Predominant Api m 10 sensitization as risk factor for treatment failure in honey bee venom immunotherapy
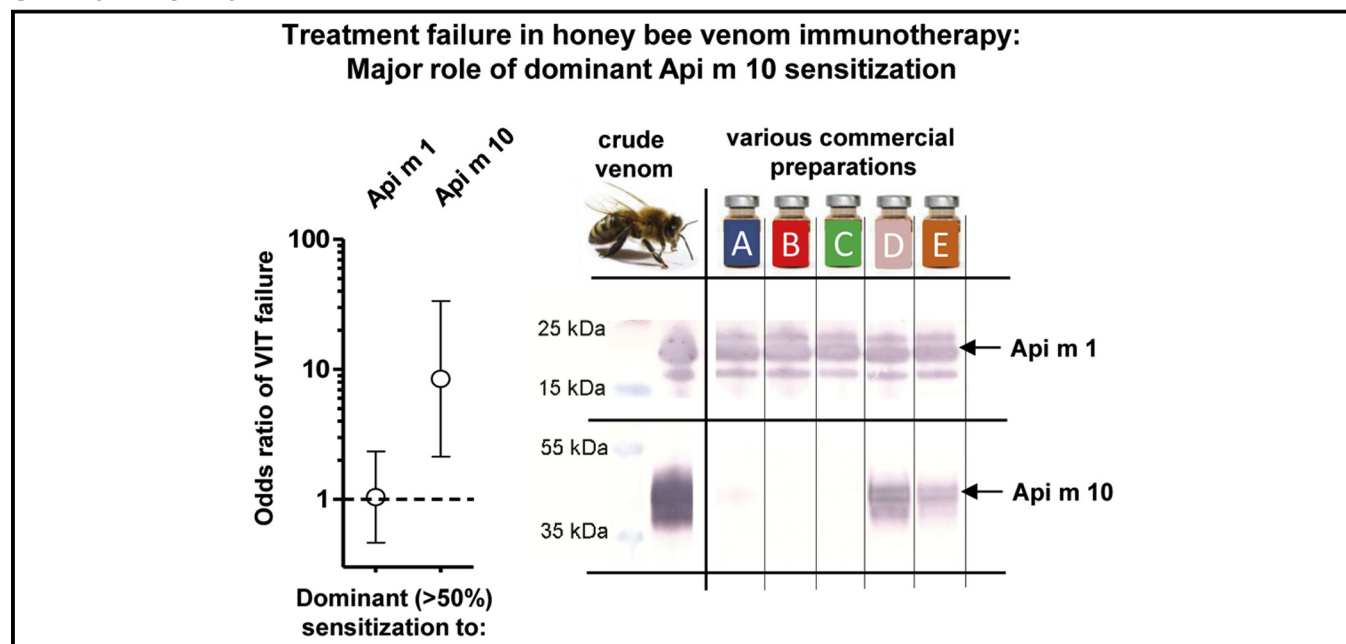
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GRAPHICAL ABSTRACT

Background: Component resolution recently identified distinct sensitization profiles in honey bee venom (HBV) allergy, some of which were dominated by specific IgE to Api m 3 and/or Api m 10, which have been reported to be underrepresented in therapeutic HBV preparations.

Objective: We performed a retrospective analysis of component-resolved sensitization profiles in HBV-allergic patients and association with treatment outcome.

Methods: HBV-allergic patients who had undergone controlled honey bee sting challenge after at least 6 months of HBV immunotherapy (n = 115) were included and classified as responder (n = 79) or treatment failure (n = 36) on the basis of absence or presence of systemic allergic reactions upon sting challenge. IgE reactivity to a panel of HBV allergens was analyzed in sera obtained before immunotherapy and before sting challenge.

Results: No differences were observed between responders and nonresponders regarding levels of IgE sensitization to Api m 1, Api m 2, Api m 3, and Api m 5. In contrast, Api m 10 specific IgE was moderately but significantly increased in nonresponders. Predominant Api m 10 sensitization (>50% of specific IgE to HBV) was the best discriminator (specificity, 95%; sensitivity, 25%) with an odds ratio of 8.444 (2.127-33.53; $P = .0013$) for treatment failure. Some but not all therapeutic HBV preparations displayed a lack of Api m 10, whereas Api m 1 and Api m 3 immunoreactivity was comparable to that of crude HBV. In line with this, significant Api m 10 sIgG$_4$ induction was observed only in those patients who were treated with HBV in which Api m 10 was detectable.

Conclusions: Component-resolved sensitization profiles in HBV allergy suggest predominant IgE sensitization to Api m 10 as a risk factor for treatment failure in HBV immunotherapy. (J Allergy Clin Immunol 2016;138:1663-71.)

Systemic allergic reaction to Hymenoptera stings affects 0.3% to 3.5% of the adult population.[1,2] Venom immunotherapy (VIT) protects allergic patients from systemic reactions to subsequent stings.[2,3] The effectiveness of VIT depends on a number of variables such as treatment duration, venom dose during maintenance therapy, and type of venom (honey bee [HB] vs vespid) used for immunotherapy.[4-6] Treatment failure is more frequent in HB VIT than in vespid VIT, ranging from 11% to 23% as compared with 0% to 9%.[4-7] A recent retrospective study on the outcome of more than 1600 sting challenges calculated an odds ratio (OR) of more than 5 for treatment failure in honey bee venom (HBV) allergy as compared with VIT in vespid venom allergy.[6] This increased risk of treatment failure in HBV allergy has been suggested to be

associated with differences in venom composition, venom dose during natural exposure conditions, and differences in sensitization profiles.[4,7,8]

Advances in proteomics and molecular biology have allowed a detailed characterization of the protein composition of HBV. The best-characterized HBV allergens are phospholipase A2 (Api m 1), hyaluronidase (Api m 2), and the basic peptide melittin (Api m 4).[9,10] Additional HBV allergens of lower abundance have been cloned and characterized such as acid phosphatase (Api m 3),[11] dipeptidylpeptidase IV (Api m 5),[12] icarapin (Api m 10),[13,14] and others as recently reviewed.[15] Analysis of different venom preparations have shown that Api m 3 and Api m 10, while present in the crude HBV, are absent or underrepresented in preparations used for HBV immunotherapy.[13] These findings were supported by subsequent observations that in patients with dominant sensitization to Api m 10, IgE reactivity to HBV could be inhibited by crude HBV preparations but not by therapeutic HBV preparations.[8] In addition, HBV-allergic patients who had undergone VIT displayed a strong induction of sIgG$_4$ to Api m 1, Api m 2, and Api m 4, whereas no or little induction of sIgG$_4$ to Api m 3 and Api m 10 could be detected.[8] On the basis of these 3 lines of evidence, we hypothesized that the absence or underrepresentation of Api m 3 and Api m 10 in therapeutic HBV preparations may have an impact on the treatment outcome of VIT and that distinct sensitization profiles, for example, with predominant IgE reactivity to Api m 3 and/or Api m 10, may represent a potential risk factor for treatment failure of VIT in HBV allergy. To address this issue, we here retrospectively analyzed the molecular sensitization profiles in HBV-allergic patients who had undergone controlled HB sting challenge after at least 6 months of HBV.

METHODS
Patients
Sera from HBV-allergic patients who had undergone controlled HB sting challenge after at least 6 months of HBV immunotherapy at a maintenance dose of 100 µg BV were included in the study (n = 115) and classified as responder,

TABLE I. Clinical data of patient population

Variable	Responders (n = 79)	Nonresponders (n = 36)
Age (y), mean (range)	40.1 (10-77)	41.4 (10-65)
Sex: F/M (%)	41.8/58.2	42.5/57.5
Total IgE (kU$_A$/L), mean (range)	329.5 (2-9360)	245.6 (8-3525)
Systemic sting reaction,* n (%)	I: 13 (16.5)	I: 2 (5.5)
	II: 49 (62.0)	II: 22 (61.1)
	III: 16 (20.3)	III: 11 (30.5)
	IV: 1 (1.3)	IV: 1 (2.7)
Tryptase > 20 μg/L, or MIS (%)	4 of 79 (5.1)	1 of 36 (2.8)
Double VIT HBV & YJV (%)	21 of 79 (26.6)	7 of 36 (19.4)
Antihypertensive therapy (%)	10 of 79 (6.3)	3 of 36 (8.3)
ACE inhibitor medication (%)	1 of 79 (1.3)	0 of 36 (0)
Treatment duration before SC (mo), median (range)	31.4 (7-127)	37.3 (7-90)

ACE, Angiotensin-converting enzyme; *F*, female; *M*, male; *MIS*, mastocytosis in the skin; *SC*, sting challenge; *YJV*, yellow jacket venom.
*Highest degree of index sting reaction before VIT, clinical grade.[16]

that is, protected (n = 79), or treatment failure, that is, not protected (n = 36), on the basis of the absence or presence of systemic allergic reactions upon sting challenge. The study was performed retrospectively using banked sera from patients initiated on HB VIT during August 1993 and November 2013 in 6 different clinical allergy centers that routinely perform sting challenge testing, and was approved by respective local ethic committees. Diagnosis of HBV allergy was based on a combination of patient's history of a systemic sting reaction, a positive skin test result, and positive specific IgE (sIgE) to HBV (≥0.35 kU$_A$/L; ImmunoCAP i1), as recently described.[8] All centers were asked to include patients at a 2:1 ratio of responders versus nonresponders for which they could provide serum samples obtained before initiation of VIT and during VIT before sting challenge. For each patient, the following data were obtained from the records: history and grade of anaphylactic HB sting reaction (according to Ring & Messmer),[16] skin test results, serology and tryptase levels at the time of initial diagnosis, date and treatment protocol of VIT, concomitant yellow jacket VIT, type, maintenance dose, and duration of HB VIT before sting challenge, systemic reactions during HB VIT, symptoms of systemic allergic reactions, and antihypertensive medication during sting challenge.

Statistical analysis

Statistical analysis was performed using the nonparametric Mann-Whitney *U* test for unpaired data and the Wilcoxon signed-rank test for paired data. ORs were calculated from contingency tables using the Fisher exact test on Graph Pad Prism 6 software. *P* values of less than .05 were regarded as significant. Calculation of diagnostic accuracy measures was performed using SPSS (IBM SPSS Statistics, Version 22.0.0.0, Ehningen, Germany) and the open statistical software environment R.[17] These were sensitivity, specificity, positive likelihood ratio (PLR) = sensitivity/(1 − specificity), negative likelihood ratio (NLR) = (1 − sensitivity)/specificity, receiver operating characteristic (ROC) curve, and area under the ROC curve (AUC).

For additional information on allergens, sIgE and sIgG$_4$ measurements, antisera, and immunoblot analysis, please see the Methods section in the Online Repository at www.jacionline.org.

RESULTS
IgE reactivity to HBV allergens in HBV-allergic patients before the start of VIT

The frequency of IgE sensitization (≥0.35 kU$_A$/L) to Api m 1, Api m 2, Api m 3, Api m 5, and Api m 10 of the entire study population (n = 115) was 81.7%, 52.2%, 49.6%, 61.7%, and 72.2%, respectively, confirming previously reported numbers in HBV-allergic patients (see Fig E1 in this article's Online Repository at www.jacionline.org).[8] Demographic and clinical data (age, sex, degree of anaphylaxis during index sting, treatment duration before sting challenge, and previously suggested risk factors for treatment failure) are summarized in Table I.

The comparison of responders (n = 79) and nonresponders (n = 36) before the initiation of VIT demonstrated no significant difference in level of sIgE to Api m 1, Api m 2, Api m 3, and Api m 5. In contrast, sIgE to HBV and to Api m 10 was moderately but significantly increased in nonresponders (Fig 1). We next compared the percentage of sIgE directed against individual allergens in relation to sIgE directed against the entire HBV (i1) in both patient groups. Although no significant differences were observed for Api m 1, Api m 2, and Api m 5, nonresponders displayed significantly increased percentage of sIgE to Api m 10 and a slightly reduced percentage of sIgE to Api m 3 in relation to sIgE to the entire HBV (Fig 2). Similarly, nonresponders displayed moderately but significantly increased percentage of sIgE to Api m 10 in relation to total IgE (see Fig E2 in this article's Online Repository at www.jacionline.org).

On analyzing the ROCs of Api m 10 sIgE (AUC, 0.687; *P* = .001) (Fig 3, *A*), the best discrimination between responders and nonresponders (ie, the highest Youden's index) was observed at values of 1.82 kU$_A$/L with a specificity of 70.9% (95% CI, 56.0% to 85.7%), a sensitivity of 69.4% (95% CI, 54.4% to 84.5%), a PLR of 2.39, and an NLR of 0.43. The cutoff of more than 0.35 kU$_A$/L provided a specificity of 34.2% (95% CI, 18.7% to 49.7%), a sensitivity of 83.3% (95% CI, 71.2% to 95.5%), a PLR of 1.27, and an NLR of 0.49. An Api m 10 sIgE level of 26.55 kU$_A$/L provided a specificity of 95% (95% CI, 90.0% to 100%), a sensitivity of 8.3% (95% CI, 0% to 17.4%), a PLR of 2.19, and an NLR of 0.95.

ROC analysis of the percentage of Api m 10 sIgE in relation to HBV sIgE (AUC, 0.667; *P* = .004) showed the best discrimination at 47.7%, with a specificity of 93.7% (95% CI, 85.7% to 100%), a sensitivity of 33.3% (95% CI, 17.9% to 48.7%), a PLR of 5.28, and an NLR of 0.71. Specificity and sensitivity levels at different percentages of Api m 10 sIgE to HBV sIgE are displayed in Fig 3, *B*. When more than 50% of HBV sIgE was directed against Api m 10, the test showed a specificity of 96.2% (95% CI, 90.0% to 100%) and a sensitivity of 25% (95% CI, 10.9% to 39.1%), with a PLR of 6.58 and an NLR of 0.78. This observation suggested that patients with a predominant sensitization to Api m 10 are at a higher risk of VIT treatment failure. To address this relationship, we analyzed the ORs for different percentage of sIgE to Api m 10 in relation to sIgE to HBV. Indeed, increasing percentages of sIgE to Api m 10 (in relation to sIgE to HBV) were associated with increasing OR for treatment failure (Fig 3, *C*). Notably, all subjects with sIgE to
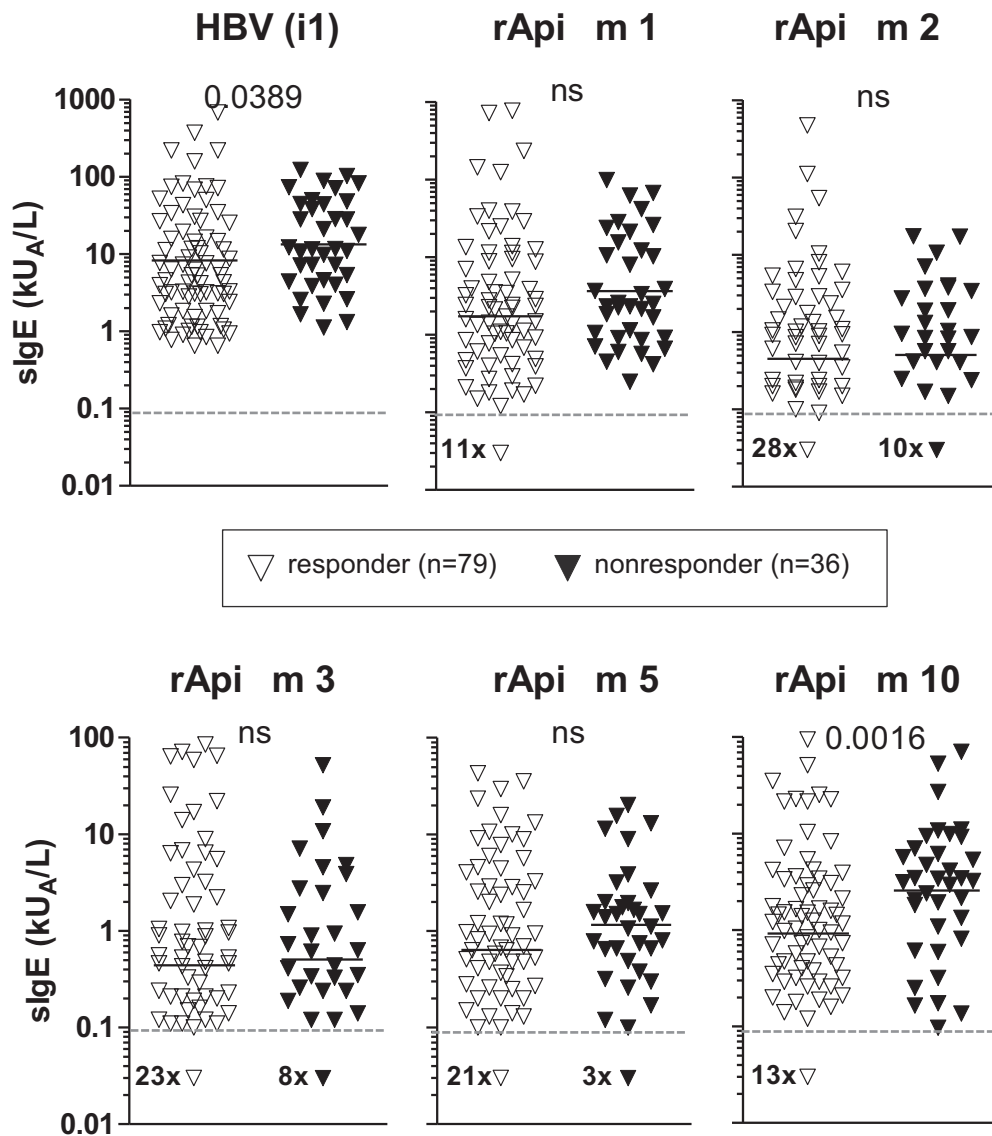
FIG 1. Serum IgE reactivity to HBV (i1), Api m 1, Api m 2, Api m 3, Api m 5, and Api m 10 in HBV-allergic patients before the initiation of HBV immunotherapy. All patients were classified as either responder (n = 79) or nonresponder (n = 36) on the basis of absence or presence of systemic allergic reactions upon sting challenge. The number of sera that displayed sIgE concentrations of less than 0.1 kU$_A$/L are displayed below the *dashed line* (*solid line*, geometric mean). *ns*, Not significant.

Api m 10 exceeding 60% of sIgE to HBV (n = 8) were treatment nonresponders. No such relationship was observed for sensitization to Api m 1, Api m 2, Api m 3, or Api m 5 (see Table E1 in this article's Online Repository at www.jacionline. org). Similarly, when comparing responders and nonresponders regarding the induction of sIgG$_4$ to HBV (i1), Api m 1, Api m 3, and Api m 10 during VIT, no significant differences were observed (see Fig E3 in this article's Online Repository at www.jacionline.org).

Analysis of Api m 1, Api m 3, and Api m 10 in crude and therapeutic HBV preparations

The initial evidence for the absence or underrepresentation of Api m 10 and Api m 3 in therapeutic HBV preparations was obtained using recombinant IgE antibodies isolated by combinatorial technologies.[13] To provide a more solid basis for analyzing the presence of allergens in crude and therapeutic HBV preparations, specific polyclonal antisera were generated in rabbits immunized with Api m 1, Api m 3, or Api m 10 (see Fig E4 in this article's Online Repository at www.jacionline. org). On applying these polyclonal antibodies in immunoblot analysis of 2 different sources of crude HBV, we found that all sera detected the corresponding proteins at the expected size and with comparable intensity. For Api m 3 and Api m 10, bands at 55 and 50 kDa, respectively, were detected (Fig 4, *A* and *B*). For Api m 1, 3 bands were detected at 15 to 20 kDa, representing the main glycoforms (Fig 4, *C*). In addition, 5 different therapeutic HBV preparations were analyzed at identical concentrations (1 mg/mL), all of which exhibited a clear reactivity with antisera directed to Api m 1 and Api m 3, comparable to that of crude HBV (Fig 4, *B* and *C*). However, in contrast to crude HBV, only 2 of 5
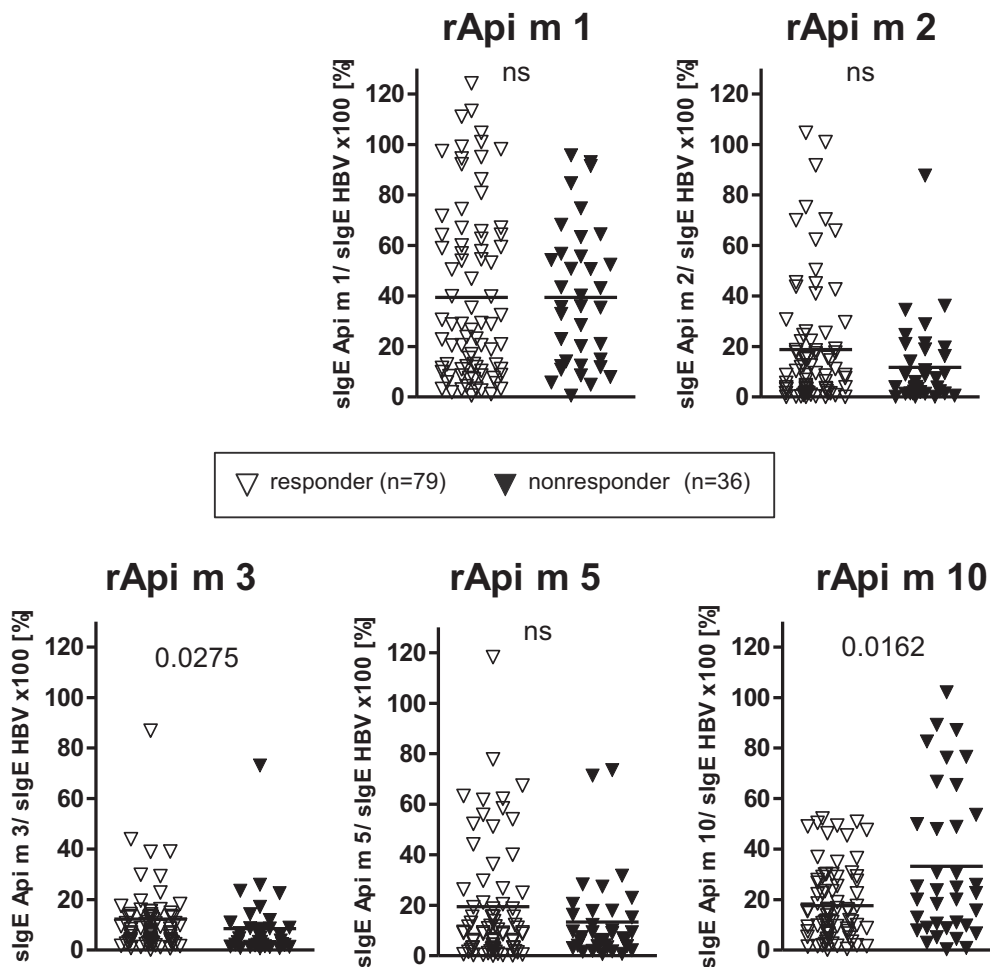
FIG 2. Percentage of sIgE directed against individual allergens in relation to IgE directed against the entire HBV (i1) in HBV-allergic patients (responders, n = 79, and nonresponders, n = 36) before the initiation of HBV immunotherapy. *ns*, Not significant.

therapeutic HBV preparations showed reactivity for the Api m 10–specific antiserum at a level comparable to that detected in crude venom when tested at a concentration of 0.3 mg/mL. These data corroborate the absence (or significant underrepresentation) of Api m 10 in some therapeutic HBV preparations (Fig 4, *A*).

Comparison of Api m 10–specific sIgE and IgG₄ responses in patients treated with different HBV preparations

Differences in Api m 10 concentrations among therapeutic HBV preparations prompted us to compare Api m 10–specific IgE and IgG₄ responses in patients who had received VIT using HBV preparations with low or no Api m 10 (group I, n = 59) and those who had received VIT using a HBV preparation with well-detectable Api m 10 immunoreactivity (group II, n = 50). VIT induced a moderate but significant reduction in sIgE to Api m 10 in both treatment groups (Fig 5, *A*). In contrast, significant induction of sIgG₄ was observed only in those patients treated with the HBV that contained detectable amounts of Api m 10 (group II, Fig 5, *B*).

DISCUSSION

Treatment failure in HB VIT has been suggested to be associated with the complexity of venom composition and the presence of individual sensitization profiles.[5-8] Additional risk factors include elevated baseline serum tryptase, concomitant mast cell disorders,[6,18,19] systemic allergic reactions during VIT build-up or maintenance phase, and angiotensin-converting enzyme inhibitor medication at the time of sting challenge.[6]

For the present study, patient charts were analyzed for evidence of known risk factors to exclude potential confounder effects. As summarized in Table I, both patient groups were comparable with regard to age, sex distribution, antihypertensive therapy, in particular ACE inhibitor medication, percentage of double VIT, and treatment duration. When analyzing the highest degree of sting reaction before VIT, in the present study nonresponders displayed a higher percentage of severe reactions (grades III and IV) as compared with responders (33.2% vs 21.6%). However, as recently demonstrated in a larger patient cohort, the degree of sting reaction before VIT does not appear to be a relevant risk factor for treatment failure.[6] A second potential difference was the percentage of patients with elevated serum tryptase concentrations and/or mastocytosis in the skin, which was lower among

FIG 3. **A,** ROC analysis of Api m 10 sIgE (AUC 0.687; *P* = .001) that display true positivity rate (sensitivity) plotted against false-positive rate (specificity) for different cutoff points. *Black circle*: WHO cutoff point for sIgE level of 0.35 kU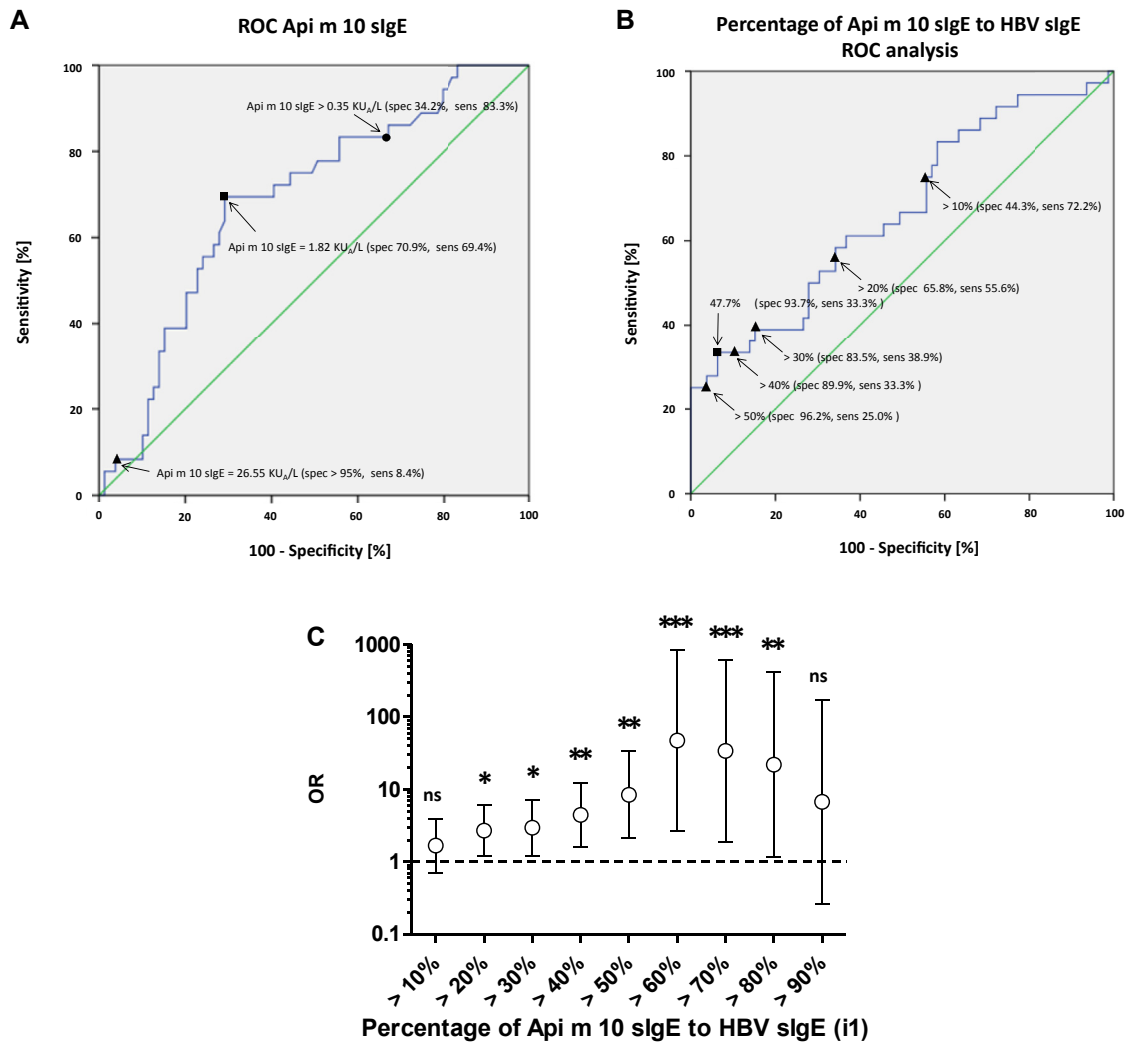$_A$/L or more; *black square*: highest Youden's index, that is, point of best discrimination between responders and nonresponders; *black triangle*: Api m 10 sIgE level at which a specificity of more than 95% is reached. **B,** ROC analysis of the percentage of Api m 10 sIgE in relation to HBV sIgE (AUC, 0.667; *P* = .004). *Black square*: highest Youden index, that is, point of best discrimination between responders and nonresponders; *black triangles*: specificity and sensitivity at different percentage levels of Api m 10 sIgE in relation to HBV sIgE. **C,** Calculated ORs for different percentage levels of sIgE to Api m 10 in relation to sIgE to HBV (i1) in HBV-allergic patients before the initiation of VIT. (*A, B, C*: responders, n = 79, nonresponders, n = 36). *P < .05, **P < .01, and ***P < .001. *ns*, Not significant; *WHO*, World Health Organization.

nonresponders than among responders (2.8% vs 5.1%). Because both parameters have been associated with increased risk of treatment failure, we would have expected the opposite and assume that this difference is random, that is, result of the relatively small study populations and thus does not constitute a relevant confounder for the present study.

A comparison of sensitization profiles to the panel of recombinant HBV allergens used in this study demonstrated an increased degree of IgE sensitization to Api m 10 among the nonresponders, while no significant differences were observed for the other allergens. The best discrimination between responders and nonresponders was calculated at Api m 10 sIgE levels of 1.82 kU$_A$/L with a specificity of 71% and a sensitivity of 70%. To obtain a specificity of 95%, an Api m 10 sIgE level of 26.55

kU$_A$/L was required, which resulted in a drop of sensitivity to 8.3%. In conclusion, the ROCs of Api m 10 sIgE as a single parameter, although significant (AUC, 0.687; *P* = .001), were far from optimal for identifying patients who were at risk to be not protected by HBV immunotherapy.

We speculated that the percentage of Api m 10 sIgE in relation to HBV sIgE (or to total serum IgE) may be more relevant. Indeed, the ROCs of the percentage of Api m 10 sIgE (in relation to HBV sIgE) displayed a better performance. When more than 50% of HBV sIgE was directed against Api m 10, the test showed a specificity of 96.2%, a sensitivity of 25%, and an OR of 8.44 for treatment failure. No such relationship was observed for Api m 1, Api m 2, Api m 3, and Api m 5 (Table E1). Similar results were obtained when the percentage of Api m 10 sIgE in relation to total
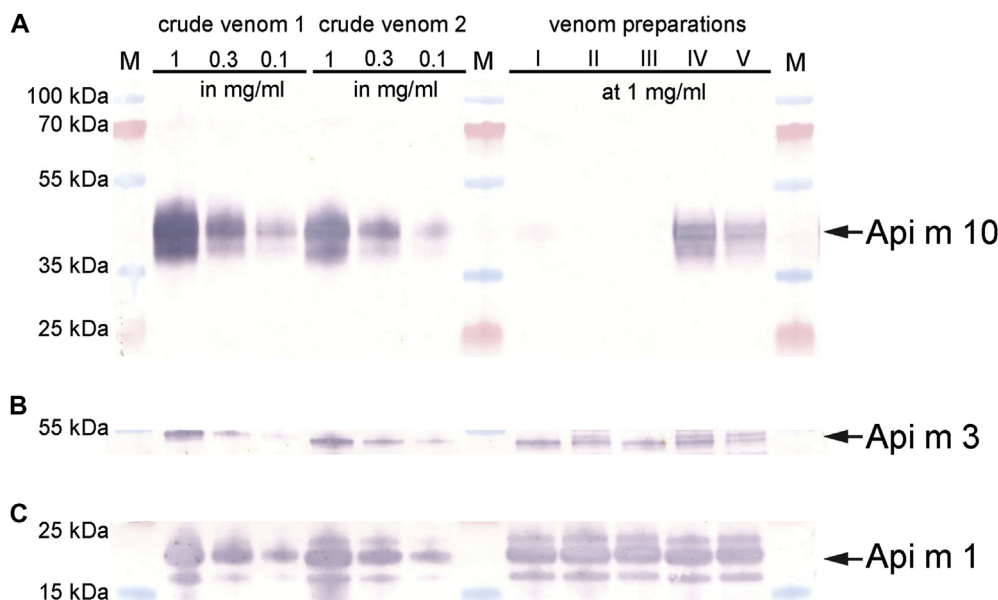
FIG 4. Presence of individual allergens in crude HBV and 5 different therapeutic HBV preparations. The presence of Api m 10 (**A**), Api m 3 (**B**), and Api m 1 (**C**) in 2 different crude and 5 different therapeutic preparations (I: Venomil, Allergy Therapeutics, Worthing, UK; II: Venomenhal, HAL Allergy, Leiden, the Netherlands; III: Aquagen, ALK-Abelló, Hamburg, Germany; IV: Pharmalgen, ALK-Abelló; V: Alyostal Venin, Stallergenes, Antony, France) using allergen-specific rabbit polyclonal anti–Api m 1, anti–Api m 3, and anti–Api m 10 antibodies was assessed by immunoblotting. Representative data of 3 different batches tested for I, III, IV, and V, and 2 different batches tested for II.

IgE was analyzed (see Figs E2 and E5 in this article's Online Repository at www.jacionline.org). However, the performance characteristics were less favorable, which may be due to a smaller group size and/or the fact that for a large proportion of the patients total IgE concentrations had not been analyzed in parallel with sIgE but in different laboratories. For most patients, serum samples were also available, which were obtained before the sting challenge. Again, nonresponders displayed moderately but significantly increased levels of sIgE to Api m 10 (see Fig E6 in this article's Online Repository at www.jacionline.org) and an increased percentage of sIgE to Api m 10 in relation to HBV sIgE (i1) (see Fig E7 in this article's Online Repository at www.jacionline.org) but not as prominently as in samples that were taken before initiation of VIT. In conclusion, the best test performance was obtained when analyzing the percentage of Api m 10 sIgE in relation to HBV sIgE before initiation of VIT. These data suggested that a predominant (>50%) sensitization to Api m 10 represents a relevant risk factor for VIT treatment failure in HBV allergy.

In this context, we reinvestigated the presence of Api m 1, Api m 3, and Api m 10 in different HBV preparations using polyclonal rabbit antisera in order to corroborate findings previously obtained using recombinant human IgE antibodies.[13] Monoclonal antibodies with high specificity for one epitope only might be more susceptible for interference by loss of epitope structures by unfolding, protein fragmentation, or isoform variation and thus might exhibit a lower sensitivity. We could demonstrate the presence of Api m 1 and Api m 3 in all tested HBV preparations. In contrast and consistent with our previous report, Api m 10 was underrepresented in 3 of 5 therapeutic HBV preparations while present in both crude HBV preparations analyzed (Fig 4).

Notably, the lack of additional bands for Api m 10 makes the presence of putative Api m 10 isoforms with significantly variant molecular mass unlikely. This finding also suggests that the absence of Api m 10 in some of the therapeutic HBV preparations is not mainly caused by protein fragmentation.

Currently we speculate that processing/purification of the crude HBV during the manufacturing process may lead to the loss of Api m 10 immunoreactivity. In this context, it is of interest that Api m 10 was detectable in nonprocessed HBV preparations (as in Pharmalgen, ALK-Abelló), whereas it was not detectable in therapeutic HBV preparations that have been processed/purified to reduce low molecular weight substances (such as Aquagen, ALK-Abelló). This step has been introduced to exclude vasoactive mediators and small bioactive peptides that have been associated with local side effects of VIT.[20,21] Because direct loss of Api m 10 on the basis of molecular size is unlikely, other indirect mechanisms during the manufacturing process may be involved and must be addressed for each product by the respective manufacturer.

The analysis of allergen-specific IgG$_4$ levels before and under VIT demonstrated robust induction of sIgG$_4$ directed against HBV or Api m 1 but no or little against Api m 3 and Api m 10 (Fig E3). This difference may be due to the quantities of allergens in the HBV. Although Api m 1 represents a highly abundant allergen that constitutes up to 12% of the HBV dry weight, both Api m 3 and Api m 10 are present in much smaller quantities (1% to 2% and <1%, respectively).[15] Induction of IgG$_4$ to any of the allergens did not allow discrimination between responders and nonresponders (Fig E3). Interestingly, when patients were stratified according to the type of venom used for VIT, a low but significant induction of sIgG$_4$ to Api m 10 was observed
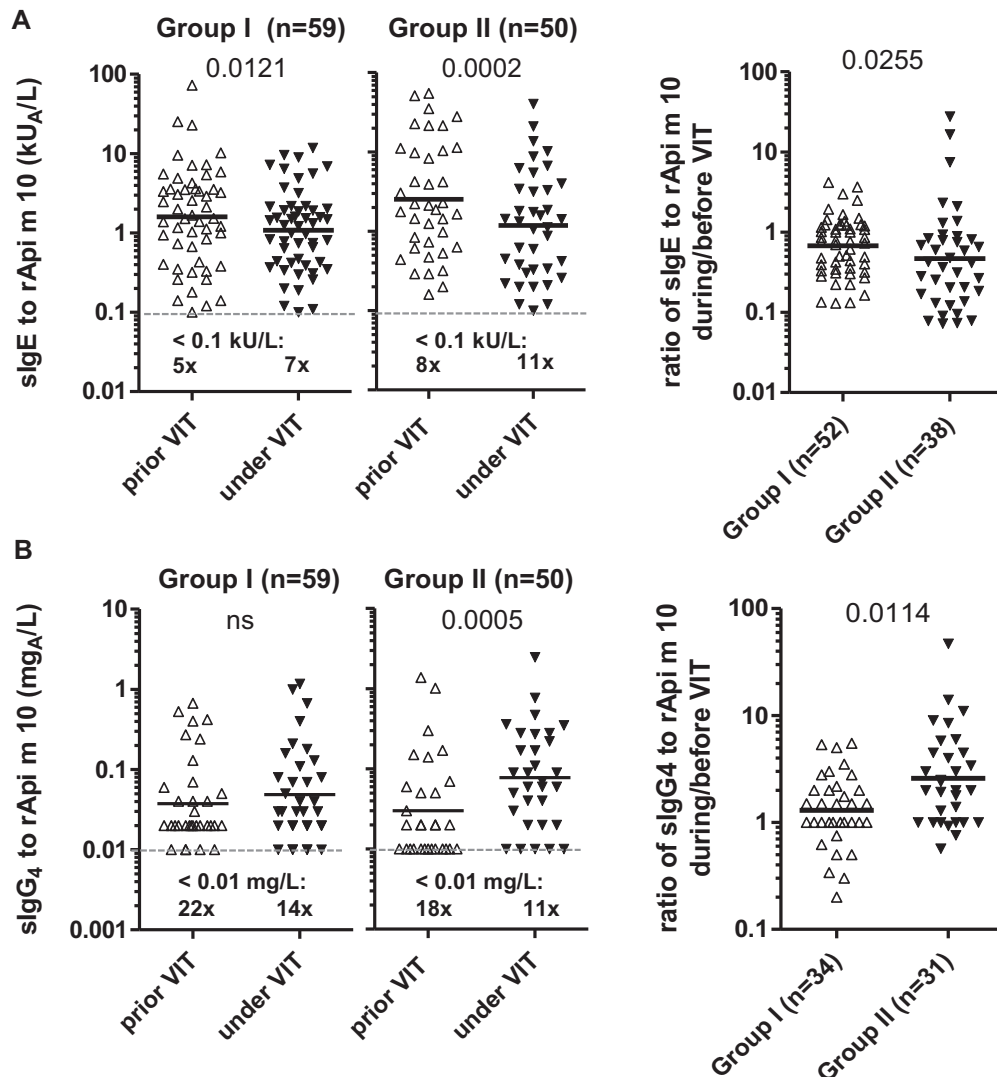
FIG 5. Api m 10–specific immune responses in HBV-allergic patients under immunotherapy with an HBV preparation that contains detectable Api m 10 immunoreactivity (group II) or with HBV preparations with no or low Api m 10 immunoreactivity (group I). **A,** sIgE to Api m 10 before VIT and under VIT *(left panels)*. Samples with sIgE levels above the LOD at both time points were used to calculate the ratio of sIgE during/before VIT *(right panel)* for both groups. **B,** sIgG$_4$ to Api m 10 before VIT and under VIT *(left panels)*. Samples with sIgG$_4$ levels above the LOD at both time points were used to calculate the ratio of sIgG$_4$ during/before VIT *(right panel)* for both groups. *LOD,* Limit of detection.

only in those patients treated with the HBV that contained detectable amounts of Api m 10.

The present study has clear limitations in that it used a retrospective study design using a limited number of stored sera and corresponding clinical data from patients seen at 6 different allergy clinics. The differences observed were relatively small but consistent regardless of the way they were calculated (Api m 10 sIgE values, percentage of Api m 10 sIgE to HBV sIgE, or percentage of Api m 10 sIgE to total IgE). The ROCs and the PLRs of these parameters are clearly less impressive than those of other risk markers, such as Ara h 2 in peanut allergy. This may be related to the fact that risk markers such as Ara h 2 are used in the diagnostic setting to detect IgE sensitizations associated with increased risk of severe allergic reactions including anaphylaxis. In contrast, we have used component-resolved diagnostics to address whether certain sensitization profiles are associated with

different treatment outcomes, that is, an increased risk not to benefit from VIT. In this setting, the hurdle may be even higher to detect significant and clinically useful differences because more variables may influence the outcome of the therapeutic approach.

Despite these limitations, we provide for the first time evidence that patients with a predominant sensitization to Api m 10 are at increased risk of not becoming protected by HBV immunotherapy. In addition, we demonstrate that some but not all therapeutic HBV preparations lack Api m10 and that sIgG$_4$ responses depend on the presence of Api m 10 in the VIT preparation. Given the quite recent availability of Api m 10 on a widely used test platform, it appears justified to recommend that (a) sIgE to Api m 10 should be tested in HBV allergy and (b) patients with a predominant Api m 10 sensitization should be treated with a HBV preparation in which the presence of a relevant amount of Api m 10 has been documented. Additional evidence to support

this notion will require prospective studies in which the treatment efficacy of HBV preparations with and without Api m 10 is compared and related to initial component-resolved sensitization profiles.

> **Clinical implications:** HBV-allergic patients with dominant sensitization to Api m 10 are at increased risk for treatment failure in HBV immunotherapy and should benefit from treatment with Api m 10–containing preparations.

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METHODS

Allergens and IgE and IgG$_4$ antibody measurements

Recombinant Api m 2, Api m 3, and Api m 5 were expressed as secreted full-length proteins by *Spodoptera frugiperda* (Sf9) insect cells as recently described.[E1-E4] Api m 10 was expressed in *Escherichia coli*. Experimental ImmunoCAP tests (Phadia AB, Uppsala, Sweden) containing the purified HBV allergens were prepared as described.[E5] In addition, commercially available ImmunoCAPs for sIgE to HBV (i1) and Api m1 (i208) and total IgE were used. All IgE and IgG$_4$ antibody measurements were performed using a Phadia 250 instrument according to the manufacturer's instructions (Thermo Fischer Scientific, Uppsala, Sweden). The cutoff level for positive/negative sIgE sensitization ($\geq$0.35 kU$_A$/L) is indicated as dotted line (Fig E1). sIgE levels below 0.1 kU$_A$/L were set at 0.1 kU$_A$/L for statistical analysis and displayed separately as less than 0.1 kU$_A$/L in Figs 1 and 5, *A*. sIgG$_4$ antibody measurements were performed at a serum dilution of 1:100 for HBV and Api m 1 according to the manufacturer's instructions. The LOD for sIgG$_4$ was calculated as 89 $\pm$ 22 μg$_A$/L as described.[E6] Thus, levels below 100 μg$_A$/L were set at 100 μg$_A$/L for statistical analysis and displayed separately as less than 100 μg$_A$/L in the figures. sIgG4 for Api m 3 was analyzed at a 1:20 and for Api m 10 at a 1:5 dilution and values were calculated and displayed accordingly.

Generation of antisera

Recombinantly produced allergens Api m 1, Api m 3, and Api m 10 were used for immunization of rabbits according to established protocols. Immunoreactivity and specificity of the resulting antisera were assessed by ELISA using recombinant HBV proteins (Fig E3). Purified recombinant Api m 1, Api m 2, Api m 3, Api m 5, and Api m 10 (10 μg/mL) were coated on 384-well microtiter plates (Greiner, Frickenhausen, Germany) at 4°C overnight and blocked with 40 mg/mL milk powder in PBS. Thereafter, antisera were diluted 1:5.000-20.000 with 2% milk powder in TBS and were incubated in a final volume of 50 μL for 2 hours at room temperature and applied to the corresponding wells. After washing 4 times with T-PBS and PBS, alkaline phosphatase–conjugated antirabbit IgG antibodies diluted 1:30.000 in 20 mg/mL milk powder in PBS were added for 1 hour at room temperature. Signals were detected after washing 4 times with T-PBS and PBS and subsequent addition of 50 μL of substrate solution.

Immunoblot analyses of venom and VIT preparations

Crude venoms were obtained from Latoxan (Valence, France) and Entomon (Florence, Italy). The VIT preparations we assessed included Pharmalgen (ALK-Abelló), Alyostal Venin (Stallergenes), Aquagen (ALK-Abelló), Venomhal (HAL Allergy), and Venomil (Allergy Therapeutics). For immunoblot procedures, crude venom as well as the therapeutic venom preparations were dissolved to a stock concentration of 1 mg/mL. Amounts of 5 μL for detection of Api m 1, Api m 3, and Api m 10 were separated under reducing conditions by SDS-PAGE using Criterion XT (12%) Bis-Tris gels and MES buffer (Biorad, Munich, Germany) and transferred by wet blotting in Towbin buffer onto nitrocellulose membranes (Roth, Karlsruhe, Germany). The membranes were blocked with 4% milk powder in TBS buffer. Allergen-specific sera were diluted 1:5.000-50.000 with 2% milk powder in TBS and applied to the corresponding membranes. Visualization of bound IgG was performed with goat antirabbit IgG conjugated to alkaline phosphatase (Sigma-Aldrich, Hamburg, Germany) diluted 1:20.000 in 2% milk powder in TBS and nitrotetrazolium blue chloride/x-phos (Sigma-Aldrich, Hamburg, Germany) according to the recommendations of the manufacturer.

RESULTS

Prevalence of IgE sensitization to HBV allergens in HBV-allergic patients

Prevalence of IgE sensitization to HBV allergen components among the 115 HBV-allergic patients included in the study (sIgE to Api m 1, Api m 2, Api m 3, Api m 5, and Api m 10) is displayed in Fig E1.

IgE reactivity to Api m 10 in relation to total IgE

Although no significant differences were observed for the percentage of sIgE to HBV, Api m 1, Api m 2, Api m 3, and Api m 5, nonresponders displayed moderately but significantly increased percentage of sIgE to Api m 10 in relation to total IgE (Fig E2).

ROC analysis of the percentage of Api m 10 sIgE in relation to total serum IgE (AUC, 0.659; *P* = .009) showed the best discrimination between responders and nonresponders at 2.9%, with a specificity of 77.3% (95% CI, 63.7% to 91.0%) and a sensitivity of 54.5% (95% CI, 38.3% to 70.8%) with a PLR of 2.40 and an NLR of 0.58. Specificity and sensitivity levels at different percentages of Api m 10 sIgE to total IgE are displayed in Fig E5. When more than 11% of total IgE was directed against Api m 10, the test showed a specificity of 95% (95% CI, 89.6% to 100%), a sensitivity of 18.2% (95% CI, 5.6% to 30.8%), a PLR of 4.55, and an NLR of 0.85.

IgG$_4$ reactivity to HBV allergens before and during VIT

For the analysis of IgG$_4$ responses, paired samples obtained before and after at least 6 months of VIT (ie, before sting challenge) were available from 73 responders and 35 nonresponders. Before VIT, sIgG$_4$ to HBV and to Api m 1 was detectable in most of the samples both in responders and in nonresponders (Fig E3, *A*). In contrast, sIgG$_4$ to Api m 3 and Api m 10 was detectable at a much lower level and a substantial proportion of the patients' sera were below the detection limit of the assay (25 of 73 responders, 10 of 35 nonresponders). Following at least 6 months of VIT, a prominent induction of sIgG$_4$ was observed for the highly abundant allergen Api m 1, comparable to that observed for whole HBV (Fig E3, *A* and *B*). In contrast, no significant induction of sIgG$_4$ to Api m 3 and only a minor increase in sIgG$_4$ to Api m 10 was observed in both responders and nonresponders (Fig E3, *A*). Patients' samples with sIgG$_4$ levels above the cutoff of the assay at both time points were used to calculate the ratio of sIgG$_4$ during/before VIT. On comparing responders and nonresponders regarding the degree of sIgG$_4$ induction, no significant difference was observed for any of the parameters (Fig E3, *B*).

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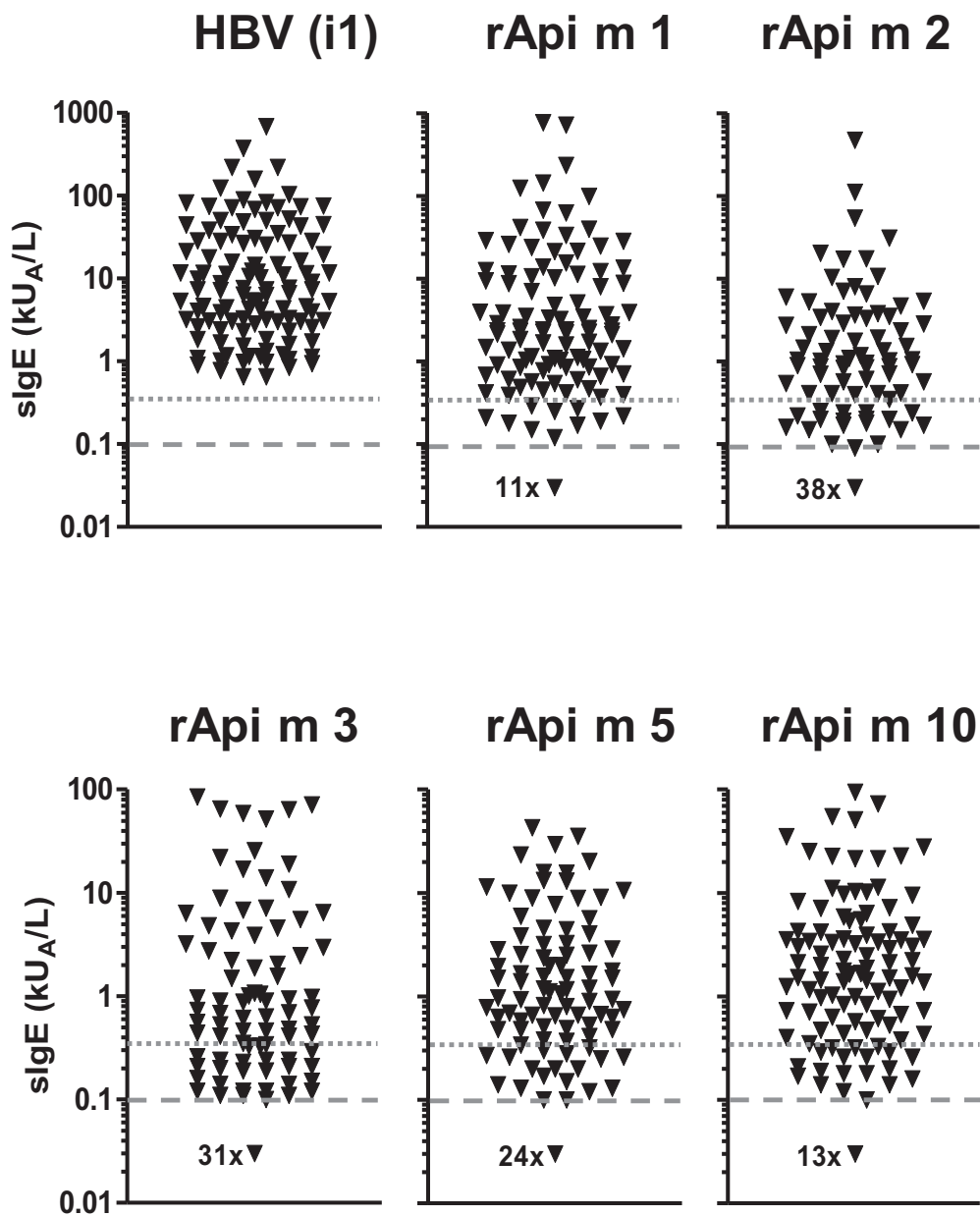
FIG E1. Prevalence of IgE sensitization to HBV allergen components among the 115 HBV-allergic patients included in the study (sIgE to Api m 1, Api m 2, Api m 3, Api m 5, and Api m 10). The cutoff level for positive sensitization (≥0.35 kU$_A$/L) is indicated as dotted line, and the lower limit of quantification (≥0.10 kU$_A$/L) is indicated as dashed line.
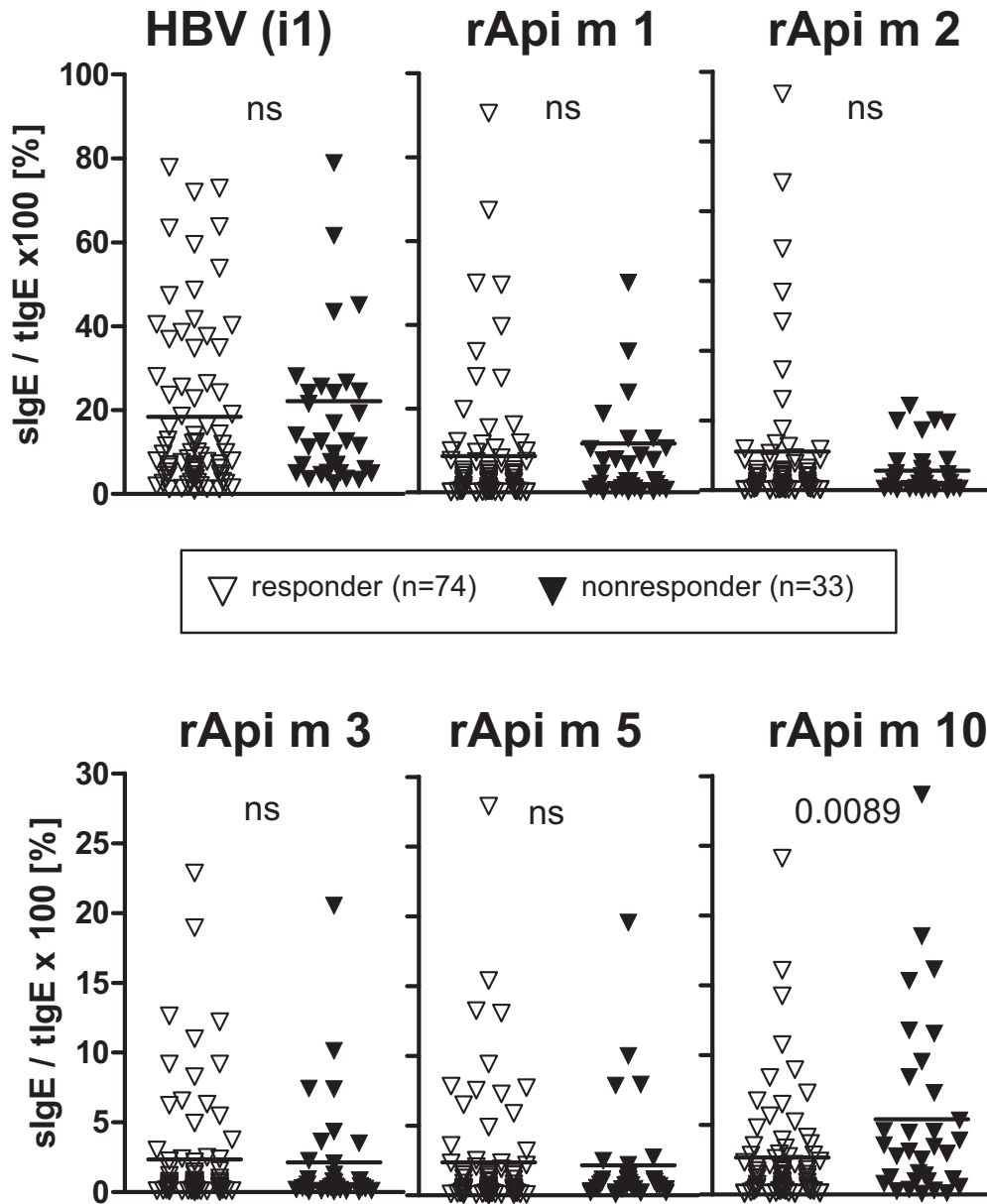
FIG E2. Percentage of sIgE to HBV, Api m 1, Api m 2, Api m 3, Api m 5, and Api m 10 in relation to total IgE in HBV-allergic patients before the initiation of VIT. All patients were classified as either responder (n = 75) or nonresponder (n = 33) on the basis of the absence or presence of systemic allergic reactions upon sting challenge. Please note that because of lacking total IgE values, 4 responder sera and 3 nonresponder sera could not be included in this calculation. *ns*, Not significant.
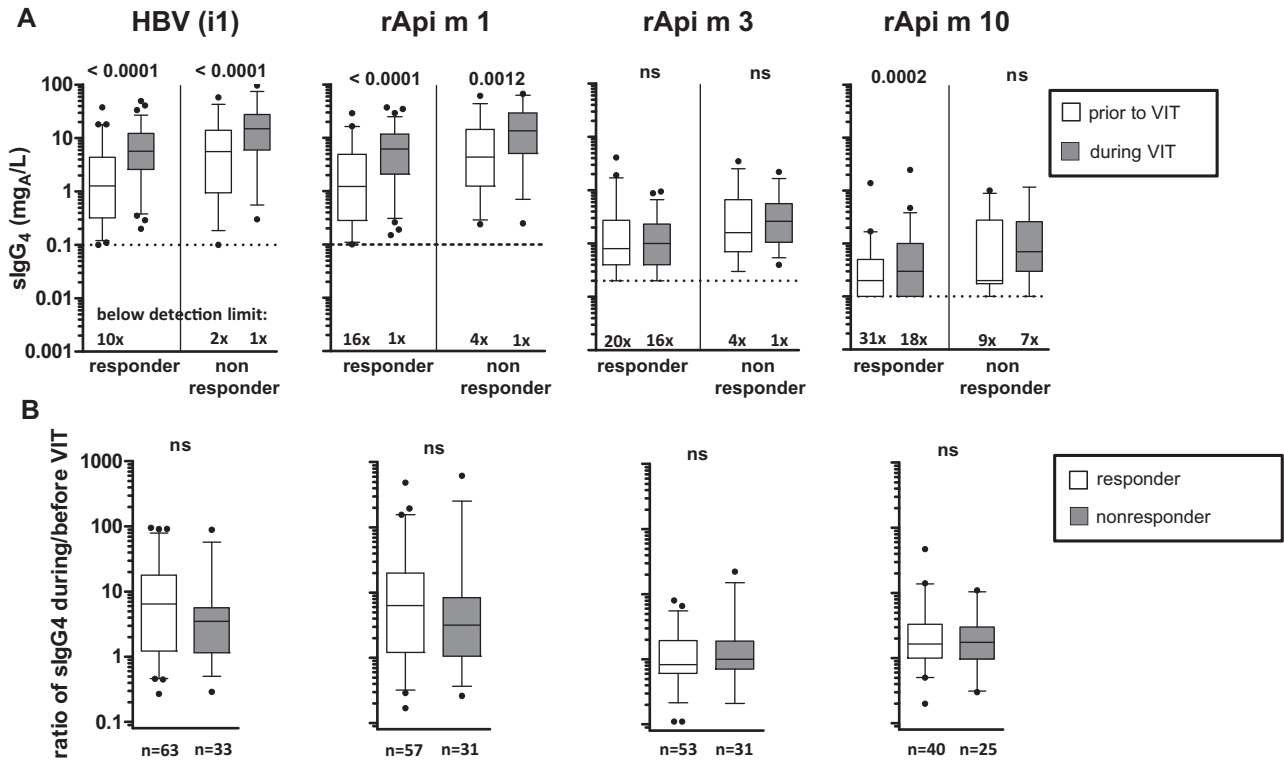
FIG E3. A, Serum IgG$_4$ reactivity to HBV (i1), Api m 1, Api m 3, and Api m 10 in HBV-allergic patients (responders, n = 73; nonresponders; n = 35) before initiation and after at least 6 months of HBV immunotherapy. The number of sera that displayed sIgG$_4$ concentrations below the LOD of the assay is displayed below the dashed line. **B,** Samples with sIgG$_4$ levels above the LOD of the assay at both time points were used to calculate the ratio of sIgG$_4$ during/before VIT. *LOD,* Limit of detection; *ns,* not significant.
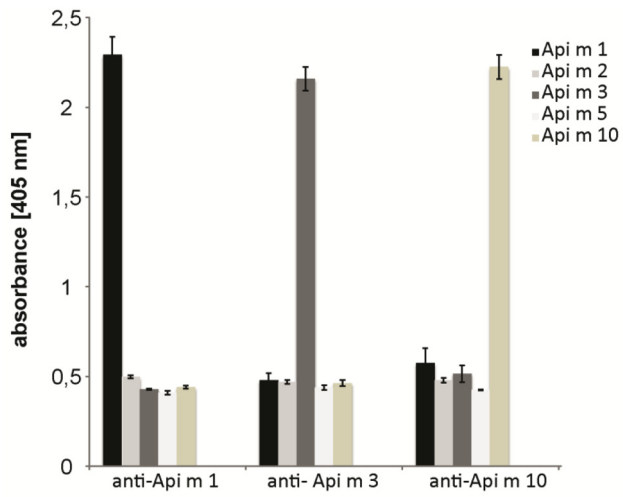
FIG E4. Specificity and immunoreactivity of antibodies against Api m 1, Api m 3, and Api m 10. The specificity of the polyclonal antisera against Api m 1, Api m 3, and Api m 10 was assessed by ELISA using recombinant HBV allergens Api m 1, Api m 2, Api m 3, Api m 5, and Api m 10.
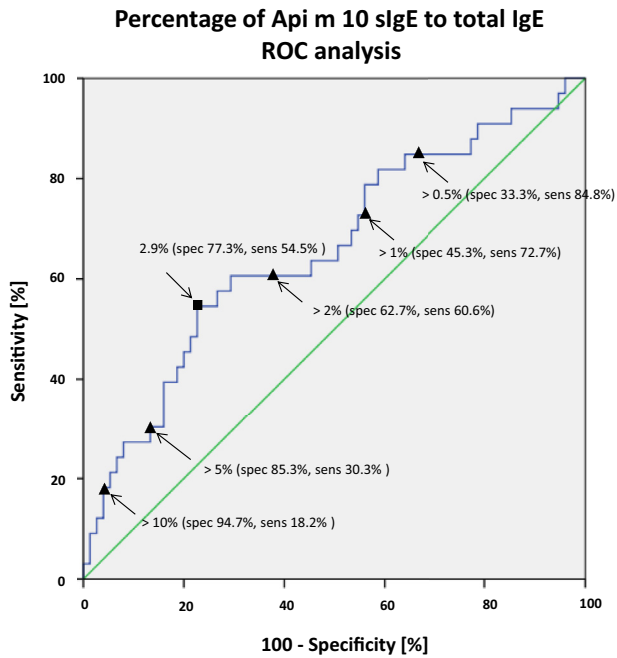
FIG E5. ROC analysis of the percentage of Api m 10 sIgE in relation to total IgE (AUC, 0.659; P = .009). *Black square*: highest Youden's index, that is, point of best discrimination between responders and nonresponders; *black triangles*: specificity and sensitivity at different percentage levels of Api m 10 sIgE in relation to total IgE.
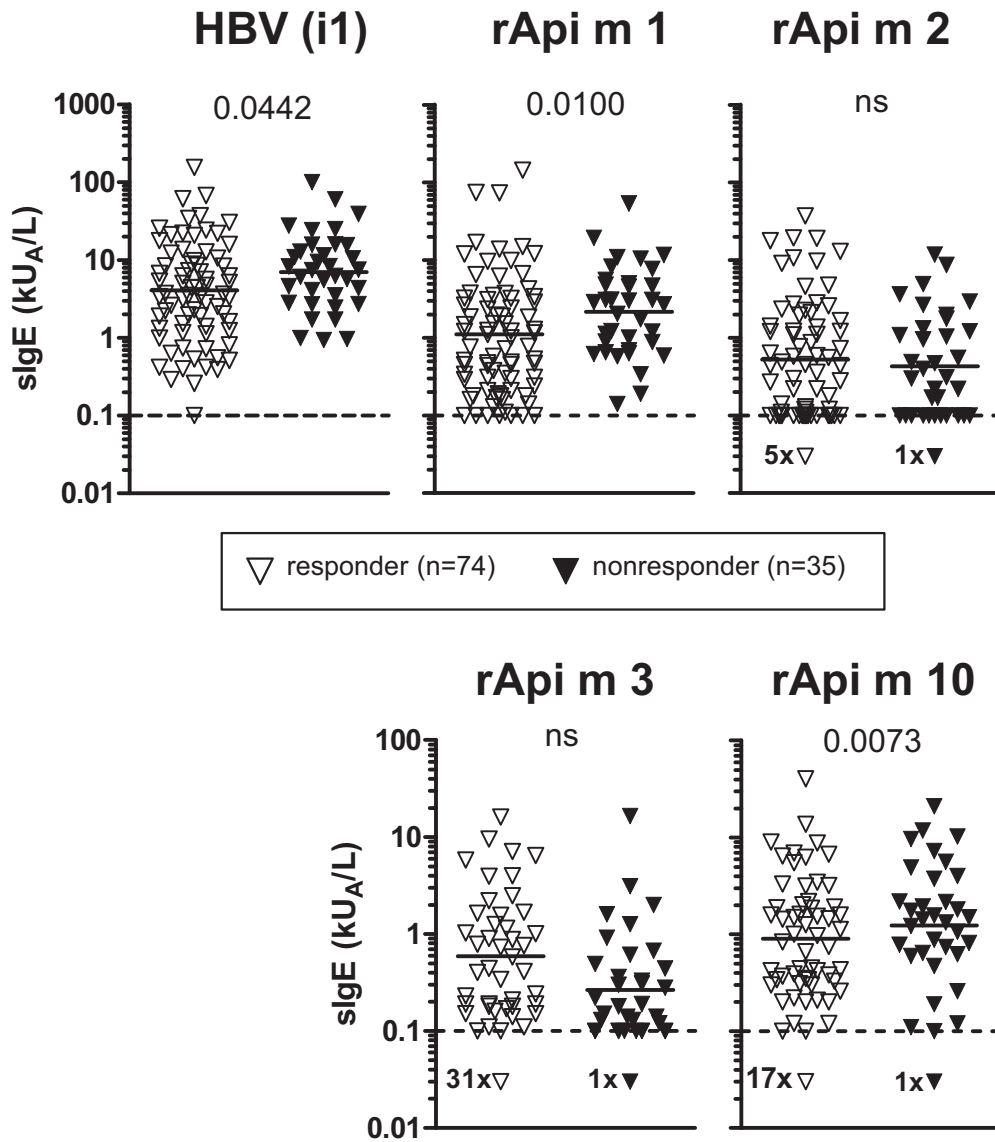
FIG E6. Serum IgE reactivity to HBV (i1), Api m 1, Api m 2, Api m 3, Api m 5, and Api m 10 in HBV-allergic patient under VIT before sting challenge. All patients were classified either as responders (n = 74) or as nonresponders (n = 35) on the basis of the absence or presence of systemic allergic reactions upon sting challenge. The number of sera that displayed sIgE concentrations of less than 0.1 kU$_A$/L is displayed below the dashed line. *ns*, Not significant.

FIG E7. Percentage of sIgE directed against individual allergens in relation to IgE directed against the entire HBV (i1) in HBV-allergic patient (responders, n = 71, and nonresponders, n = 35) under VIT before sting challenge. Please note that 3 responder sera were excluded because sIgE to HBV (i1) had fallen below 0.35 kU$_A$/L. *ns*, Not significant.

TABLE E1. ORs for nonresponding to VIT were calculated for different percentages of sIgE directed against individual HBV allergens (Api m 1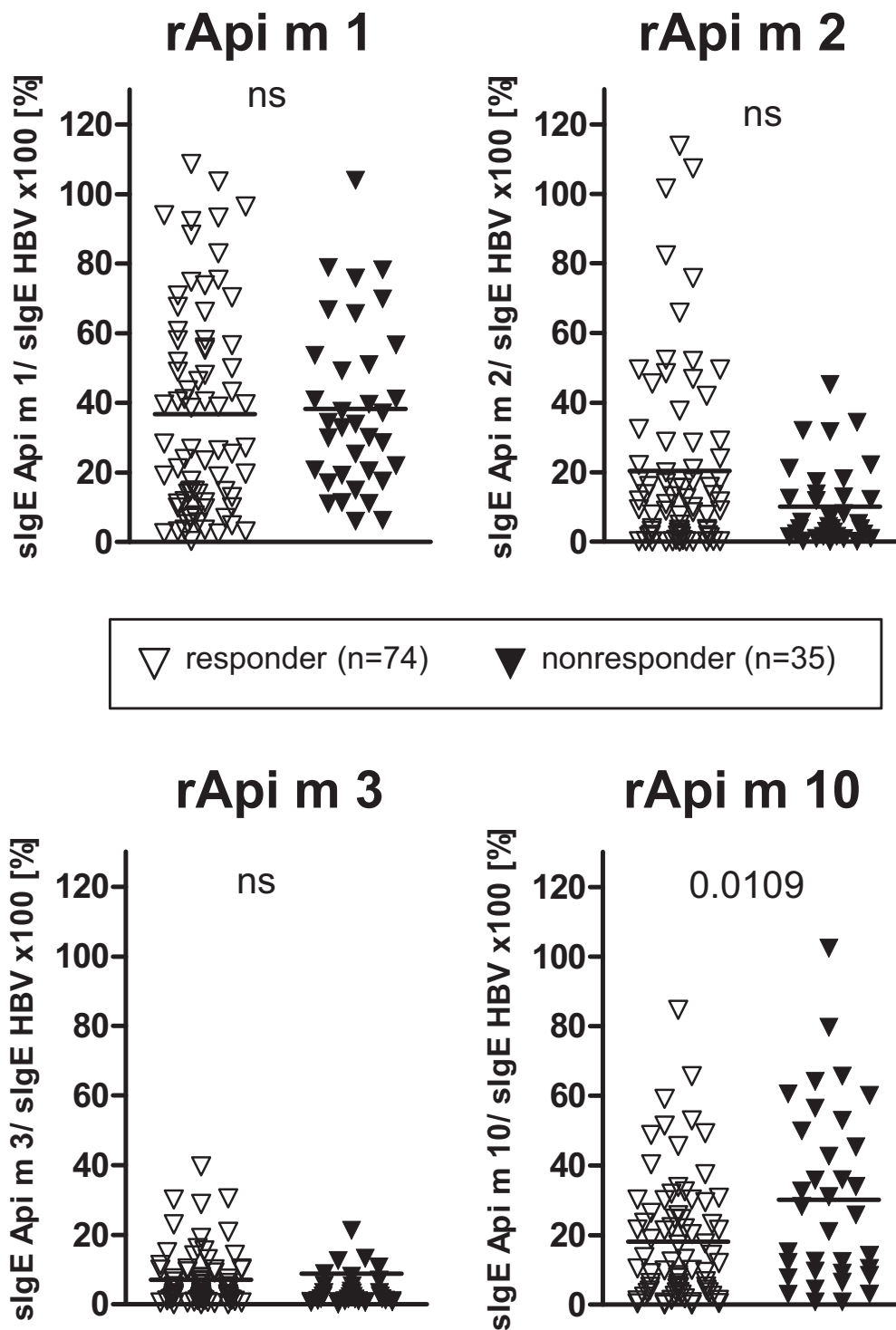, Api m 2, Api m 3, Api m 5, and Api m 10) in relation to sIgE to the entire HBV (i1) in HBV-allergic patients before the initiation of VIT

sIgE Api m 1 in relation to sIgE HBV (i1)	n of responders	n of nonresponders	OR	CI	*P* value
>10%	60 of 79	31 of 36	1.963	0.669-5.762	NS
>20%	48 of 79	25 of 36	1.468	0.633-3.403	NS
>30%	36 of 79	21 of 36	1.672	0.754-3.711	NS
>40%	31 of 79	17 of 36	1.385	0.625-3.069	NS
>50%	30 of 79	14 of 36	1.039	0.463-2.336	NS
>60%	22 of 79	8 of 36	0.740	0.293-1.871	NS
>70%	15 of 79	5 of 36	0.688	0.229-2.066	NS
>80%	13 of 79	4 of 36	0.635	0.192-2.102	NS
>90%	11 of 79	3 of 36	0.544	0.147-2.153	NS

sIgE Api m 2 in relation to sIgE HBV (i1)	n of responders	n of nonresponders	OR	CI	*P* value
>10%	38 of 79	12 of 36	0.539	0.237-1.227	NS
>20%	21 of 79	7 of 36	0.667	0.254-1.750	NS
>30%	15 of 79	3 of 36	0.388	0.105-1.436	NS
>40%	14 of 79	1 of 36	0.133	0.017-1.052	.0347
>50%	9 of 79	1 of 36	0.222	0.027-1.826	NS
>60%	8 of 79	1 of 36	0.254	0.030-2.109	NS
>70%	5 of 79	1 of 36	0.423	0.048-3.759	NS
>80%	3 of 79	1 of 36	0.724	0.073-7.211	NS
>90%	3 of 79	0 of 36	0.299	0.015-5.954	NS

sIgE Api m 3 in relation to sIgE HBV (i1)	n of responders	n of nonresponders	OR	CI	*P* value
>10%	31 of 79	8 of 36	0.442	0.179-1.095	NS
>20%	8 of 79	4 of 36	1.109	0.311-3.954	NS
>30%	5 of 79	1 of 36	0.423	0.048-3.759	NS
>40%	3 of 79	1 of 36	0.724	0.073-7.211	NS
>50%	2 of 79	1 of 36	1.10	0.096-12.55	NS
>60%	2 of 79	1 of 36	1.10	0.096-12.55	NS
>70%	2 of 79	1 of 36	1.10	0.096-12.55	NS
>80%	2 of 79	0 of 36	0.425	0.019-9.080	NS
>90%	1 of 79	0 of 36	0.717	0.028-18.04	NS

sIgE Api m 5 in relation to sIgE HBV (i1)	n of responders	n of nonresponders	OR	CI	*P* value
>10%	38 of 79	13 of 36	0.609	0.271-1.372	NS
>20%	21 of 79	7 of 36	0.667	0.254-1.750	NS
>30%	15 of 79	3 of 36	0.388	0.105-1.436	NS
>40%	13 of 79	2 of 36	0.299	0.064-1.401	NS
>50%	12 of 79	2 of 36	0.328	0.069-1.552	NS
>60%	7 of 79	2 of 36	0.605	0.119-3.069	NS
>70%	3 of 79	2 of 36	1.490	0.238-9.334	NS
>80%	2 of 79	0 of 36	0.425	0.019-9.080	NS
>90%	2 of 79	0 of 36	0.425	0.019-9.080	NS

sIgE Api m 10 in relation to sIgE HBV (i1)	n of responders	n of nonresponders	OR	CI	*P* value
>10%	48 of 79	26 of 36	1.679	0.712-3.960	NS
>20%	27 of 79	21 of 36	2.696	1.200-6.059	.0242
>30%	14 of 79	14 of 36	2.955	1.220-7.157	.0194
>40%	8 of 79	12 of 36	4.438	1.620-12.15	.0064
>50%	3 of 79	9 of 36	8.444	2.127-33.53	.0013
>60%	0 of 79	8 of 36	47.42	2.649-848.8	.0001
>70%	0 of 79	6 of 36	33.89	1.851-620.3	.0007
>80%	0 of 79	4 of 36	22.02	1.151-421.0	.0085
>90%	0 of 79	1 of 36	6.718	0.267-169.1	NS

NS, Not significant.