**Component-resolved IgE and IgG4 profiling reveals robust IgG4 responses primarily to abundant Hymenoptera allergens during venom immunotherapy and in beekeepers**

**Running title: IgE & IgG4 responses to Hymenoptera allergens**

Simon Blank1,\*, Britta Dorn2, Peter Seiringer3, Johanna Grosch1, Benjamin O. Slusarenko1, Michael Dittmar1, Robert Kaczmarcyk3, Danielle Rogner3, Andreas Jung2, Laura Plail3, Bernadette Eberlein3, Tilo Biedermann3, Ulf Darsow3, Knut Brockow3, Carsten B. Schmidt-Weber1, Thilo Jakob2,\*

1Center of Allergy and Environment (ZAUM), Technical University of Munich, School of Medicine and Health & Helmholtz Munich, German Research Center for Environmental Health, Munich, Germany

2Experimental Dermatology and Allergy Research Group, Department of Dermatology and Allergology, Justus-Liebig-University Gießen, Gießen, Germany

3Department of Dermatology and Allergy Biederstein, Technical University of Munich, School of Medicine and Health, Munich, Germany

\*Corresponding authors

**Address correspondence to:**

Prof. Dr. Simon Blank

Center of Allergy and Environment (ZAUM)

Technical University of Munich & Helmholtz Munich

Ingolstädter Landstraße 1, 85764 Munich, Germany

Phone: +49-89-318-726-25, Email: simon.blank@tum.de

Prof. Dr. Thilo Jakob

Experimental Dermatology and Allergy Research Group

Department of Dermatology and Allergology

Justus-Liebig-University Gießen

Gaffkystr. 14, 35385 Gießen, Germany

Phone: +49-641-985-43201, E-mail: thilo.jakob@derma.med.uni-giessen.de

**Word count:** 3697 (including >100 times Api m x or Ves v x counted as 3 words)

**Funding**

This study was funded in part by a research grant (CABAL 1848) from Thermo Fisher Scientific to SB. The company had no influence on the study design, the collection, analysis, and interpretation of data, or the decision to submit the article for publication. Moreover, this work was supported by the Helmholtz Association, Future Topic “Immunology and Inflammation” (ZT-0027) to SB and CBS-W and by the Von Behring-Röntgen-Stiftung, Marburg (66-0004) to TJ.

**Conflict of interest**

SB has given advice or has received honorarium for talks or research grants from Bencard Allergie, Thermo Fisher Scientific, Allergy Therapeutics, Allergopharma, LETI Pharma, and the Helmholtz Association of German Research Centers, outside the submitted work. BE reports financial support from Bühlmann Laboratories, outside the submitted work. TB has given advice or has received honorarium for talks or research grants from ALK-Abelló, Almirall, GSK, Leo Pharma, Lilly, Novartis, Sanofi-Genzyme, Regeneron Boehringer-Ingelheim, Viatris, and Celgene-BMS, outside the submitted work. CBS-W has given advice or has received honorarium for talks or research grants from Allergopharma, DFG, BMBF, Zeller, German Center for Lung Research, Pinsent Masons, and LETI Pharma, outside the submitted work. TJ reports grants and personal fees from Allergy Therapeutics/Bencard Allergie, ALK-Abelló, Leo-Pharma, Novartis, Sanofi-Genzyme, and Thermo Fisher Scientific, outside the submitted work. The other authors declare no competing interests.

**Author contributions**

SB and TJ initiated and supervised the study, analyzed data, and wrote the manuscript. BD performed experiments, analyzed data, and critically revised the manuscript. PS, RK, DR, AJ, and LP recruited patients, collected and analyzed patient data, and critically revised the manuscript. JG performed experiments. BOS, BE, and TB discussed the data and critically revised the manuscript. MD recruited beekeepers and collected and analyzed data. UD, KB, and CBS-W supervised the study, discussed the data, and critically revised the manuscript.

**Abstract**

**Background:** Venom immunotherapy (VIT) and natural exposure to Hymenoptera venoms induce immunological tolerance in allergic patients and beekeepers, respectively. Specific IgE (sIgE) and IgG4 (sIgG4) antibodies play crucial roles in allergic reactions and immune tolerance.

**Objective:** To investigate the dynamics of sIgE and sIgG4 responses to Hymenoptera venoms in patients undergoing VIT and in non-allergic beekeepers at a component-resolved level.

**Methods:** Serum samples from patients allergic to honeybee venom (HBV) or yellow jacket venom (YJV) and from beekeepers were collected during the first year of VIT and around the beekeeping season, respectively. sIgE and sIgG4 levels to whole venom and molecular allergens were measured using the ImmunoCAP platform.

**Results:** Pronounced sIgE and sIgG4 responses to Ves v 1 and 5 in YJV-allergic patients were accompanied by an increased detection of Ves v 1 sensitization after up-dosing. While sIgE profiles in HBV-allergic patients were highly diverse, with a particular contribution of Api m 1 and Api m 10 sIgE, the sIgG4 response during VIT was strongly dominated by Api m 1. Different VIT preparations did not significantly affect the sIgG4 response to low abundance HBV allergens. In beekeepers, allergen sIgG4 induction was dependent on sting frequency and also dominated by Api m 1.

**Conclusion:** Robust IgG4 induction during VIT and natural venom exposure occurs primarily to abundant allergens and is unaffected by the choice of VIT preparation. The effectiveness of VIT and beekeepers' tolerance to HBV indicate that strong sIgG4 responses to low abundance allergens are not crucial for immunological tolerance.

**Keywords:** beekeeper, component-resolved diagnostics, IgE, IgG4, immune tolerance, natural exposure, venom allergy, venom immunotherapy

**Summary box**

*What do we know about this topic?*

Venom immunotherapy and natural venom exposure induce immunological tolerance in allergic patients and beekeepers, respectively, with sIgE and sIgG4 antibodies playing key roles in allergic reactions and immune tolerance.

*How does this study impact our current understanding and/or clinical management of this topic?*

This study reveals that sIgG4 induction during venom immunotherapy and natural exposure occurs primarily to high-abundance allergens and is independent of venom immunotherapy preparation choice, suggesting that robust sIgG4 responses to low-abundance allergens are not essential for effective immunological tolerance.

**Abbreviations**

AIT, allergen-specific immunotherapy

CRD, component-resolved diagnostics

HBV, honeybee venom

HVA, Hymenoptera venom allergy

sIgE, specific immunoglobulin E antibodies

sIgG4, specific immunoglobulin G4 antibodies

V, visit

VIT, venom immunotherapy

YJV, yellow jacket venom

**Introduction**

Hymenoptera venom allergy (HVA) represents one of the most severe IgE-mediated hypersensitivity reactions, with the risk of fatal outcomes [1]. Despite its severity, it stands out as the allergic condition most effectively treated by allergen-specific immunotherapy (AIT), specifically venom immunotherapy (VIT) [2], which demonstrates success rates for honeybee venom (HBV) allergy and yellow jacket venom (YJV) allergy of 77-95% and 91-99%, respectively [3-5]. This not only highlights the potential of AIT in effectively treating severe allergies but additionally renders profound insights into the mechanisms of AIT-induced tolerance. On the other hand, high-dose HBV exposure in frequently stung non-allergic beekeepers represents a model to study allergen tolerance in healthy individuals [6].

The role of IgE in allergic reactions leading to the clinical symptoms of allergy is well-documented [7]. In contrast, IgG4 is believed to play a protective role by blocking allergen-IgE interactions and reducing inflammation [8]. The regulation of IgG4 production relies on assistance from T-helper type 2 cells, a fact linking IgG4 and IgE responses [9].

AIT regulates both IgE and IgG4 antibodies. During therapy, sIgE levels initially rise but subsequently decrease. In contrast, sIgG4 titers increase and remain elevated throughout the treatment. This shift towards a higher IgG4/IgE ratio is a hallmark of successful AIT [10, 11]. Although increasing levels of allergen sIgG4 are indicative of a favorable response to AIT, IgG4 levels alone do not consistently correlate with clinical success [12-14]. A notable parallel to VIT-induced allergen tolerance may be the natural tolerance to HBV observed in non-allergic beekeepers, who were demonstrated to display HBV-specific functional IgG4 antibodies [15].

To address the significant gap in understanding how specific allergens shape the humoral immune response in both induced and natural tolerance to Hymenoptera venoms, this study seeks to comprehensively investigate the sIgE and sIgG4 responses in venom-allergic patients during VIT and in non-allergic beekeepers at a molecular allergen-resolved level.

**Methods**

**Study participants**

HVA patients (n=86) from two study centers (Justus-Liebig-University Giessen, Giessen, Germany & Technical University of Munich, Munich, Germany) wereincluded in the study. The diagnosis of HVA was based on a combination of clinical history and skin testing and/or sIgE measurement to venom extracts and molecular allergens. According to diagnosis, 36 and 50 patients have been recommended for VIT with HBV and YJV, respectively. VIT was performed following 3-day rush up-dosing protocols. Patients from Munich received maintenance doses one, two, four, six, nine, and twelve weeks after completing the up-dosing phase, followed by 4-week intervals (Figure 1A). In Munich, patients were treated with Venomil (Bencard Allergie GmbH, Munich, Germany) or ALK-lyophilized SQ (ALK-Abelló, Hamburg, Germany). The patients from Giessen received maintenance doses one, three, and six weeks after the up-dosing phase, followed by intervals of 4 weeks (Figure 1A). Treatment was performed either with Venomil HBV or ALK-lyophilized SQ HBV. Patients with a predominant, IgE-mediated sensitization to Api m 10 or Api m 1 were treated with Venomil HBV or ALK lyophilized SQ HBV, respectively. Demographic data of patients are given in Table 1. Detailed clinical data of YJV- and HBV-allergic patients can be found in supplemental Tables S1 and S2, respectively.

Blood samples were taken directly before the start of the up-dosing phase (V1; HBV: n=36; YJV: n=50), 1-2 weeks after completing the up-dosing phase before a maintenance injection (V2; HBV: n=29; YJV: n=40), and approx. 3-6 months (V3; HBV: n=20) and one year (V4; HBV: n=14) after therapy initiation (Figure 1A). Unfortunately, due to logistic reasons, the YJV-allergic patients could not be followed up at V3 and V4.

Moreover, 61 beekeepers were enrolled in the study. None of the beekeepers reported or was diagnosed with systemic reactions following Hymenoptera stings. For subgroup analyses, beekeepers were divided into different groups according to the number of self-reported honeybee stings during the beekeeping season. Demographic data of beekeepers are given in Table 1.

Blood samples of beekeepers from cohort I (Munich, Germany; n=29) were taken in spring before the beekeeping season and any seasonal bee stings as well as after the season in autumn. Blood samples of beekeepers from cohort II (Freiburg, Germany; n=32) were collected at the end of the beekeeping season (Figure 1B).

Signed written consent was obtained from all participants before enrolment in the study. The study was conducted in accordance with the Declaration of Helsinki. The protocol was approved by the ethics committees of the Faculties of Medicine of the Technical University of Munich, Germany (538/17S), the Justus-Liebig-University Giessen, Germany (218/16), and the Albert-Ludwigs-University Freiburg, Germany (390/12).

**Antibody measurements**

The levels of sIgE and sIgG4 antibodies to HBV (i1) and YJV (i3) and the allergens Api m 1 (i208; phospholipase A2), Api m 2 (i214; hyaluronidase), Api m 3 (i215; acid phosphatase), Api m 4 (u1273; melittin), Api m 5 (i216; dipeptidyl peptidase IV), Api m 10 (i217; icarapin), Ves v 1 (i211; phospholipase A1), and Ves v 5 (i209; antigen 5) were determined using the ImmunoCAP platform (Thermo Fisher Scientific, Uppsala, Sweden) according to the recommendations of the manufacturer. All serum samples were stored at -80 °C immediately after acquisition and analyzed together at the end of the study sampling period.

**Statistical analysis**

Statistical analyses were conducted using GraphPad Prism 10 (GraphPad Software, La Jolla, CA, USA). Unpaired analyses were performed using either the Kruskal-Wallis test or the Mann-Whitney test. Paired analyses were conducted using the Wilcoxon test or Mixed-effects analysis with Geisser-Greenhouse correction. p-values of ≤0.05, ≤0.01, ≤0.001, and ≤0.0001 are shown as \*, \*\*, \*\*\*, and \*\*\*\*, respectively.

**Results**

**Allergen-resolved IgE responses in HBV- and YJV-allergic patients during VIT**

In HBV-allergic patients, no significant changes in sIgE levels between V1 (baseline; immediately before up-dosing) and V2 (1-2 weeks after up-dosing) were observed (Figure 2A). However, a VIT-induced increase in sIgE could be demonstrated by higher sensitization rates of the patient population at V2 compared to V1 (Figure 2B). At V3 (3-6 months after start of VIT) and/or V4 (1 year after start of VIT), sIgE levels for most allergens and whole HBV had decreased again (Figure 2A). Notably, the relative molecular allergen sIgE response in relation to whole HBV sIgE was dominated by Api m 1 and Api m 10 sIgE at all visits (Figure 2C). Interestingly, the Api m 4 contribution to the HBV sIgE pool increased over the first 3 visits but then dropped to pre-treatment levels. The sIgE delta shifts from V2 to V1 revealed no significant differences between the molecular allergens (Figure 2D).

In YJV-allergic patients, the increase in sIgE levels from V1 to V2 was significant for whole YJV and the allergens Ves v 1 and Ves v 5 (Figure 2E). While sIgE sensitization rates (cut-off 0.35 kUA/L) only slightly increased from V1 to V2 for YJV (from 98% to 100%) and Ves v 5 (from 86% to 90%), the sensitization rate for Ves v 1 drastically increased from 46% to 87.5% (Figure 2F). A similar pattern was seen when applying the 0.1 kUA/L cut-off. The sIgE delta shifts from V2 to V1 revealed no significant differences between the allergens (Figure 2G).

**Allergen-resolved IgG4 responses in HBV- and YJV-allergic patients during VIT**

 In HBV-allergic VIT patients, a substantial sIgG4 response was found exclusively for Api m 1, contributing dominantly to the observed increase in total HBV sIgG4 response (Figure 3A, D). sIgG4 levels were already detectable before VIT initiation (V1) and significantly increased at V2, with only minor increases during further visits. Interestingly, these changes were specific for Api m 1, whereas for remaining HBV allergens many measurements remained below the detection limit of 0.3 mgA/L. While there was a tendency for increased values over the treatment course for Api m 2 and Api m 4, the levels for Api m 3 decreased. Api m 5 and Api m 10 sIgG4 was not detectable, except for the latter in one patient (Figure 3A). Accordingly, the sIgG4 delta shift from V2 to V1 for Api m 1 was significantly higher compared to all other allergens and most likely accounted for the majority of the change observed for whole HBV (Figure 3B). The sIgG4/sIgE ratio significantly increased for whole HBV and Api m 1 from V1 to V2 but not for the other HBV allergens (Figure 3C). Similarly, the relative allergen sIgG4 response in relation to whole HBV sIgG4 was dominated by Api m 1 at all visits. While Api m 1 was followed by Api m 3 and Api m 2 at V1, the relative contribution of Api m 4 increased over the following visits (Figure 3D).

For YJV-allergic patients, the sIgG4 levels significantly increased from V1 to V2 for whole YJV as well as for Ves v 1 and Ves v 5 (Figure 3E), with the sIgG4 delta shift being slightly lower for Ves v 1 compared to Ves v 5 (Figure 3F). The sIgG4/sIgE ratio increased from V1 to V2 for whole YJV, Ves v 1, and Ves v 5, with a significant change observed only for whole YJV and Ves v 5 (Figure 3G).

Patients with HBV allergy were treated with either a VIT preparation that had undergone a gel filtration step to reduce small molecule substances [16] (ALK lyophilized SQ HBV) or a VIT preparation without this procedure (Allergy Therapeutics, Venomil HBV). In a subgroup analysis, the course of sIgG4 titers during VIT with the two different preparations was compared, and no major differences between the treatment groups were demonstrated (Figure 3H). Venomil HBV and ALK lyophilized SQ HBV induced slightly higher levels of Api m 2 sIgG4 and Api m 4 sIgG4 at V4, respectively. sIgG4 responses to low abundance allergens Api m 3, 5, and 10 were minor and did not show a time-dependent induction on average for both preparations. Interestingly, one patient treated with ALK lyophilized SQ HBV displayed a clear time-dependent induction of sIgG4 to Api m 10 (Figure 3H).

**Allergen-resolved IgE and IgG4 responses in non-allergic HBV-exposed beekeepers**

 In cohort I of beekeepers (Figure 4A-F), serum samples were taken before the beekeeping season (BS) and the first seasonal honeybee sting in spring and after the season (AS) in autumn. The majority of beekeepers displayed sIgE to HBV and various HBV allergens. Changes in sIgE levels from BS to AS were generally minor, although the slight increase in sIgE levels for Api m 4 and Api m 5 was significant (Figure 4A).

 Interestingly, the composition of the sIgE response (Figure 4A and B) differed from that observed in HBV-allergic patients (Figure 2A and C). In beekeepers, the relative allergen sIgE response in relation to whole HBV sIgE was not dominated by Api m 1 and Api m 10 sIgE. Instead, sIgE to other allergens such as Api m 2, 3, and 5 had a higher impact on the overall HBV sIgE response (Figure 4B). Notably, the high relative contribution of Api m 3 sIgE in beekeepers AS was mainly due to a few individuals with exceptionally high titers.

 The sIgG4 response in non-HBV-allergic beekeepers exposed to honeybee stings is particularly interesting as it reflects the humoral immune response to pure, unprocessed HBV. Here, sIgG4 levels significantly increased during the season for HBV and all allergens (Figure 4C). Interestingly, low sIgG4 levels were observed for the main component of HBV, Api m 4, both BS and AS and the low abundance allergens Api m 5 and Api m 10 BS. The sIgG4 increase during the season was significantly higher for Api m 1 (and HBV) compared to these allergens (Figure 4D).

To assess the impact of sting frequency on antibody titers, the beekeepers from cohort I were assigned to three different subgroups based on the number of self-reported honeybee stings during the season (0-10, 10-50, and >50). Significant differences in the sIgG4 levels between the subgroups receiving 0-10 and >50 stings were demonstrated for whole HBV, Api m 1, 2, 3, and 5, BS and AS. However, despite a visible tendency for increase, the differences were not significant for Api m 4 and Api m 10 (Figure 4E). Notably, most beekeepers reported a similar sting frequency in the season before the one investigated in this study. The sIgG4 increase over the season was significantly higher for all allergens in beekeepers with >50 stings compared to those who received 0-10 stings. This was also true for whole HBV, Api m 2, and Api m 3 when comparing the groups receiving 0-10 and 10-50 stings (Figure 4F). There were no relevant differences between the subgroups regarding the sIgE levels to the individual allergens (Figure S1).

The dependency of sIgG4 levels on sting frequency was confirmed in cohort II of beekeepers, where sera were collected exclusively at the end of the season (Figure 4G). In beekeepers receiving >50 stings, sIgG4 levels to all allergens, except Api m 3, were significantly higher compared to beekeepers receiving <50 stings. However, IgG4 induction to low abundance allergens, particularly to Api m 10, was minor, even in the highly sting-exposed subgroup. In this cohort, sIgE levels were lower compared to cohort I but did not greatly differ in composition (Figure S2).

In both cohorts, the relative allergen sIgG4 response in relation to whole HBV sIgG4 was comparable and dominated by Api m 1 (Figure 4H).

**Discussion**

 The results of this study highlight the dynamic nature of allergen sIgE and sIgG4 responses during VIT and natural allergen exposure. Component-resolved diagnostics (CRD) utilizing individual allergens in addition to whole venom extracts have become an integral part of clinical routine [17, 18]. It represents a significant advancement in diagnostics, especially for patients with positive test results for different venoms or in cases of discrepancies between clinical history and classical diagnostic tests [19-21]. Moreover, CRD is discussed for its potential use in patient risk stratification, *e.g.*, side effects or treatment failure [22, 23].

Although the determination of sIgE und sIgG4 levels to Hymenoptera venom extracts cannot be regarded as reliable biomarkers for the success of VIT or the status of immune tolerance [14, 24], changes in both can be indicative of treatment response and immunological changes [25-27]. Therefore, the investigation of allergen-resolved antibody responses during VIT and natural exposure can further help to shed light on the mechanisms of allergen tolerance and the contribution of individual allergens to the immunologic response.

The sensitization rates to individual allergens found for HBV-allergic patients are in line with previously published data [22, 28], and the initially observed slight rise and subsequent decrease of sIgE levels was expected during therapy [29, 30]. Interestingly, the initial increase of sIgE at the start of VIT was more pronounced in YJV-allergic patients. Notably, the increase in detectable sensitization (cut-off 0.1 kUA/L) to Ves v 1 from 58% to 90% from V1 to V2 was particularly striking. Most likely, many of these patients had a latent sensitization to Ves v 1 that was below the detection limit of the assay but then boostered by the up-dosing. Nevertheless, a *de novo* sensitization to Ves v 1 by VIT in individual patients cannot be completely excluded [31]. However, given the high efficacy of YJV VIT (91-99% [3, 4]), the clinical relevance of this rise in detectable Ves v 1 sensitization seems neglectable.

In HBV-allergic patients, the usual HBV sIgE response is dominated by Api m 1 and Api m 10, allergens of particular high and low abundance, respectively. Since the allergenic potency of these allergens is not driven by abundance, detailed studies of their molecular characteristics might help to shed further light on the properties that determine the allergenicity of proteins. The possibility that immunodominant and pro-anaphylactic venom epitopes are critical factors in distinguishing clinically relevant Hymenoptera venom sensitization from asymptomatic cases, similar to Ara h 2 epitopes in peanut allergy [32], seems intriguing. The importance of allergen/epitope specificity of the antibody response as a significant regulator for disease versus tolerance may be supported by the divergent composition of the sIgE response in non-allergic beekeepers, which is not dominated by Api m 1 and 10.

Although in YJV allergy, the sIgE response is slightly dominated by Ves v 5 [33], an unbiased conclusion about their contribution to whole YJV sIgE cannot be drawn as the YJV ImmunoCAP used in this study is spiked with Ves v 5 [34].

In contrast to the highly diverse sIgE sensitization profiles observed in HBV-allergic patients, the sIgG4 response during the first year of VIT is strongly dominated by Api m 1 sIgG4 antibodies, which likely reflects the majority of the HBV sIgG4 response. Similar results have been published previously [30]. Although not significant and by far not comparable to Api m 1, the contribution of sIgG4 to the most abundant Allergen, Api m 4, starts to rise over time. This is in line with a previous study demonstrating that 36 months after the initiation of HBV VIT, prominent IgG4 induction was restricted to the highly abundant allergens Api m 1 and Api m 4 [28]. Nevertheless, Api m 4, despite its high abundance in HBV [35] and its principal ability to induce IgE and IgG4 antibodies, seems to be a poor immunogen. In YJV-allergic patients, the IgG4 responses to Ves v 1 and Ves v 5, which are present in the venom in comparable amounts, were considerable. However, Ves v 5 seemed to be a slightly more potent inducer. An increase in the IgG4/sIgE ratio has been described as a hallmark of successful AIT [11]. In our study, we analyzed the sIgG4/sIgE ratios at an earlier time point during VIT rather than after completing therapy. We observed an increase in these ratios for the whole venoms and the major allergens Api m 1 and Ves v 5, but not for the low-abundance HBV allergens. It is important to note that this early time point may not fully capture the dynamics of immune modulation, and higher IgG4/IgE ratios for low-abundance allergens could potentially establish later in therapy; a question that should be addressed in future studies on a component-resolved level with extended follow-up. Our findings suggest that the high success rates of HBV VIT, as widely reported in the literature [3-5], may not necessarily rely on the induction of sIgG4 to low-abundance allergens. However, as we did not perform sting challenge tests or systematically capture field sting reactions in this study, our conclusions regarding the relationship between immunological parameters and clinical outcomes must be interpreted with caution. Nevertheless, our data may underline the fact that other factors beyond IgG4 induction may be the critical drivers of immunological tolerance to allergens. However, this does not exclude the possibility that the low abundance of several of the relevant HBV allergens might be one factor contributing to the lower efficacy observed for HBV compared to YJV VIT [3-5]. This is supported by the fact that patients still reacting to a sting challenge while receiving conventional VIT are protected by increased venom doses [36].

A retrospective study suggested that a predominant sensitization to Api m 10 represents a risk factor for failure of HBV VIT [22]. Therefore, it was speculated that the lack of sIgG4 to Api m 10 could be a contributing factor to therapy failure [37]. The reported underrepresentation of low abundance allergens such as Api m 3, Api m 5, and Api m 10 [22, 38, 39] in ALK lyophilized SQ HBV and the hypothesis that therapeutic success of VIT might be associated with the venom preparation and its concentration of allergens a patient is sensitized [20] prompted us to compare the IgG4 response in HBV-allergic patients treated with different VIT products. IgG4 induction to these allergens was low compared to Api m 1, regardless of which preparation was used for VIT. Despite this observation, it cannot be entirely excluded that the choice to treat patients from Giessen, who showed predominant sensitization to Api m 10, with Venomil - due to its higher content of intact Api m 10 [38] - may have introduced a potential confounding factor. Notably, one patient showed significant and time-dependent induction of sIgG4 to Api m 10. This patient was treated with ALK lyophilized SQ HBV, had a history of beekeeping for more than ten years prior to the beginning of VIT, and received up-dosing from 100µg to 200µg maintenance dose between visits 2 and 3. The patient confirmed that he had not received a field sting during the observation period, suggesting that treatment with ALK lyophilized SQ HBV was sufficient to induce a robust IgG4 response to Api m 10 in this patient. Whether this response was the result of high HBV exposure due to his beekeeping history or the up-dosing during therapy can only be speculated.

Although IgG4 induction is often regarded as a hallmark of responsiveness to allergen-specific immunotherapy, the role of these “blocking antibodies”, which are thought to compete with the IgE-allergen interaction [40-42], in establishing immune tolerance remains unclear, as no consistent correlation with clinical improvement has been observed [12-14]. Additionally, studies have shown that 2 years after VIT withdrawal, high allergen-specific IgG4 levels and inhibition of facilitated allergen-IgE binding are not maintained [43]. Whether this decline increases the risk of renewed allergic reactions remains unknown. Beyond IgG4, other immunological changes, such as regulatory T cell (Treg) induction, increased IL-10 and TGF-β secretion, and downregulation of pro-inflammatory cytokines in effector cells, likely contribute to VIT success [41, 44, 45]. Notably, a recent study highlighted the expansion of inducible Helios-negative Tregs in the periphery as central to natural and VIT-induced HBV tolerance [30, 46]. Another study linked immune tolerance following VIT to reduced allergen-specific basophil responsiveness [47]. However, for individual patients, tailoring the maintenance dose and VIT preparation may be essential to achieve these tolerance-inducing immunological shifts [48].

 The discussions about the impact of allergen content, particularly of low abundance allergens, in different HBV VIT preparations [22, 38, 49] prompted us to evaluate the allergen-specific antibody responses in non-allergic beekeepers who are exposed to natural, unprocessed HBV. The venom in VIT preparations, in contrast, has undergone an extraction procedure and, in some cases, a gel filtration step. While the increase of sIgE levels over the beekeeping season was negligible, the bee stings obtained during the season led to significant increases of sIgG4 levels to all allergens, including those of low abundance. This is a major difference to allergic patients undergoing VIT. However, as in HBV-allergic patients, the IgG4 response was dominated by Api m 1 sIgG4 antibodies, and their increase was significantly higher than those measured for Api m 5 and Api m 10. The slightly more pronounced sIgG4 response to low abundance allergens compared to HBV-allergic VIT patients might be due to a variable composition of the natural venom [38] or to a higher exposure of beekeepers on average. Interestingly, another study addressing IgG4 responses in tolerant beekeepers observed higher levels of Api m 2 and Api m 4 sIgG4 [30]. However, in that study, 80% of beekeepers reported receiving more than 200 honeybee stings per year, whereas this high sting frequency was an exception in our study population.

 Previous studies demonstrated a negative correlation between sting frequency and the risk of acquiring HBV allergy in beekeepers [50, 51]. Hence, beekeepers from cohort I, for whom serum samples before and after the beekeeping season were collected, were divided into three different subgroups according to the number of self-reported honeybee stings during the season. Already before the season, beekeepers from the group with >50 stings exhibited significantly higher sIgG4 levels to whole HBV, Api m 1, 2, 3, and 5. This might be explained by the fact that most of the beekeepers also reported the same sting frequency for the season prior to the one investigated in this study. Given the reported average biologic half-life of IgG4 of 21 days [52], the IgG4 levels before the season probably result from long-lived plasma cells, which primarily reside in the bone marrow [53]. Whether the higher pre-seasonal sIgG4 titers depend on exposure from the previous year or result from several years of beekeeping is speculative, especially considering that behavioral changes occur and beekeepers tend to receive more stings the longer they practice beekeeping. A similar pattern was observed after the beekeeping season. Although not statistically significant, there was a more pronounced trend for higher sIgG4 levels to Api m 4 and Api m 10 in the group with the highest sting exposure. Notably, the increase in sIgG4 levels over the season was significantly higher for all allergens in the subgroup with the highest compared to that with the lowest sting frequency. Significantly higher sIgG4 levels to the low abundance allergen Api m 10 after the beekeeping season in highly sting-exposed beekeepers were confirmed in beekeeper cohort II.

This study demonstrates that antibody responses in YJV-allergic VIT patients are very consistent. The two major allergens, Ves v 1 and Ves v 5, dominate the sIgE response. Notably, the rise in detectable sensitization to Ves v 1 during VIT, likely due to boostering of existing sensitization, was interesting. Similarly, these two allergens induce a robust sIgG4 response during VIT. However, the picture might become more complex if additional YJV allergens were included in antibody measurements. This increased complexity is reflected in the humoral responses to HBV allergens. The sIgE sensitization profiles of HBV-allergic patients are highly diverse, and Api m 1 and Api m 10 appear to be particularly potent inducers of IgE. In stark contrast, the sIgG4 response in HBV VIT patients and non-allergic beekeepers is strongly dominated by Api m 1 sIgG4. sIgE to low abundance allergens such as Api m 5 and Api m 10 is scarcely induced by VIT, independent of which VIT preparation is used. The findings in frequently stung beekeepers show that a robust sIgG4 response to Api m 10 is dependent on highly frequent exposure. However, the high success rates of HBV VIT and the tolerogenic status of investigated beekeepers suggest that immunological tolerance to low abundance allergens does not necessarily require robust sIgG4 induction.

**Acknowledgment**

The authors would like to thank Benjamin Schnautz for technical assistance with the compilation of raw data.

**References**

1. Perez-Codesido S, Rosado-Ingelmo A, Privitera-Torres M, Pérez Fernández E, Nieto-Nieto A, Gonzalez-Moreno A, et al. Incidence of Fatal Anaphylaxis: A Systematic Review of Observational Studies*.* J Investig Allergol Clin Immunol. 2022;32(4):245-260.

2. Sturm GJ, Varga EM, Roberts G, Mosbech H, Bilo MB, Akdis CA, et al. EAACI guidelines on allergen immunotherapy: Hymenoptera venom allergy*.* Allergy. 2018;73(4):744-764.

3. Müller U, Helbling A, Berchtold E. Immunotherapy with honeybee venom and yellow jacket venom is different regarding efficacy and safety*.* J Allergy Clin Immunol. 1992;89(2):529-35.

4. Rueff F, Vos B, Oude Elberink J, Bender A, Chatelain R, Dugas-Breit S, et al. Predictors of clinical effectiveness of Hymenoptera venom immunotherapy*.* Clin Exp Allergy. 2014;44(5):736-46.

5. Kranert P, Forchhammer S, Volc S, Stenger F, Schaller M, Fischer J. Safety and Effectiveness of a 3-Day Rush Insect Venom Immunotherapy Protocol*.* Int Arch Allergy Immunol. 2020;181(2):111-118.

6. Meiler F, Zumkehr J, Klunker S, Ruckert B, Akdis CA, Akdis M. In vivo switch to IL-10-secreting T regulatory cells in high dose allergen exposure*.* J Exp Med. 2008;205(12):2887-98.

7. Galli SJ, Tsai M. IgE and mast cells in allergic disease*.* Nat Med. 2012;18(5):693-704.

8. Sahiner UM, Giovannini M, Escribese MM, Paoletti G, Heffler E, Alvaro Lozano M, et al. Mechanisms of Allergen Immunotherapy and Potential Biomarkers for Clinical Evaluation*.* J Pers Med. 2023;13(5):845.

9. Aalberse RC, Stapel SO, Schuurman J, Rispens T. Immunoglobulin G4: an odd antibody*.* Clin Exp Allergy. 2009;39(4):469-77.

10. Akdis M, Akdis CA. Mechanisms of allergen-specific immunotherapy: multiple suppressor factors at work in immune tolerance to allergens*.* J Allergy Clin Immunol. 2014;133(3):621-31.

11. Nikolov G, Todordova Y, Emilova R, Hristova D, Nikolova M, Petrunov B. Allergen-Specific IgE and IgG4 as Biomarkers for Immunologic Changes during Subcutaneous Allergen Immunotherapy*.* Antibodies (Basel). 2021;10(4):49.

12. James LK, Shamji MH, Walker SM, Wilson DR, Wachholz PA, Francis JN, et al. Long-term tolerance after allergen immunotherapy is accompanied by selective persistence of blocking antibodies*.* J Allergy Clin Immunol. 2011;127(2):509-516.e1-5.

13. Müller U, Akdis CA, Fricker M, Akdis M, Blesken T, Bettens F, et al. Successful immunotherapy with T-cell epitope peptides of bee venom phospholipase A2 induces specific T-cell anergy in patients allergic to bee venom*.* J Allergy Clin Immunol. 1998;101(6 Pt 1):747-54.

14. Ewan PW, Deighton J, Wilson AB, Lachmann PJ. Venom-specific IgG antibodies in bee and wasp allergy: lack of correlation with protection from stings*.* Clin Exp Allergy. 1993;23(8):647-60.

15. Varga EM, Kausar F, Aberer W, Zach M, Eber E, Durham SR, et al. Tolerant beekeepers display venom-specific functional IgG4 antibodies in the absence of specific IgE*.* J Allergy Clin Immunol. 2013;131(5):1419-21.

16. Bilò MB, Cinti B, Brianzoni MF, Braschi MC, Bonifazi M, Antonicelli L. Honeybee venom immunotherapy: a comparative study using purified and nonpurified aqueous extracts in patients with normal Basal serum tryptase concentrations*.* J Allergy (Cairo). 2012;2012(869243.

17. Blank S, Korošec P, Slusarenko BO, Ollert M, Hamilton RG. Venom component allergen IgE measurement in the diagnosis and management of insect sting allergy*.* J Allergy Clin Immunol Pract. 2024;13(1):1-14.

18. Vega-Castro A, Rodríguez-Gil D, Martínez-Gomariz M, Gallego R, Peña MI, Palacios R. Api m 6 and Api m 10 as Major Allergens in Patients With Honeybee Venom Allergy*.* J Investig Allergol Clin Immunol. 2022;32(2):116-123.

19. Blank S, Bilò MB, Grosch J, Schmidt-Weber CB, Ollert M, Jakob T. Marker allergens in Hymenoptera venom allergy - Characteristics and potential use in precision medicine*.* Allergo J Int. 2021;30(1):26-38.

20. Blank S, Bilo MB, Ollert M. Component-resolved diagnostics to direct in venom immunotherapy: Important steps towards precision medicine*.* Clin Exp Allergy. 2018;48(4):354-364.

21. Dramburg S, Hilger C, Santos AF, de Las Vecillas L, Aalberse RC, Acevedo N, et al. EAACI Molecular Allergology User's Guide 2.0*.* Pediatr Allergy Immunol. 2023;34 Suppl 28(e13854.

22. Frick M, Fischer J, Helbling A, Rueff F, Wieczorek D, Ollert M, et al. Predominant Api m 10 sensitization as risk factor for treatment failure in honey bee venom immunotherapy*.* J Allergy Clin Immunol. 2016;138(6):1663-1671 e9.

23. Ruiz B, Serrano P, Moreno C. IgE-Api m 4 Is Useful for Identifying a Particular Phenotype of Bee Venom Allergy*.* J Investig Allergol Clin Immunol. 2016;26(6):355-361.

24. Peternelj A, Silar M, Erzen R, Kosnik M, Korosec P. Basophil sensitivity in patients not responding to venom immunotherapy*.* Int Arch Allergy Immunol. 2008;146(3):248-54.

25. Vachova M, Panzner P, Kopac P, Bidovec Stojkovic U, Korosec P. Routine clinical utility of honeybee venom allergen components*.* J Allergy Clin Immunol Pract. 2018;6(6):2121-2123 e1.

26. Zemelka-Wiacek M, Agache I, Akdis CA, Akdis M, Casale TB, Dramburg S, et al. Hot topics in allergen immunotherapy, 2023: Current status and future perspective*.* Allergy. 2024;79(4):823-842.

27. Brasch J, Maidusch T. Immunotherapy with wasp venom is accompanied by wide-ranging immune responses that need further exploration*.* Acta Derm Venereol. 2009;89(5):466-9.

28. Köhler J, Blank S, Muller S, Bantleon F, Frick M, Huss-Marp J, et al. Component resolution reveals additional major allergens in patients with honeybee venom allergy*.* J Allergy Clin Immunol. 2014;133(5):1383-9, 1389 e1-6.

29. Akdis CA, Akdis M. Mechanisms of allergen-specific immunotherapy and immune tolerance to allergens*.* World Allergy Organ J. 2015;8(1):17.

30. Navas A, Ruiz-Leon B, Serrano P, Martí M, Espinazo ML, Blanco N, et al. Natural and Induced Tolerance to Hymenoptera Venom: A Single Mechanism? Toxins (Basel). 2022;14(7):426.

31. Gellrich D, Eder K, Högerle C, Becker S, Canis M, Gröger M. De novo sensitization during subcutaneous allergen specific immunotherapy - an analysis of 51 cases of SCIT and 33 symptomatically treated controls*.* Sci Rep. 2020;10(1):6048.

32. Croote D, Wong JJW, Pecalvel C, Leveque E, Casanovas N, Kamphuis JBJ, et al. Widespread monoclonal IgE antibody convergence to an immunodominant, proanaphylactic Ara h 2 epitope in peanut allergy*.* J Allergy Clin Immunol. 2024;153(1):182-192.e7.

33. Blank S, Bazon ML, Grosch J, Schmidt-Weber CB, Brochetto-Braga MR, Bilo MB, et al. Antigen 5 Allergens of Hymenoptera Venoms and Their Role in Diagnosis and Therapy of Venom Allergy*.* Curr Allergy Asthma Rep. 2020;20(10):58.

34. Vos B, Kohler J, Muller S, Stretz E, Rueff F, Jakob T. Spiking venom with rVes v 5 improves sensitivity of IgE detection in patients with allergy to Vespula venom*.* J Allergy Clin Immunol. 2013;131(4):1225-7, 1227 e1.

35. Habermann E. Bee and wasp venoms*.* Science. 1972;177(4046):314-22.

36. Rueff F, Wenderoth A, Przybilla B. Patients still reacting to a sting challenge while receiving conventional Hymenoptera venom immunotherapy are protected by increased venom doses*.* J Allergy Clin Immunol. 2001;108(6):1027-32.

37. Jakob T, Rauber MM, Perez-Riverol A, Spillner E, Blank S. The Honeybee Venom Major Allergen Api m 10 (Icarapin) and Its Role in Diagnostics and Treatment of Hymenoptera Venom Allergy*.* Curr Allergy Asthma Rep. 2020;20(9):48.

38. Blank S, Etzold S, Darsow U, Schiener M, Eberlein B, Russkamp D, et al. Component-resolved evaluation of the content of major allergens in therapeutic extracts for specific immunotherapy of honeybee venom allergy*.* Hum Vaccin Immunother. 2017;13(10):2482-2489.

39. Blank S, Seismann H, Michel Y, McIntyre M, Cifuentes L, Braren I, et al. Api m 10, a genuine A. mellifera venom allergen, is clinically relevant but underrepresented in therapeutic extracts*.* Allergy. 2011;66(10):1322-9.

40. James LK, Shamji MH, Walker SM, Wilson DR, Wachholz PA, Francis JN, et al. Long-term tolerance after allergen immunotherapy is accompanied by selective persistence of blocking antibodies*.* J Allergy Clin Immunol. 2011;127(2):509-516 e1-5.

41. Nouri-Aria KT, Wachholz PA, Francis JN, Jacobson MR, Walker SM, Wilcock LK, et al. Grass pollen immunotherapy induces mucosal and peripheral IL-10 responses and blocking IgG activity*.* J Immunol. 2004;172(5):3252-9.

42. van Neerven RJ, Wikborg T, Lund G, Jacobsen B, Brinch-Nielsen A, Arnved J, et al. Blocking antibodies induced by specific allergy vaccination prevent the activation of CD4+ T cells by inhibiting serum-IgE-facilitated allergen presentation*.* J Immunol. 1999;163(5):2944-52.

43. Varga EM, Francis JN, Zach MS, Klunker S, Aberer W, Durham SR. Time course of serum inhibitory activity for facilitated allergen-IgE binding during bee venom immunotherapy in children*.* Clin Exp Allergy. 2009;39(9):1353-7.

44. Till SJ, Durham SR. Immunologic responses to subcutaneous allergen immunotherapy*.* Clin Allergy Immunol. 2008;21(59-70.

45. Pilette C, Nouri-Aria KT, Jacobson MR, Wilcock LK, Detry B, Walker SM, et al. Grass pollen immunotherapy induces an allergen-specific IgA2 antibody response associated with mucosal TGF-beta expression*.* J Immunol. 2007;178(7):4658-66.

46. Ruiz-Leon B, Navas A, Serrano P, Espinazo M, Guler I, Alonso C, et al. Helios-Negative Regulatory T Cells as a Key Factor of Immune Tolerance in Nonallergic Beekeepers*.* J Investig Allergol Clin Immunol. 2022;32(6):451-459.

47. Eržen R, Košnik M, Silar M, Korošec P. Basophil response and the induction of a tolerance in venom immunotherapy: a long-term sting challenge study*.* Allergy. 2012;67(6):822-30.

48. Ruiz-León B, Navas A, Serrano P, Espinazo M, Labrador-Horrillo M, Monsalve RI, et al. Successful Adaptation of Bee Venom Immunotherapy in a Patient Monosensitized to Api m 10*.* J Investig Allergol Clin Immunol. 2020;30(4):296-298.

49. Sturm GJ, Arzt-Gradwohl L, Čerpes U, Koch L, Bokanovic D, Laipold K, et al. Prospective studies are needed to elucidate the clinical impact of predominant Api m 10 sensitization*.* Allergy. 2022;77(2):687-689.

50. Muller UR. Bee venom allergy in beekeepers and their family members*.* Curr Opin Allergy Clin Immunol. 2005;5(4):343-7.

51. Bousquet J, Ménardo JL, Aznar R, Robinet-Lévy M, Michel FB. Clinical and immunologic survey in beekeepers in relation to their sensitization*.* J Allergy Clin Immunol. 1984;73(3):332-40.

52. Morell A, Terry WD, Waldmann TA. Metabolic properties of IgG subclasses in man*.* J Clin Invest. 1970;49(4):673-80.

53. Nutt SL, Hodgkin PD, Tarlinton DM, Corcoran LM. The generation of antibody-secreting plasma cells*.* Nat Rev Immunol. 2015;15(3):160-71.

**Tables**

**Table 1.** Demographic data of venom-allergic patients and non-allergic beekeepers.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | HBV-allergicpatients | YJV-allergic patients | Beekeeperscohort I Munich | Beekeeperscohort II Freiburg |
| Total | 36 | 50 | 29 | 32 |
| Sex |  |  |  |  |
| Female | 18 | 30 | 15 | 3 |
| Male | 18 | 20 | 14 | 29 |
| Median age (range) | 47 (22-73) | 50 (22-80) | 51 (20-72) | 61.5 (41-80) |

**Figure legends**

**Figure 1.** Overview of the study. (A) Dosing and sampling scheme for Hymenoptera venom-allergic patients. The majority of patients received a maintenance dose of 100 µg, while a small subset of patients was up-dosed to a maintenance dose of 200 µg. d, day; w, week; y, year. (B) Sampling scheme for non-allergic beekeepers.

**Figure 2.** Component-resolved IgE responses in patients undergoing venom-specific immunotherapy. (A) HBV- and allergen sIgE levels in HBV-allergic patients before therapy (V1; n=36) and 3-6 weeks (V2; n=29), 3-6 months (V3; n= 20), and approx. one year (V4; n=14) after initiation of VIT. Statistical analysis was performed separately for each allergen across the respective visits using Mixed-effects analysis with Geisser-Greenhouse correction. (B) sIgE sensitization rates in percent of HBV-allergic patients at visits 1 and 2 using the cut-offs of 0.1 and 0.35 kUA/L.(C) Proportion of allergen sIgE titers normalized to the total HBV sIgE titer depending on patient visits. The graphs depict the average proportion of the individual allergens over all patients. sIgE values below 0.1 kUA/L were considered non-detectable and counted as zero. (D) Changes in HBV- and allergen sIgE titers from visit 1 to visit 2 represented as calculated delta values. For the calculation, sIgE values below 0.1 kUA/L were considered non-detectable and counted as zero. Statistical analysis was performed using Kruskal-Wallis test. (E) YJV- and allergen sIgE levels in YJV-allergic patients before therapy (V1; n=50) and 3-6 weeks after initiation of VIT (V2; n=40). The paired values of the two visits were compared for each allergen separately using Wilcoxon test. (F) sIgE sensitization rates of YJV-allergic patients at visits 1 and 2 using the cut-offs of 0.1 and 0.35 kUA/L. (G) Changes in YJV- and allergen sIgE titers from visit 1 to visit 2 represented as calculated delta values. For the calculation, sIgE values below 0.1 kUA/L were considered non-detectable and counted as zero. Statistical analysis was performed using Kruskal-Wallis test. Data is displayed either as box-and-whisker plots showing minimum to maximum values or as part of whole plots. HBV, honeybee venom; V, visit; VIT, venom-specific immunotherapy; YJV, yellow jacket venom.

**Figure 3:** Component-resolved IgG4 responses in patients undergoing venom-specific immunotherapy. A) HBV- and allergen sIg4 levels in HBV-allergic patients before therapy (V1; n=36) and 3-6 weeks (V2; n=28), 3-6 months (V3; n= 20), and approx. one year (V4; n=14) after initiation of VIT. The dashed line indicates the cut-off of 0.3 mgA/L. Statistical analysis was performed separately for each allergen across the respective time points using Mixed-effects analysis with Geisser-Greenhouse correction. (B) Changes in HBV- and allergen sIgG4 titers from visit 1 to visit 2 represented as calculated delta values. For the calculation, sIgG4 values below 0.3 mgA/L were considered non-detectable and counted as zero. Statistical analysis was performed using Kruskal-Wallis test. (C) HBV- and allergen sIgG4/sIgE ratios at visits 1 and 2 (n=28). sIgG4 and sIgE values below 0.3 mgA/L and 0.1 kUA/L, respectively, were considered non-detectable and counted as zero. Statistical analysis was performed using Wilcoxon test. (D) Proportion of allergen sIgG4 titers normalized to the total HBV sIgG4 titer depending on patient visits. The graph depicts the average proportion of individual allergens in all patients. sIgG4 values below 0.3 mgA/L were considered non-detectable and counted as zero. (E) YJV- and allergen sIgG4 levels in YJV-allergic patients before therapy (V1; n=50) and 3-6 weeks after initiation of VIT (V2; n=39). The dashed line indicates the cut-off of 0.3 mgA/L. The paired values of the two time points were compared for each allergen separately using Wilcoxon test. (F) Changes in YJV- and allergen sIgG4 titers from visit 1 to visit 2 represented as calculated delta values. For the calculation, sIgG4 values below 0.3 mgA/L were considered non-detectable and counted as zero. Statistical analysis was performed using Kruskal-Wallis test. (G) YJV- and allergen sIgG4/sIgE ratios at visits 1 and 2 (n=39). sIgG4 and sIgE values below 0.3 mgA/L and 0.1 kUA/L, respectively, were considered non-detectable and counted as zero. Statistical analysis was performed using Wilcoxon test. (H) IgG4 responses during VIT in HBV-allergic patients treated with either ALK lyophilized SQ® (n=13) or Venomil® (n=12). Statistical analysis for differences between the two VIT preparations at each visit was performed using Mann-Whitney test. The dashed line indicates the cut-off of 0.3 mgA/L. Data is displayed as box-and-whisker plots showing minimum to maximum values, part of whole plots, or XY plots. HBV, honeybee venom; V, visit; VIT, venom-specific immunotherapy; YJV, yellow jacket venom.

**Figure 4.** Component-resolved IgE and IgG4 responses in non-allergic beekeepers. (A) HBV- and allergen sIgE levels before (BS) and after (AS) the beekeeping season (n=29). Differences between the two time points were compared for each allergen separately using Wilcoxon test. (B) Proportion of allergen sIgE titers normalized to the total HBV sIgE titer before and after the beekeeping season. The graph depicts the average proportion of individual allergens among all beekeepers. sIgE values below 0.1 kUA/L were considered non-detectable and counted as zero. (C) HBV- and allergen sIgG4 levels before (BS) and after (AS) the beekeeping season (n=29). Differences between the time points were compared for each allergen separately using Wilcoxon test. The dashed line indicates the cut-off of 0.3 mgA/L. (D) Changes in HBV- and allergen sIgG4 titers during the beekeeping season are represented by calculated delta values. For the calculation, sIgG4 values below 0.3 mgA/L were considered non-detectable and counted as zero. Statistical analysis was performed using Kruskal-Wallis test. (E) HBV- and allergen sIgG4 levels before (BS) and after (AS) the beekeeping season in beekeepers reporting 0-10 (n=11), 10-50 (n=6), and >50 (n=10) honeybee stings during the season. Statistical analysis was performed for each allergen separately using Kruskal-Wallis test. The dashed line indicates the cut-off of 0.3 mgA/L. (F) Changes in HBV- and allergen sIgG4 titers during the beekeeping season in beekeepers of the different sting frequency categories represented as calculated delta values. For the calculation, sIgG4 values below 0.3 mgA/L were considered non-detectable and counted as zero. Statistical analysis was performed for each allergen separately using Kruskal-Wallis test. (G) sIgG4 levels at the end of the beekeeping season in a second cohort of non-allergic beekeepers categorized by the sting frequency in the past season (<50 stings: n=20; >50 stings: n= 12). The two sting frequency categories were compared for each allergen separately using Mann-Whitney test. The dashed line indicates the cut-off of 0.3 mgA/L. (H) Proportion of allergen sIgG4 titers normalized to the total HBV sIgG4 titer after the beekeeping season in the two cohorts of beekeepers. The graphs present the average proportion of individual allergens among all beekeepers. sIgG4 values below 0.3 mgA/L were considered non-detectable and counted as zero. Data is displayed either as box-and-whisker plots showing minimum to maximum values or as part of whole plots. AS, after season; BS, before season; HBV, honeybee venom.