 2, 3* and *6*. *HVB* honeybee venom, *YJV* yellow jacket venom, *Api m 1 Apis mellifera* allergen number 1, *Ves v 1 Vespula vulgaris* allergen number 1

Table 4 Overview of Hymenoptera venom allergens relevant in Europe (adapted from [32])

Allergen	Name/function	MW (kDa)	Potential N-glycosylations
Honeybee allergens (Apis spp.)			
Api m 1	Phospholipase A2	17	1
Api m 2[a]	Hyaluronidase	45	3
Api m 3	Acid phosphatase	49	2
Api m 4	Melittin	3	–
Api m 5[b]	Allergen C/DPP IV	100	6
Api m 6	Protease inhibitor	8	–
Api m 7[c]	Protease	39	3
Api m 8	Carboxylesterase	70	4
Api m 9	Carboxypeptidase	60	4
Api m 10	CRP/Icarapin	55	2
Api m 11.0101	MRJP 8	65	6
Api m 11.0201	MRJP 9	60	3
Api m 12[d]	Vitellogenin	200	1
Bumblebee allergens (Bombus spp.)			
Bom p 1, Bom t 1	Phospholipase A2	16	1
Bom p 4, Bom t 4	Protease	27	0.1
Yellow jacket allergens (Vespula spp.)			
Ves v 1	Phospholipase A1	35	–
Ves v 2.0101[a]	Hyaluronidase	45	4
Ves v 2.0201[a]	Hyaluronidase	45	2
Ves v 3[b]	DPP IV	100	6
Ves v 5	Antigen 5	25	–
Ves v 6 [d]	Vitellogenin	200	4
Bald-faced hornet allergens (Dolichovespula spp.)			
Dol m 1	Phospholipase A1	34	2
Dol m 2	Hyaluronidase	42	2
Dol m 5	Antigen 5	23	0
Hornet allergens (Vespa spp.)			
Vesp c 1	Phospholipase A1	34	0
Vesp ma 2	Hyaluronidase	35	4
Vesp c 5	Antigen 5	23	0
European wasp allergens (Polistes spp.)			
Pol d 1	Phospholipase A1	34	1
Pol d 4	Protease	33	6
Pol d 5	Antigen 5	23	0

MW molecular weight
[a,b,d]Refer to homologous allergens
[c]A homologous yellow jacket protease and further honeybee proteases were identified but these have not been described as allergens

Hymenoptera. These are termed marker allergens as they serve as a marker of genuine sensitization to HBV. Examples of honeybee marker allergens are phospholipase A2 (Api m 1), acid phosphatase (Api m 3), melittin (Api m 4), and icarapin (Api m 10). In YJV, phospholipase A1 (Ves v 1) and antigen 5 (Ves v 5) are marker allergens specific to yellow jacket. In addition, some allergens in HBV are similar to allergens in YJV resulting from a high sequence identity. Such allergens are termed homologous or cross-reactive allergens as sIgE of individuals sensitized to one of these allergens in HBV might show cross-reactivity with the homologous allergens in YJV. Cross-reactive allergens in HBV and YJV are the hyaluronidases (Api m 2 and Ves v 2), the dipeptidylpeptidases IV (Api m 5 and Ves v 3), and the vitellogenins (Api m 12 and Ves v 6). Fig. 1 illustrates marker and cross-reactive allergens present in HBV and YJV, respectively. The majority of HBV and YJV allergens are glycoproteins containing one or more oligosaccharides linked to the protein. These carbohydrates often contain an alpha 1.3-linked fucose residue on the N-glycan core that is produced by insects and plants. The resulting structure is known as cross-reactive carbohydrate determinant (CCD) and does not exist in mammals. As a result and due to their widespread prevalence, CCD

are highly immunogenic epitopes that can give rise to the production of sIgE. The clinical relevance of CCD is disputed but the consensus in the case of HVA is that CCD are clinically irrelevant. Table 4 shows an overview of Hymenoptera venom allergens relevant in Germany and Europe including the number of potential glycosylations sites (adapted from [32]).

Between 45 and 50% of Hymenoptera venom allergic patients display double sensitization to both HBV and YJV on diagnostic testing with skin tests and venom sIgE. This makes choosing the correct venom for immunotherapy difficult, when the culprit insect is unknown [6]. Double sensitization to HBV and YJV occurs for three reasons and molecular allergy diagnostics can help clarify the relevance of sensitizations by measuring sIgE to individual allergens present in whole HBV and YJV.

First, double sensitization can represent a genuine double sensitization to both HBV and YJV marker allergens, resulting from previous stings from both of these insects. The clinical relevance of genuine double sensitization depends on the patient history. Second, double sensitization can result from IgE-mediated sensitization to cross-reactive, homologous venom allergens present in HBV and YJV, resulting from a sting from either of the insects. The third and most common cause of double sensitization to HBV and YJV is the presence of sIgE to CCD. This accounts for up to 50% of double sensitizations to HBV and YJV [33]. Specific IgE to CCD can be measured using horseradish peroxidase (HRP) or the glycan structure from pineapple stem bromelain (MUXF3) as test allergen and should be included in the investigation of double sensitized patients. However, sensitization to CCD does not rule out a simultaneous clinically relevant sensitization to an allergen protein epitope [32, 34].

Molecular allergy diagnostics in HVA

Recombinant expression of allergens has enabled the production of CCD-free allergens for diagnostic purposes [35]. As a result, molecular allergy diagnostics have become an integral part of HVA diagnostics. Currently, a limited number of recombinant allergens are commercially available: rVes v 1 and rVes v 5 in the case of YJV and rApi m 1, rApi m 2 and recently rApi m 10 in the case of HBV.

With currently available test systems, sensitization rates of between 85 and 90% for rVes v 5 [28, 36–40] and between 39 and 79% for rVes v 1 are reported [35, 36, 39, 40]. Combining both allergens resulted in a sensitivity of 92–96% for the detection of YJV allergic patients [28, 35, 36, 38–40]. It was previously shown that yellow jacket allergic patients not sensitized to whole YJV subsequently tested positive for rVes v 5 [37, 41]. An increased diagnostic sensitivity of 97% was reported for the detection of sIgE to a rVes v 5 supplemented whole YJV extract compared to 83% using conventional whole YJV [28]. These results led to the spiking of YJV with rVes v 5 by one manufacturer and since October 2012 this spiked YJV preparation has fully replaced that previously commercially available. The remaining currently identified YJV allergens show potential cross-reactivity with homologous allergens in HBV. Studies assessing improved diagnostic precision by detecting sIgE to cross-reactive allergens gave mixed results. Diagnostic testing with ImmunoCAPs and ELISA for the detection of sensitizations to rVes v 1, 2, 3, and 5 allowed a YJV sensitization to be found in 84% of YJV allergic patients who had tested negative using whole YJV extract (n = 19). In HBV allergic patients serologically nonreactive to whole HBV extract, the same study detected sensitizations to rApi m 1, 2, 3, and 5 in 100% (n = 8) [36], suggesting that testing with single components may be more sensitive to detect IgE sensitizations in Hymenoptera venom allergy. This assumption, however, could not be confirmed in a follow-up study with a higher number of patients tested at our center [42]. Currently no marker allergens specific to *Polistes* or *Dolichovespula* have been identified so that patients primarily sensitized to these Hymenoptera venoms will easily be misdiagnosed as allergic to yellow jacket but subsequently inadequately protected by yellow jacket VIT [43].

Phospholipase A 2 (Api m 1) was the first marker allergen to be identified in HBV. Compared to Ves v 5 in the case of YJV allergic patients the sensitivity of Api m 1 in HBV allergy is low. In HBV allergic patients, the prevalence of sensitization to Api m 1 is reported to range between 57 and 97% [26, 37, 44–47]. Based on this, lack of sensitization to Api m 1 in patients suspected of having HBV allergy is insufficient to rule out genuine HBV sensitization. The reported difference in Api m 1 sensitization rates may reflect regional differences as suggested by some [48] or may reflect differences in the definition of the patient population as suggested by others [37, 40]. In addition, the sensitivity of Api m 1 may partly depend on the test system used. Recently, direct comparison of sIgE levels to Api m 1 measured on the Immulite fluid phase test system and the ImmunoCAP solid phase test system suggested a higher sensitivity for the Immulite system [49, 50]. It was speculated that IgE binding capacity of the recombinant Api m 1 used in the ImmunoCAP system may be diminished due to altered protein folding [49, 50]. However, this seems rather unlikely, since direct comparison of IgE reactivity to natural Api m 1 and to the recombinant Api m 1 on the ImmunoCAP system has been shown to be identical in CCD-negative sera [51]. Another suggested cause is possible variance in the interpolation calibration algorithm between the assays [49]. Indeed, two comparative studies using chimeric mouse human IgE antibodies to a variety of different recombinant allergens have provided convincing evidence that the Immulite system tends to overestimate the actual levels

Table 5 Depicts sensitization rates to honeybee and yellow jacket venom allergens in Hymenoptera venom allergic patients as reported in the literature

Allergen source/ allergens	Name/function	Sensitization frequency (%)	No. of patients	Reference
Apis mellifera				
rApi m 1	Phospholipase A2	79 57 78 78 72 97	34 175 100 23 144 100	Hofmann 2011 [37] Korosec 2011 [45] Sturm 2011 [47] Muller 2012 [44] Kohler 2014 [46] Muller 2009 [26]
rApi m 2	Hyaluronidase	46 52 48	82 40 144	Hofmann 2011 [37] Sturm 2011 [47] Kohler 2014 [46]
rApi m 3	Acid phosphatase	38 50	40 144	Grunwald 2006 [55] Kohler 2014 [46]
nApi m 4	Melittin	27 42 23	82 40 144	Hofmann 2011 [37] Sturm 2011 [47] Kohler 2014 [46]
rApi m 5	Dipeptidylpeptidase IV	60 58	35 144	Blank 2010 [56] Kohler 2014 [46]
rApi m 6	Serine protease inhibitor	26	31	McIntyre 2012 [57]
rApi m 10	Icarapin	49 62	68 144	Blank 2011 [58] Kohler 2014 [46]
rApi m 11a (0101) rApi m 11b (0201)	Major royal jelly protein 8/9	15/34	47	Blank 2012 [59]
rApi m 12	Vitellogenin	44	45	Blank 2013 [60]
Combination	rApi m 1, rApi m 2, rApi m 3, nApi m 4, rApi m 5, rApim 10	94	144	Kohler 2014 [46]
Combination	rApi m 1, rApi m 2, rApi m 3, rApi m 5	92	86	Cifuentes 2014 [36]
Vespula vulgaris				
rVes v 1	Phospholipase A1	79 54 39 58	14 148 86 109	Seismann 2010 [35] Ebo 2013 [39] Cifuentes 2014 [36] Hofmann 2011 [40]
rVes v 2a (0101) rVes v 2b (0201)	Hyaluronidase Hyaluronidase**inactive isoform	5 28 20	41 86 41	Seismann 2010 [35] Cifuentes 2014 [36] Seismann 2010 [35]
rVes v 3	Dipeptidylpeptidase IV	57 50	35 86	Blank 2010 [56] Cifuentes 2014 [36]
rVes v 5	Antigen 5	90 90 90 87 85 90	59 148 308 86 200 109	Hofmann 2011 [37] Ebo 2013 [39] Vos 2013 [28] Cifuentes 2014 [36] Korosec 2012 [38] Hofmann 2011 [40]
rVes v 6	Vitellogenin	39	28	Blank 2013 [59]
Combination	rVes v 1 + r Ves v 5	93 92 98 96 96	14 200 148 308 109	Seismann 2010 [35] Korosec 2012 [38] Ebo 2013 [39] Vos 2013 [28] Hofmann 2011 [40]
Combination	rVes v 1, rVes v 2, Ves v 3, rVes v 5	95	86	Cifuentes 2014 [36]

of sIgE to a given allergen approximately 3–5 fold [52, 53]. Thus, as concluded by one of the studies [52], just because two systems present their results in the same units does not mean that the results are necessarily correct or interchangeable.

Further allergens occurring in lesser abundance in HBV have since been identified as major allergens including Api m 3 and Api m 10. Sensitizations to these allergens are present in 50 and 62% of HBV allergic patients, respectively. An extended repertoire of HBV marker allergens (Api m 1, Api m 3, Api m 4, and Api m 10) significantly increased the diagnostic sensitivity for detection of HBV sensitization and reached nearly 90% compared to 72% for Api m 1 alone [46]. In addition, a high individual heterogeneity of sensitization profiles to HBV allergens was found. Similarly

in patients double sensitized to HBV and YJV that had not identified the culprit insect, the combination of Api m 1, Api m 3, and Api m 10 increased the diagnostic sensitivity to 78.6% compared with 54% using Api m 1 alone. Sensitizations to Api m 3 and Api m 10 were detected in two thirds of patients that had tested negative to Api m 1, thus, providing evidence of the need for treatment with both honeybee and yellow jacket VIT in these patients [54]. In Table 5 reported sensitization rates to HBV and YJV allergens and combinations of allergens are shown.

From our own data, up to 10% of patients with a convincing history of HVA will have negative skin tests. In some of these, venom sIgE will also be negative. In a previous study, 14% of patients with a convincing history of HVA with both negative skin tests and nondetectable venom sIgE subsequently suffered anaphylaxis following Hymenoptera sting challenge [61]. Initial findings suggested molecular allergy diagnostics could improve the diagnostic sensitivity in the detection of HVA in patients testing negative for HBV- and YJV-sIgE [36]. A subsequent study failed to verify this and found no diagnostic benefit of molecular allergy diagnostics in patients with negative skin tests and lack of venom sIgE [42].

Cellular tests

The basophil activation test (BAT) is not a first-line test but its role in the diagnostics of HVA is well established. It requires expertise with respect to both its practical implementation and interpretation of results and is usually reserved for use in secondary care centers. CD203c and CD63 molecules are both expressed on basophil granule membranes. Following allergen-induced activation, basophils express these molecules on the cell surface and can be quantified by flow cytometry. The sensitivity for the BAT measuring CD63 expression is reported as 89%, for CD203c expression 97% [62]. The use of the CD63 BAT is more widespread. Negative controls in basophil activation tests show a background basophil activation of approx. 10%. As a result a level of 15% basophil activation has been chosen as the cut-off level to identify Hymenoptera venom sensitizations [63].

In patients with no detectable venom sIgE but a convincing history of HVA, an IgE-mediated sensitization can be detected with the BAT in 80% [64] and in 60% of those also negative in skin tests, making it a particularly useful diagnostic tool in this subgroup [65]. Similarly where diagnostics and history show contradictory results, the BAT may detect missed Hymenoptera venom sensitizations. In a study of 63 patients with mastocytosis and a history of HVA but no evidence of sensitization to Hymenoptera venom with sIgE or skin testing, the BAT did not detect any further sensitization [66]. This suggests that the efficacy of the BAT may be reduced in mastocytosis patients or possibly in those with low total IgE levels.

As with skin tests and venom sIgE, the ability of the BAT to provide reliable results is hampered by the presence of CCD in whole venom extracts [67]. BAT has been suggested to be helpful in the investigation of double sensitized patients who reacted to only one sting in the past or in those where molecular-based allergy diagnostics are ambiguous. In particular the BAT using CCD-free species-specific allergens (Ves v 1 and Ves v 5) was shown to improve diagnostic precision in the detection of YJV allergy [63]; however, it is unclear if, in the case of sensitization to cross-reactive allergens such as Ves v 2 and Api m 2 or Ves v 3 and Api m 5, any differentiation between primary and cross-reactive sensitizations is possible. As the BAT is not fully standardized, the results of different studies are difficult to compare. False-positive BAT results may be caused by high venom concentrations. False-negative results may occur with the absolute number of basophils evaluated are less than 150, or as with other diagnostic tests, a long interval between sting event and diagnostic work-up.

In the histamine release test, a precursor of the BAT, histamine released by activated basophils was quantified. The finding that not only basophils but also platelets contributed to histamine release reduced the diagnostic reliability of this test. The histamine release test is laborious, expensive, and has largely been replaced by the BAT. The cellular antigen stimulation test (CAST) measures sulfidoleukotriene release by activated basophils and may be helpful in isolated cases.

Further diagnostic tests

In some countries, an intentional sting challenge is included in the diagnostic work-up of patients with suspected HVA. If a systemic reaction occurs, an intentional sting challenge confirms the clinical relevance of a sensitization; however, it can lead to severe systemic reactions. The diagnostic sting challenge is therefore highly controversial [68]. It has been argued that a diagnostic sting challenge reduces the socioeconomic burden of HVA. Using a diagnostic sting challenge to confirm the clinical relevance of a sensitization, one study group argued that VIT could be withheld from 83% of YJV and 56% of HBV allergic patients, due to tolerance of the diagnostic sting challenge [69]. Another study from the same time showed the diagnostic sting challenge to be unreliable as 21% of patients tolerating an initial sting challenge developed anaphylaxis following a second sting challenge [70]. Importantly, half of those reacting to the second challenge suffered severe anaphylaxis. In Germany, diagnostic sting challenges in the case of HVA are no longer recommended as the risks clearly outweigh the benefits [5].

IgE-inhibition tests with whole venom are expensive and time consuming. Due to the complexity of individual patient sensitization profiles, the added benefit of inhibition tests is probably minimal in most

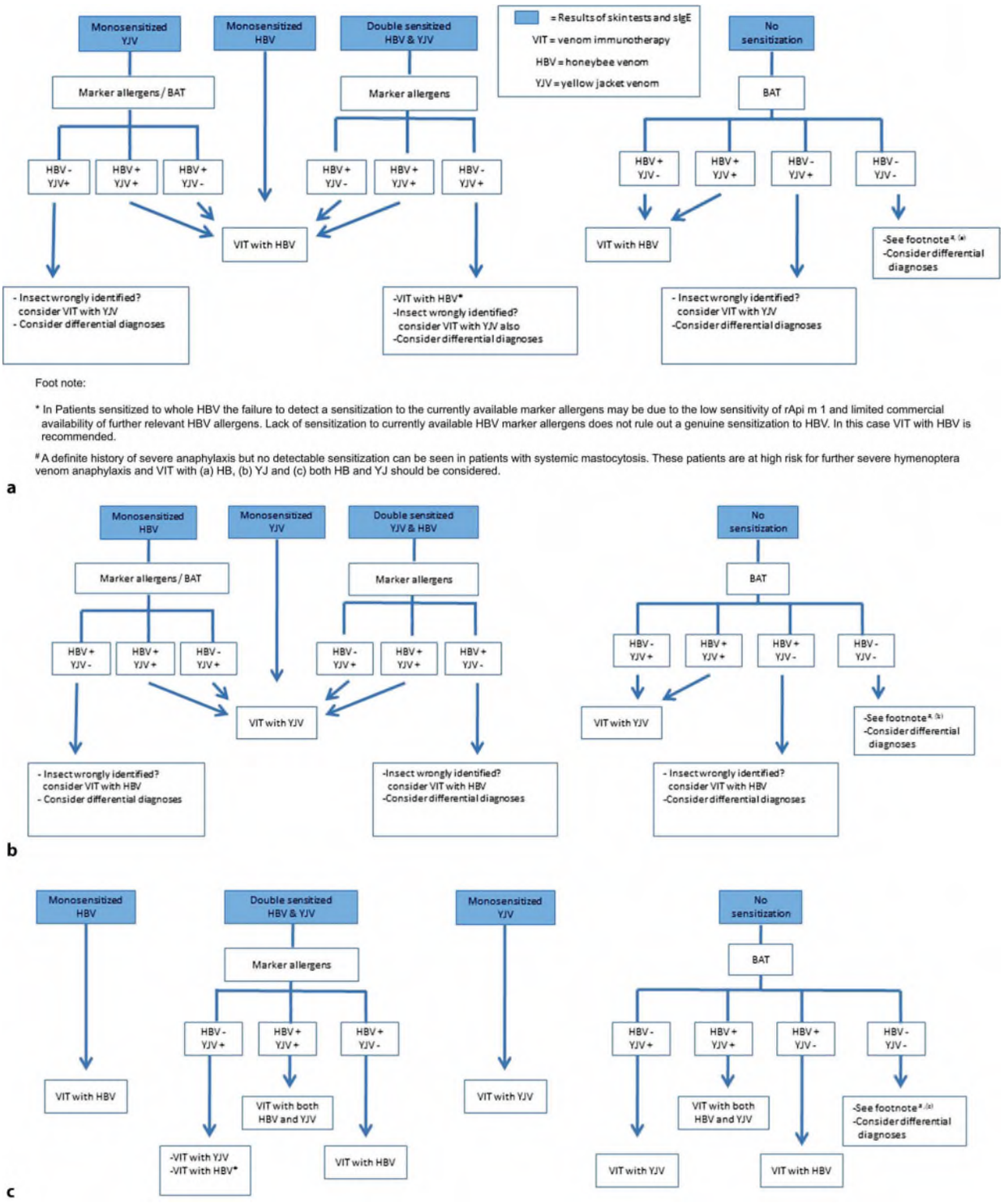

Fig. 2 Recommended diagnostic algorithm for the investigation of Hymenoptera venom allergic patients. **a** Insect honeybee (as reported by the patient), **b** Insect yellow jacket (as reported by the patient), and **c** Insect not identified by the patient

cases. In addition, their use in patients with low sIgE is limited. IgE-inhibition tests may be useful in isolated cases, e. g., for detecting primary sensitizations in patients double sensitized to *Polistes* and YJV, where discriminating marker allergens are not yet available [71].

Diagnostic algorithm

All patients with a history of HVA require a basic diagnostic work-up encompassing a medical history, clinical examination, skin testing, and detection of total and venom sIgE to HBV and YJV. For risk stratification, it is useful to determine baseline serum tryptase. The following management algorithm guides the clinician through the steps required to make a competent diagnosis of HVA. The algorithm assists the choice of venom for VIT based on the test results and patient history. The algorithm is summarized in Fig. 2 and assumes differential diagnoses in Table 1 are considered unlikely.

Culprit insect honeybee according to the patient

Patients reporting honeybee as the culprit insect that are monosensitized to HBV require no further diagnostics and receive honeybee VIT (Fig. 2a). Patients with the same history but monosensitized to YJV should undergo further investigation with HBV marker allergens/BAT. If a HBV sensitization is detected, honeybee VIT is indicated. If a genuine monosensitization to YJV is the only finding, the reason may be incorrect identification of the culprit insect. VIT with YJV should be considered.

Patients double sensitized on basic diagnostic tests, need further investigation with marker allergens in order to clarify the cause of double sensitization. Those with a genuine sensitization to HBV should receive VIT with HBV. Sensitization to YJV can be considered irrelevant. Where only a genuine sensitization to YJV marker allergens is detected, the possibility that the culprit insect was in fact a yellow jacket must be considered. The low sensitivity of rApi m 1 and limited availability of further relevant HBV allergens means a relevant genuine sensitization to HBV cannot be ruled out. VIT with HVB is recommended and VIT with YJV should be considered.

In patients with a definite history of sting-related anaphylaxis but negative diagnostics, VIT with HBV may still be considered, in particular in patients at high risk for severe sting-related anaphylaxis, e. g., mastocytosis patients or those having suffered anaphylaxis with cardiorespiratory arrest. In patients with mast cell disease, the history alone may be the only indication of HVA due to very low levels of circulating IgE.

Culprit insect yellow jacket according to the patient

Patients reporting yellow jacket as the culprit insect that are monosensitized to YJV receive VIT with YJV (Fig. 2b). Those reporting a yellow jacket but monosensitized to honeybee require further diagnostics with marker allergens/BAT. If a genuine sensitization to YJV is detected, then VIT with YJV is indicated. If diagnostics with marker allergens/BAT contradict the history and detect only a genuine HBV sensitization, the insect may have been wrongly identified and VIT with HBV should be considered.

Patients double sensitized on routine diagnostics require further investigation with marker allergens. Detection of a genuine sensitization to yellow jacket or genuine double sensitization provides a rational for prescribing VIT with YJV. Again if a genuine sensitization to HBV marker allergens is the only finding, then the option of VIT with HBV should be discussed with the patient.

Patients with the same history but no evidence of any sensitization should be further investigated with a BAT. Those sensitized to YJV in the BAT receive VIT with YJV. A HBV sensitization alone suggests the insect was incorrectly identified and VIT with HBV should be considered. In patients with a definite history of severe sting-related anaphylaxis but entirely negative diagnostics, VIT with YJV may be considered in patients at high risk for severe sting-related anaphylaxis.

Culprit insect not identified by the patient

Patients that were unable to identify the culprit insect and that are monosensitized to HBV on routine diagnostic work-up should receive VIT with HBV (Fig. 2c).

Those double sensitized at this level require testing with marker allergens. Those with evidence only of a genuine sensitization to HBV marker allergens require VIT with HBV; those genuinely double sensitized receive double VIT. In those only genuinely sensitized to YJV marker allergens, the relatively low sensitivity of Api m 1 and Api m 10, and limited availability of further marker allergens means VIT with YJV is indicated and additional VIT with HBV should still be considered.

Patients that are monosensitized to YJV on routine diagnostics receive VIT with YJV. When basic diagnostics reveal no sensitizations, a BAT should be performed and VIT chosen according to the sensitization profile obtained. If no sensitizations are detected with this step, but there is a definite history of severe sting-related anaphylaxis, VIT with HVB and YJV may be considered in patients at high risk for severe sting-related anaphylaxis.

Perspective

Optimal management of HVA patients can be challenging. Molecular allergy diagnostics have signifi-

cantly improved the diagnostic precision in HVA but a diagnostic gap remains. Until recently, the main limiting factor has been the commercial availability of only a few marker allergens (Ves v 1, Ves v 5, Api m 1, and Api m 10). The release of Api m 2, Api m 3, and Api m 5 as additional HBV allergens in 2016 will further improve diagnostic accuracy in the future. Our own data showed that the combination of Api m 1, 2, 3, 4, 5, and 10 detected 94% of honeybee venom allergic patients. We speculate that the commercial availability of further, albeit cross-reactive HBV allergens may help to further differentiate primary honeybee and yellow jacket sensitizations. The homology between cross-reactive allergens of HBV and YJV reaches 45–50%. We hypothesize that comparing the magnitude of sensitizations to cross-reactive homologous allergens, e. g., Ves v 2 and Api m 2 or Ves v 3 and Api m 5, may help to identify primary sensitizations, as a greater degree of sensitization, i. e., IgE reactivity, would be expected to the clinically relevant venom. Similarly it remains to be seen what role the BAT with CCD-free cross-reactive allergens may have in improving the diagnostic sensitivity in patients double sensitized to HBV and YJV. Finally, molecular sensitization profiles may not only help us to improve diagnostic precision, but may also prove to be useful as risk markers for treatment failure in VIT, as quite recently demonstrated for dominant Api m 10 sensitization in HBV allergy [72].

Conflict of interest T. Jakob has received research grants and speakers honoraria from Thermo Fischer/Phadia Freiburg, Germany and Uppsala, Sweden in addition to research support from ALK-Abello, Allergy Therapeutics, Allergopharma, Cormetics Europe and Novartis. D. Rafei-Shamsabadi declares that he has no competing interests. E. Spillner is cofounder of PLS-Design. S. Müller has received speakers honoraria from Novartis, Bencard and Travel reimbursement from ALK Abello.

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Alpha-tryptasemia: is there a link with wasp venom allergy, mastocytosis, and mast cell activation syndrome?

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Tryptase is a protein contained in mast cells and less abundantly in basophils. Upon activation of mast cells enzymatically active mature tryptases, that are tetrameric proteases stored in granules, are released. On the other hand, monomeric pro-tryptases are constitutelvy secreted in to serum and contribute to the basal serum tryptase (BST).

Elevated BST can be found in end-stage renal disease or in myeloid clonal expansion including mastocytosis. Moreover it has been recently discovered that elevated BST could inherited in an autosomal dominant pattern.

The tryptase locus, which is located on the short arm of chromosome 16 at position 13.3, contains 4 tryptase-encoding genes (TPSG1, TPSB2, TPSAB1, and TPSD1). The isoforms of tryptases that contribute to the serum tryptase measured by the clinical laboratories are encoded by TPSB2 and TPSAB1 that encode for beta and either alfa or beta respectively.

Increased mono-allelic TSAB1 copy number leads to increased BST and define a condition called hereditary alpha-tryptasemia. Affected individuals display BST above 8 ng/ml and express a wide spectrum of clinical phenotypes. How increased copy number of TSAB1 alleles determine the clinical manifestations needs still to be elucidated.

Clinical symptoms reported by individuals with hereditary alpha-tryptasemia include functional gastrointestinal complaints, flushing and pruritus and less frequently urticaria. Although these symptoms are suggestive of mast cell mediatore release, in most of the cases there is no biochemical evidence of mast cell activation (MCA), namely increase in serum tryptase from the individual's baseline to plus 20% + 2 ng/ml in concomitance with occurence of symptoms, as estabilished by the consensus criteria for mast cell activation syndrome

(MCAS). Inherited TSAB1 quintuplicaion, has been recently reported in a non atopic Belgian family presenting with elevated SBT, recurrent episodic severe abdominal cramping and diarrhea in concomitance with biochemical evidence of MCA.

The association between elevated SBT and severe anaphylaxis in the context of IgE-mediated hymenoptera venom allergy is mainly attributable to an underlying clonal mast cell disease. Noticeably, in mastocytosis patients hymenoptera venom allergy prevalence does not increase constantly with increasing levels SBT. On the other hand these systemic reactions are reported in 20% of patients with alpha-tryptasemia; a prevalence that is higher than general population. Whether increased TPSAB1 copy number contribute to increased risk of systemic reactions to hymenoptera venom allergy it requires further studies.

In a small study, mastocytosis patients were reported to be twice as likely to have two alpha-tryptase containing alleles compared to the general population. One of the the Belgian individual carrying a TSAB1 quintuplication presented the missense KIT D816V mutation and hepatosplenomegaly consistent with the diagnosis of monoclonal mast cell activation syndrome.

Whether alpha-tryptasemia can actually modify or contribute to clonal mast cell disease in vivo, however, requires additional investigation. Indeed, because of the gene-dosage effect upon both basal tryptase levels and expressivity in patients with hereditary alpha tryptasemia, should such a relationship be substantiated, we would expect a proportional effect of this genetic lesion on mast cell clonal expansion.

In conclusion, elevated BST can occur with clonal expansion of mast cells and other myeloid cells, but may occur more commonly with inherited increases in TPSAB1 copy number. Distinguishing between these causes has potential diagnostic value and clinical implications. Tryptase genotyping should be considered for inclusion as part of the work-up of patients with elevated BST.

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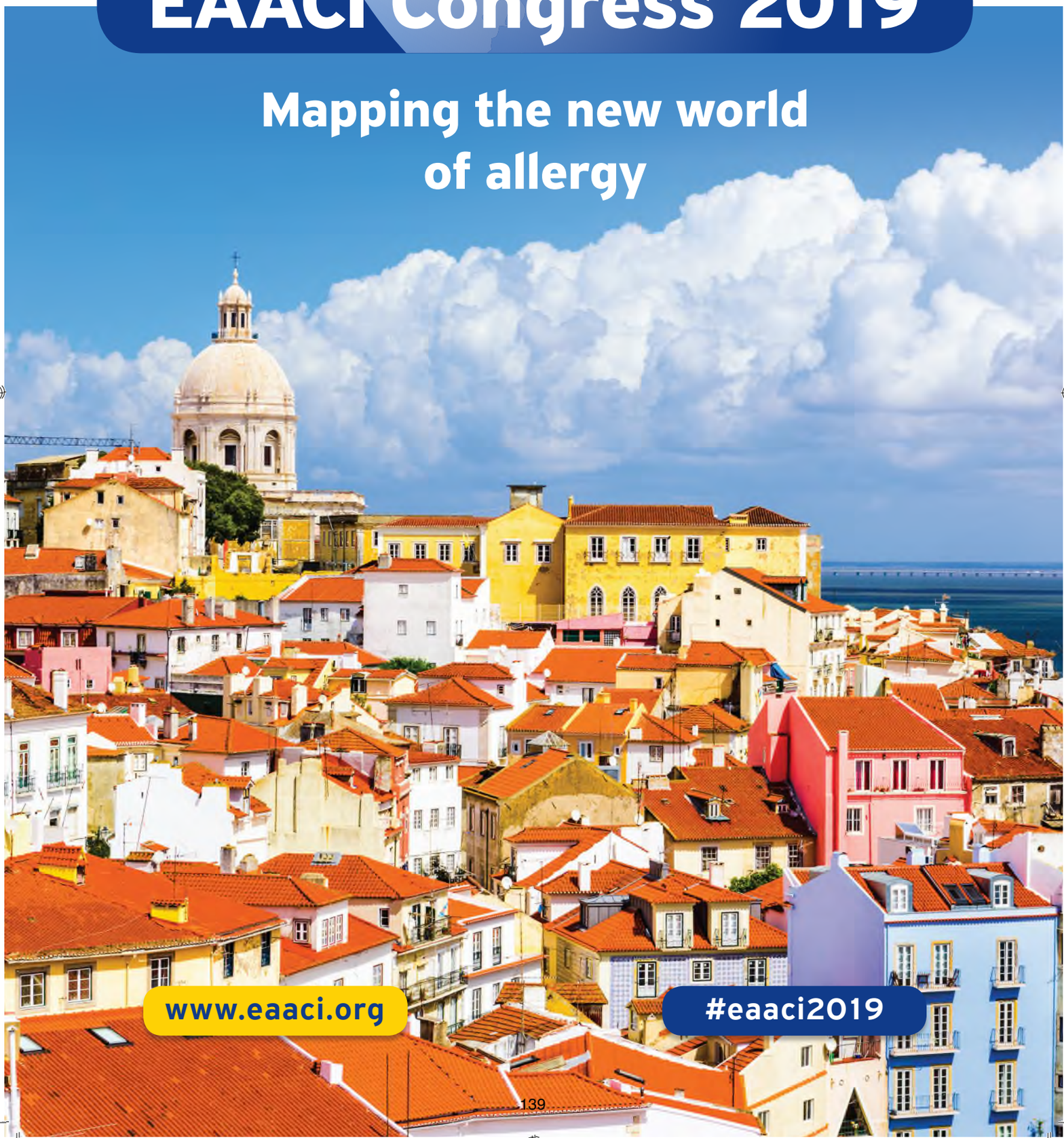

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