

Basophil Activation Test for the Diagnosis of Hymenoptera Venom Allergy in Childhood: a Pilot Study

Basophilenaktivierungstest zur Diagnostik der Insektengiftallergie im Kindesalter – eine Pilotstudie

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Key words

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- basophil activation test
- CD63

Schlüsselwörter

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Bibliography

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Abstract

Introduction: Cellular in vitro tests such as the CD63-based basophil activation test (BAT) have been successfully used to diagnose hymenoptera venom sensitization in adult patients while this has not been investigated in children so far.

Methods: 15 children (9 male, 6 female; 12.7±3.5 years) with suspected allergy to vespa (VE) or honey bee (HB) venom entered this study. Besides serum tryptase (ST) levels, sensitisation against VE and HB was assessed by titrated skin testing and determination of venom-specific serum IgE (sIgE) in all patients. After stimulation with 50 ng of insect venom, CD63-expression of activated basophils was measured by flow cytometry.

Results: Skin testing permitted identification of the culprit insect in 7 patients, 3 cases were diagnosed by additional sIgE measurements. In addition, BAT identified mono-sensitization in 3 further patients with double sensitization upon skin and sIgE testing. Test sensitivity was lower for the BAT (67–75%) than for skin testing (89–100%) and sIgE determination (100%). Neither basophil activation nor sIgE serum levels were identified as reliable predictors of sting reaction severity. In all patients, ST measurements yielded values below the upper reference value.

Conclusion: The current pilot study suggests a possible clinical benefit of BAT analysis in the diagnostic workup of pediatric insect venom allergy. However, further large-scale trials are required to investigate whether the BAT reliably contributes to the correct identification of the culprit insect venom. Due to its comparatively low sensitivity, the BAT should currently not be used in isolation from, but only in combination with established diagnostic instruments.

Zusammenfassung

Einleitung: Der CD63-basierte Basophilenaktivierungstest (BAT) wird erfolgreich zum Nachweis bei adulten Patienten eingesetzt; entsprechende Daten für das Kindesalter liegen bisher nicht vor. Ziel dieser Pilotstudie war es daher, den klinischen Nutzen des BAT bei Kindern mit Insektengiftallergie zu charakterisieren.

Methoden: Retrospektiv wurden 15 Kinder und Jugendliche (9 männlich, 6 weiblich; 12.7±3.5 Lebensjahre) untersucht, die innerhalb von 3 Jahren wegen einer kutanen oder systemischen Bienen- oder Wespenstichreaktion vorgestellt wurden. Es wurden die Reaktionsschwelle durch Hauttestungen mit ansteigenden Hymenopterengiftkonzentrationen ermittelt, insektengiftspezifische IgE-Antikörper (sIgE) und die Serum-tryptase (ST) bestimmt. Die Messung aktivierter (CD63⁺) basophiler Granulozyten erfolgte durchflusszytometrisch nach Stimulation mit je 50 ng Bienen- und Wespengiftextrakt.

Ergebnisse: Bei 7 Patienten erlaubten bereits Hauttestungen eine Bestimmung der auslösenden Insektenart, in 3 weiteren Fällen ließ sich das klinisch relevante Allergen durch zusätzliche sIgE-Bestimmung identifizieren. Ergänzend ergab der BAT bei 3 weiteren Patienten, die in Haut- und FEIA-Testung eine Doppelsensibilisierung aufwiesen, den Befund einer Monosensibilisierung. Die Sensitivität des BAT war mit 67–75% niedriger als die der Hauttestungen (89–100%) und des sIgE (100%). Ein Zusammenhang der Basophilenaktivierung, des sIgE oder der ST mit dem Schweregrad der insektengiftallergischen Reaktion ließ sich nicht nachweisen.

Schlussfolgerung: Der BAT stellt auch im Kindesalter eine vielversprechende Ergänzung der etablierten allergologischen Diagnostik dar. Aktuell sollte er jedoch nur komplementär zu den etablierten Testverfahren eingesetzt und in zukünftigen, prospektiven Studien größerer Patientenpopulationen weitergehend evaluiert werden.

Abbreviations

BAT	basophil activation test
FEIA	fluorescence enzyme immunoassay
FITC	fluorisoithiocyanate
HB	honey bee
SPT	skin prick test
IDT	intradermal test
PE	phycoerythrine
slgE	allergen-specific immunoglobulin E
ST	serum tryptase
tlgE	total serum IgE
VE	vespula

Introduction

In Europe and North America, up to 5% of the general population is affected by hymenoptera venom anaphylaxis. In contrast, only 0.8% of all children have been shown to reveal systemic hymenoptera sting reactions, although insect venom represents the second most common cause of anaphylaxis in this age group [9, 15, 17]. Whereas more than 60% of affected children reveal exclusively cutaneous symptoms such as urticaria or angioedema, a maximum of 10% show more severe reactions such as cardiocirculatory depression or anaphylactic shock [14]. Additionally, fatal sting reactions have been reported in children and adolescents [12, 24]. Thus, if suspicion arises, an adequate diagnostic workup based on a thorough medical history is required in order to identify the culprit insect and facilitate successful specific immunotherapy.

In daily clinical practice, diagnostic standard procedures comprise skin prick and intradermal tests with venom extracts. Additionally, highly-standardized enzyme immunoassays are commercially available for the detection of insect venom-specific serum IgE antibodies (slgE) [1]. However, these conventional in vivo and in vitro methods yield false negative results in 6–20% of investigated individuals [7, 9]. Moreover, the obtained data are often ambiguous, because both honey bee and wasp venom-specific IgE are detected in the same patient, particularly in serological investigations [2, 11, 25].

Against this background, ancillary cellular test systems for the diagnosis of insect venom sensitization have been evaluated in recent years [6, 8, 21, 22, 26]. In particular, basophil activation tests (BATs) relying on the flow cytometric analysis of the cell surface marker CD 63 revealed a promising clinical performance in studies of adult patients with insect venom allergy [4, 23].

Surprisingly, comparable data regarding the application of BAT in pediatric patients have not been published so far. Hence, it was the aim of this retrospective pilot study to evaluate the feasibility and the potential clinical usefulness, i.e. diagnostic sensitivity and specificity, of the BAT in the diagnostic workup of systemic insect sting reactions in children and adolescents.

Materials and methods

Patients

We identified 15 children and adolescents who had presented to our outpatient departments over a period of 3 years (January 2006 to December 2008) for further allergological assessment of suspected immediate-type allergy against honey bee (HB; *Apis*

mellifera) or wasp (VE; *Vespula germanica* and *Vespula vulgaris*). A standardized questionnaire was used to obtain a detailed medical history including co-morbidities, concomitant medication as well as the time course and clinical characteristics of the original sting reaction. None of the included patients presented with a history suggestive of successive systemic reactions to both HB and VE venom, as recalled by the patients or their caregivers.

Skin tests

In accordance with current national and European guidelines, skin prick tests were performed with serial 10-fold dilutions of HB and VE venom extracts (ALK-Abelló, Frederiksberg, Denmark) at concentrations ranging from 0.1, 1 and 10–100 µg/ml. In case of negative skin prick tests, additional intradermal tests were performed with 1 µg/ml of insect venom; alternatively, intradermal tests were performed with venom concentrations of 0.001, 0.01, 0.1 and 1 µg/ml, respectively [1, 19].

Fluorescence enzyme immunoassay (FEIA)

Upon first visit, venous blood samples of all patients were collected prior to skin testing and obtained serum probes were aliquoted and stored at – 80° C until further analysis.

In patient sera, slgE to HB and VE venom, total serum IgE (tlgE) and serum tryptase (ST) were determined with a commercially available fluorescence enzyme immunoassay (UniCAP™ FEIA, Phadia, Uppsala, Schweden). As recommended by the manufacturer, slgE and tlgE were quantified in kU/l, whereas ST was measured in µg/l.

Basophil activation test (BAT)

The BAT used in the current study (Flow-Cast®, Bühlmann Laboratories, Switzerland) is based on the flow cytometric detection of activated basophils, which are characterized by an enhanced surface expression of CD 63 upon allergen stimulation. In all patients, BATs were performed within the first 6 months, but not earlier than 6 weeks after the original sting reaction.

According to the product manual, the leukocyte fraction of EDTA whole blood (3 ml per patient) was isolated by centrifugation within 30 min after sample collection. After careful resuspension with 300 µl of stimulation buffer (heparin, interleukin-3) leukocytes were incubated with a) 50 ng of HB and VE venom (ALK-Abelló, Frederiksberg, Denmark), b) stimulation buffer (negative control) and c) a monoclonal anti-FcεRI antibody (positive control) in pyrogen-free test tubes at 37° C over 40 min.

After termination of the stimulation phase by incubation on ice, the obtained cells were incubated with an antibody reagent containing monoclonal phycoerythrine (PE)-marked anti-CD63 antibodies as well as monoclonal fluorisoithiocyanate (FITC)-marked anti-IgE-antibodies. After erythrocyte lysis, probe fixation and terminal centrifugation, the fraction of activated (CD63⁺) basophils within the total number of all (IgE⁺) basophils was determined with a flow cytometer (FACSan®, Beckton Dickinson Immunocytometry System, San Jose, USA) (○ Fig. 1). As recommended by the manufacturer and current publications, a percentage of > 10% CD63⁺ cells was considered as a positive result [3].

Statistics

Data obtained by FEIA analysis were expressed as mean values with corresponding standard deviations (SD), whereas the

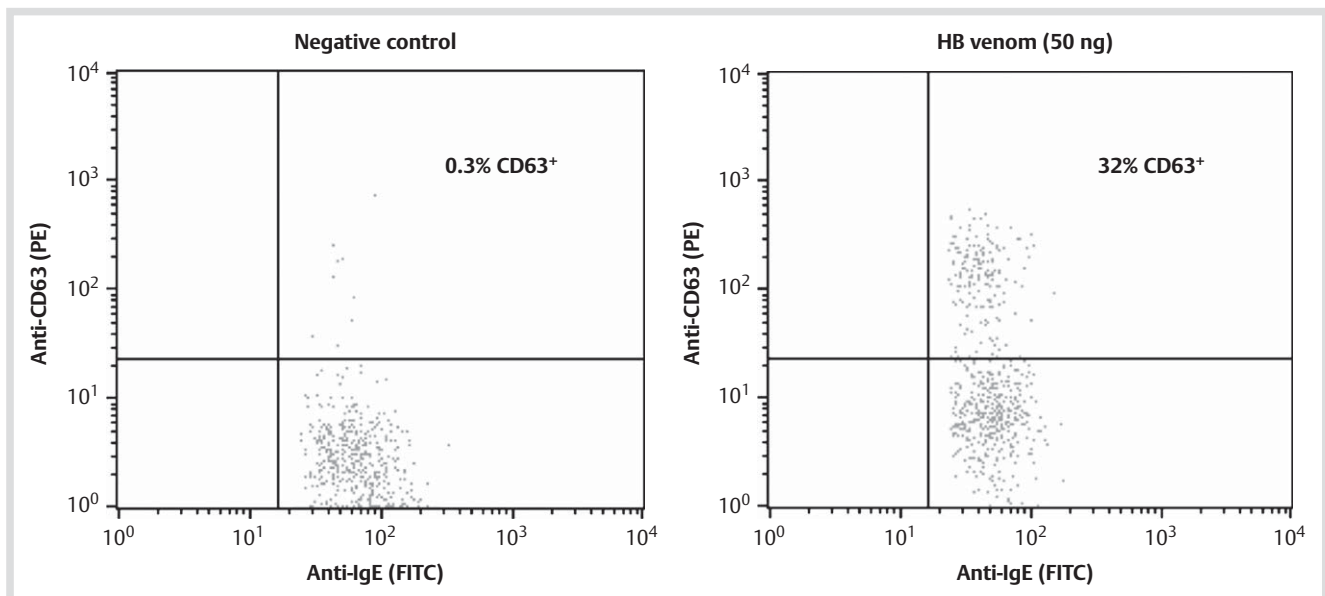


Fig. 1 Positive CD63-based BAT of a child with HB allergy. After detection of basophils with a fluorescence-tagged anti-IgE antibody, (Anti-IgE FITC) and incubation with 50 ng of HB extract CD63 was significantly upregulated (32 % CD63⁺) as compared to the negative control (0.3 % CD63⁺).

results of BAT analyses were described as percentage of activated basophils (% CD63⁺). The association of serum parameters with BAT results was illustrated by Spearman correlation coefficients (r_s).

The 2 sided, unpaired Student's t-test was used for group comparison between patients with distinct clinical characteristics (HB vs. VE venom allergy, atopic vs. non-atopic, moderate vs. severe anaphylaxis). Statistical significance was assumed at $\alpha < 0.05$. Statistical analyses were performed with the S Plus® 6.1 software package (Insightful Corp., Seattle, USA) and the Sigma-Plot 2004® Version 9.01 software (Systat Software, Erkrath, Germany) was used for graphing histograms.

Results



Patient characteristics

15 children and adolescents (9 male, 6 female) with a mean age of 12.7 ± 3.5 years (range: 6–17 years) were included in the current study (● **Table 1**). Systemic medications with antihistamines, leukotriene receptor antagonists, glucocorticoids or other immunosuppressive agents for a period of at least 4 weeks prior to allergological testing could be excluded in all patients.

The severity of insect venom-induced anaphylaxis was graded according to Messmer and Ring [20]: 6 patients (40%) presented with a history of grade I, i.e. exclusively cutaneous reactions (pruritus, flush, generalized urticaria, angioedema), whereas 8 patients (53%) showed additional extracutaneous symptoms corresponding to grade II anaphylaxis (dyspnea, dizziness). Only 1 patient revealed a more severe reaction (grade III) and had to be treated in an intensive care unit due to unconsciousness and anaphylactic shock. HB allergic patients developed more severe reactions significantly more often than VE allergic individuals (71 vs. 50%). In addition, 8 patients (53%) reported at least one atopic co-morbidity, e.g. asthma, atopic dermatitis, allergic rhinoconjunctivitis or food allergy.

Tab. 1 Demographic and clinical characteristics of the included children and adolescents with hymenoptera venom allergy (n = 15).

	n (%)	mean value (SD*)
age (years)		12.7 (± 3.5)
sex		
male	8 (53)	
female	7 (47)	
anaphylaxis-severity [§]		
I: cutaneous symptoms	6 (40)	
II: extracutaneous symptoms	8 (53)	
III: anaphylactic shock	1 (7)	
IV: cardiocirculatory arrest	0 (0)	
total serum IgE (kU/l)		537 (± 585.8)
specific IgE (kU/l)		
honey bee		13.8 (± 24.7)
vespula		6.8 (± 11.0)
serum tryptase ($\mu\text{g/l}$)		4.5 (± 2.4)

[§] modified according to Messmer and Ring [21]

* SD: standard deviation

Skin testing

In 11 patients (73%) skin prick and/or intradermal testing yielded positive reactions to HB venom, whereas 10 patients (67%) reacted to VE venom. In 7 cases (47%) a double-sensitization to both HB and VE venom could be detected, while skin tests remained completely negative in 1 patient only (● **Table 2**).

Fluorescence enzyme immunoassay

At a mean tIgE value of 537 ± 585.8 kU/l. (39.9–2246 kU/l) VE-specific IgE antibodies could be detected in 13 serum samples at a mean concentration of 6.8 ± 11.0 kU/l (0–39.8 kU/l). HB-sIgE was present in 13 patients at a mean concentration of 13.8 ± 24.7 kU/l (0–100.0 kU/l). 11 patients (73%) exhibited antibodies against both insect venoms. ● **Table 2** summarizes the obtained data in 7 semiquantitative classes. In all cases, ST measurements yielded values below the upper reference value of 11.4 $\mu\text{g/l}$ (mean value: 4.50 ± 2.42 $\mu\text{g/l}$; range: 2.14–10.10 $\mu\text{g/l}$).

Tab. 2 Results of the basophil activation test (BAT), skin prick (SPT) and intradermal (IDT) tests as well as allergen-specific serum IgE (sIgE) antibodies in 15 children and adolescents with insect venom allergy.

ID	Culprit insect [§]	Clinical severity	BAT neg. contr. [%CD63]	BAT pos. contr. [%CD63]	BAT HB venom [%CD63]	BAT VE venom [%CD63]	SPT/IDT HB venom	SPT/IDT VE venom	sIgE HB venom [CAP-class*]	sIgE VE venom [CAP-class*]	Total serum IgE [kU/l]
1	VE	1	4.7	21.8	4.4	4.0	–	+	0	2	97.5
2	VE	2	0.9	41.9	3.5	37.0	+	+	3	3	134
3	VE	1	1.3	98.2	2.3	58.2	–	+	2	3	838
4	VE	2	6.3	82.8	4.5	10.6	+	+	0	2	333
5	VE	2	0.4	71.9	1.2	24.7	+	+	2	4	817
6	VE	1	3.4	55.3	4.4	37.4	–	+	6	4	1050
7	HB	2	3.8	69.3	69.6	2.2	+	–	3	1	39.9
8	HB	1	2.5	15.5	6.7	0.9	–	–	3	0	80
9	HB	3	4.4	74.7	4.8	0.9	+	+	2	0	476
10	HB	2	9.1	81.0	75.5	2.3	+	–	3	2	536
11	HB	2	2.4	18.4	10.2	5.0	+	+	2	2	134
12	HB	1	4.9	90.7	87.0	5.3	+	–	4	3	1125
13	HB	2	2.6	61.9	38.5	3.4	+	–	3	2	2246
14	HB, VE	2	0.3	10.6	31.9	27.6	+	+	3	3	83.5
15	HB, VE	2	5.1	20.7	5.8	4.6	+	+	2	2	64.5

[§]Culprit insect identified by combined analysis of medical history, BAT, sIgE, IDT and SPT data

*CAP-class (CAP) 0: <0.35 kU/l, CAP 1: 0.35–0.70 kU/l, CAP 2: 0.70–3.50 kU/l, CAP 3: 3.50–17.50 kU/l, CAP 4: 17.50–50.00 kU/l, CAP 5: 50.00–100 kU/l, CAP 6: >100 kU/l

Basophil activation test

Individual BAT results are displayed in **Table 2**. After incubation with the negative control, the mean patient background was determined at $3.5 \pm 2.3\%$ CD63⁺ basophils. After stimulation with monoclonal anti-FcεRI antibodies (positive control) a CD63⁺-fraction >10% (mean value: $54.3 \pm 29.3\%$) could be detected in all specimen.

In the total study population, BAT analysis with HB and VE venom induced a mean basophil stimulation of $23.3 \pm 29.1\%$ CD63⁺ (4.8 – 75.5% CD63⁺) and $14.9 \pm 17.1\%$ CD63⁺ (40.9 – 58.0% CD63⁺), respectively. Serum levels of sIgE antibodies to insect venom and BAT results were found to correlate moderately with regard to VE venom ($r_s=0.37$) whereas correlation of the 2 parameters was strong with regard to HB venom ($r_s=0.87$). Comparison between patients with exclusively cutaneous symptoms and patients with concurrent extracutaneous reactions or between children with and without atopic co-morbidities did not reveal significantly different BAT, sIgE or ST results.

Identification of the culprit insect venom

Skin testing alone permitted the identification of the suspected culprit insect in 7 mono-sensitized patients (47%). Additional determination of sIgE antibodies by FEIA analysis facilitated the recognition of the clinically relevant insect venom in 3 further cases (20%). Furthermore, BAT analysis disclosed the predominant sensitization in another 3 individuals who had revealed cross-sensitization in skin and FEIA testing. In 2 patients, FEIA and skin tests yielded positive results for both insect venoms that could be verified as true double sensitization by additional RAST inhibition (data not shown). This was corroborated by BAT in 1 patient whereas the BAT remained negative in the other individual (**Table 2**). Considering the 2 double-sensitized patients, immediate-type allergy against VE venom could be diagnosed in 8 patients, whereas 9 individuals were shown to be allergic to HB venom. BAT results of both groups are further delineated in **Fig. 2**.

BAT sensitivity was calculated in relation to the culprit insect venom as identified by combined analysis of medical history, skin tests and all performed in vitro analyses. In HB allergic

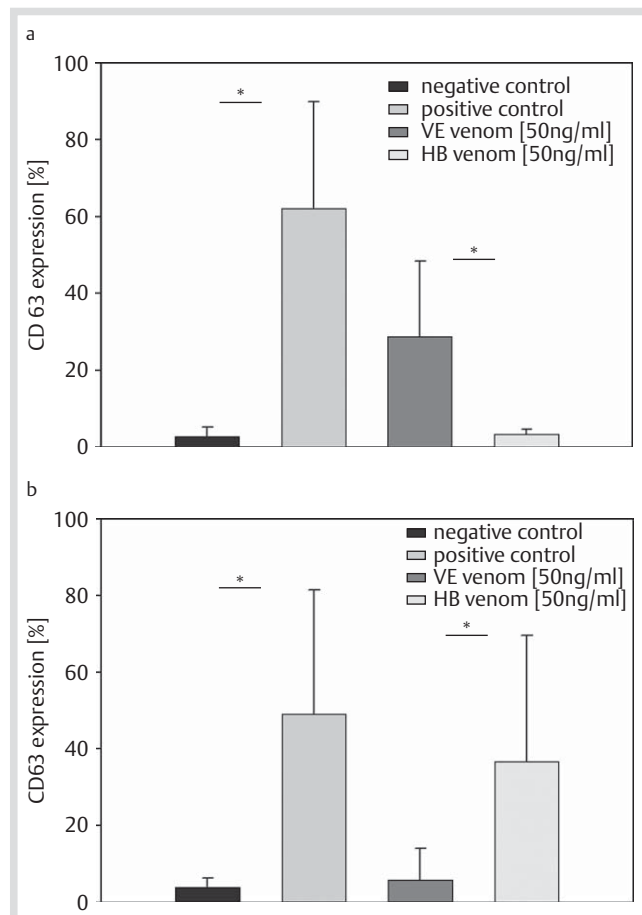


Fig. 2 (a) Mean values of basophil CD 63 expression after incubation with stimulation buffer (negative control), anti-FcεRI antibodies (positive control), 50 ng VE venom and 50 ng HB venom extract in the group of VE allergic patients (n=8). (b) Mean values of basophil CD 63 expression after incubation with stimulation buffer (negative control), anti-FcεRI antibodies (positive control), 50 ng VE venom and 50 ng HB venom extract in the group of HB allergic patients (n=9).

patients, BAT sensitivity (67%) was lower than that of skin testing (89%) and FEIA analysis (100%) in HB allergic children. Likewise, BAT sensitivity reached 75% in patients with VE allergy, whereas skin and FEIA testing had a sensitivity of 100%, respectively. Although a global test specificity could not be calculated in the absence of a control group, the BAT reached a “relative specificity” of 100% with reference to the group of patients identified as mono-sensitized to either HB or VE venom by skin and FEIA testing.

Discussion

The flow cytometric measurement of allergen-driven basophil activation has been demonstrated to be a clinically useful diagnostic tool in adult patients with insect venom allergy. Moreover, the BAT has been successfully used to identify hydroxyethyl starch as the causative agent of severe anaphylaxis in a child undergoing general anesthesia [10]. Surprisingly, comparable investigations have not been performed in pediatric patients with insect venom allergy so far [6,8,21–23,26]. Hence, it was the aim of the present pilot study to delineate the possible clinical role of BAT in the diagnosis of allergic insect sting reactions in children and adolescents.

First, we were able to demonstrate that the utilized insect venom BAT can be performed successfully even with small sample volumes of preschool and older children. In contrast to previous investigations, where up to 15% of patients revealed impaired basophil reactivity (“non-releaser”), adequate BAT reactions to the implemented positive control were detectable in all patients included in our study [13].

The diagnostic gold standard of sting provocation tests was neither used in the present study nor in the majority of comparable previous investigations. This is mainly due to current national and international guidelines which do not recommend sting provocation tests in patients who have not yet undergone specific immunotherapy, because these individuals run a particularly elevated risk of severe reactions. At the same time the negative predictive value of sting testing is still considered insufficient by the majority of investigators [19].

Related to conventional in vivo- and in vitro-diagnostic tools, we found BAT sensitivity to be lower in HB (67%) and VE (75%) allergic children than in previously described adult populations in whom comparable CD63-based assays reached sensitivities of 87–100% [21,23]. Moreover, in contrast to earlier investigations, the eliciting insect venom could not be identified by BAT analysis in our patients with negative skin test results [7,26]. These discrepancies might be attributable to the fact that we performed basophil stimulation with only one concentration of hymenoptera venom, whereas previous studies in adults used serial dilutions of HB and VE venom extracts. However, the establishment of these diagnostic dose-response curves is critical, particularly in early childhood, due to the required sample volumes. In addition, as demonstrated in the majority of previous investigations, positive BAT results can be obtained using comparatively low concentrations of insect venom (<100 ng/l) [26]. Still, further titrated BAT studies are warranted to determine the optimal test concentrations in children and adolescents with insect venom allergy. Moreover, in order to avoid false-negative results, the BAT should only be used as an adjunct to conventional diagnostic methods (skin tests, FEIA) which have been shown

to be more sensitive (>90–100%) in the current and previous investigations [3].

Up to 40% of adult patients with insect venom allergy are known to reveal serological cross-reactivity against HB and VE venom which has been observed more frequently in atopic patients with elevated tIgE serum levels [2,11,25,27]. Accordingly, 73% of our pediatric study population with a high proportion of atopic individuals and elevated tIgE levels expressed sIgE against both hymenoptera venoms as determined by one conventional test method. In 5 patients (33%), both skin testing and sIgE measurements implied cross-sensitization. Interestingly, BAT demonstrated mono-sensitization to only 1 insect venom in 3 of these patients (60%) which is in line with earlier publications reporting similar BAT results in 44–83% of adults with positive skin or sIgE reactions to both venoms [7,26]. However, further controlled studies are warranted in order to elucidate whether the BAT possesses sufficient discriminatory power to differentiate between mere cross-sensitization and genuine, clinically relevant double-reactivity [26].

Whether recombinant, species-specific venom allergen components might be helpful in this context remains to be elucidated. Of note, up to 90% of adult HB allergic patients were shown to express sIgE against the bee venom major allergen Api m 1 and nearly 90% of adults with VE allergy revealed sIgE against the wasp venom major allergen Ves v 5. False positive results were detected in only 17% of cases, respectively [16]. Therefore, the application of recombinant HB and VE allergen components in BAT-based test systems represents a novel diagnostic option warranting further investigation. However, the corresponding allergen components have not been commercially available so far and, therefore, could not be evaluated in our and previous investigations.

Comparing children with exclusively cutaneous sting reactions and those with additional extracutaneous symptoms, no significant differences could be identified with regard to in vitro test results (BAT, sIgE, ST). This is in line with previous publications illustrating that neither basophil activation nor sIgE serum levels may serve as reliable predictors of sting reaction severity [5,6,26].

In contrast, elevated ST levels are widely recognized as an established risk factor for severe anaphylactic reactions in adults with insect venom allergy. Consequently, its measurement is recommended in all affected adults by national and international guidelines [1,18]. Yet, our results suggest that ST is not a valid predictor of reaction severity in children and adolescents with insect venom allergy. However, due to the comparable low sample size of our study, further investigations are required to clarify whether ST determination is generally unnecessary in insect venom allergic children and adolescents.

In conclusion, the results of our retrospective pilot study suggest a possible clinical benefit of BAT analysis in the diagnostic workup of insect venom allergy in childhood and adolescence. However, further large-scale prospective trials are required to investigate whether the BAT might contribute to the correct identification of the clinically relevant insect venom allergen if routine diagnostics remain inconclusive. Due to its limited diagnostic sensitivity and the lack of association between test results and clinical sting reaction severity, BATs should currently not be used in isolation from, but only in combination with established diagnostic instruments in the context of a comprehensive allergological anamnesis.

Conflict of interest: The authors have no conflict of interest to disclose.

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