LAD2 mast cell activation test associates with the reaction severity and diagnoses BAT nonresponders in *Hymenoptera* venom allergy

Short title: LAD2 MAT in *Hymenoptera* venom allergy

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ABSTRACT

Background: The usefulness of the mast cell activation test (MAT) in diagnosing patients with uninterpretable basophil activation test (BAT) caused by nonresponding basophils has not yet been addressed. It should be further evaluated if the results of MAT are associated with the severity of the allergic reaction.

Methods: We recruited 39 *Hymenoptera* venom allergic (HVA) patients, 22 non-sensitized controls, and 37 BAT nonresponding HVA patients. Specific IgE levels for honey bee venom (HBV), yellow jacket venom (YJV) and total IgEs were quantified using the Immulite system. BAT and MAT with LAD2 cells in response to HBV and YJV were performed.

Results: We first optimized the susceptibility of LAD2 cells to IgE-mediated degranulation in HVA and showed that prestimulation with IL-33 and IL-6 significantly increased the LAD2 cells' responsiveness to allergen stimulation ($P<0.01$). LAD2 MAT results correlated with BAT results, and patients with severe sting reactions (Mueller grades IV or III) had a median 2-fold higher LAD2 MAT than the patients with nonsevere sting reactions (Mueller grades II, I or LLR) ($P<0.05$). Further, LAD2 MAT provided conclusive results in 54.1% (20 of 37) of HVA patients with nonresponding basophils in the BAT.

Conclusions: The LAD2 MAT represents a new diagnostic tool for HVA patients with nonresponding basophils. Further, LAD2 MAT can identify patients at risk of severe sting reactions and thus can help guide recommendations for venom immunotherapy and improve the management of patients with HVA.

Key words: Basophil activation test nonresponders. *Hymenoptera* venom allergy. LAD2. Mast cell activation test. Reaction severity.

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RESUMEN

Antecedentes: Aún no se conoce la utilidad del test de activación de mastocitos (MAT) en el diagnóstico de pacientes que tienen el test de activación de basófilos (BAT) no interpretable, debido a que sus basófilos son no respondedores. Por lo que debería evaluarse si los resultados del MAT están asociados con la gravedad de la reacción alérgica.
Métodos: Se reclutaron 39 pacientes alérgicos al veneno de himenópteros (HVA), 22 controles no sensibilizados y 37 pacientes HVA que no respondieron a la BAT. Se cuantificaron los niveles de IgE específica frente all veneno de abeja melífera (VMH), el veneno de chaqueta amarilla (VJA) y la IgE tota mediante el sistema Immulite. Se realizaron BAT y MAT con células LAD2 (MAT/LAD2) en respuesta al VHB y al YJV.
Resultados: En primer lugar, optimizamos la susceptibilidad de las células LAD2 a la degranulación mediada por IgE en HVA y demostramos que la preestimulación con IL-33 e IL-6 aumentaba significativamente la capacidad de respuesta de las células LAD2 a la estimulación con alérgenos ($P<0,01$). Los resultados del MAT/LAD2 se correlacionaron con los resultados de BAT, y los pacientes con reacciones graves a la picadura (grados IV o III de Mueller) tuvieron una mediana en el MAT/LAD2, 2 veces mayor que los pacientes con reacciones no graves a la picadura (grados II, I o LLR de Mueller) ($P<0,05$). Además, el MAT/LAD2 proporcionó resultados concluyentes en el 54,1% (20 de 37) de los pacientes HVA con basófilos no respondedores en el BAT.
Conclusiones: El MAT/LAD2 representa una nueva herramienta diagnóstica para pacientes HVA con basófilos no respondedores. Además, el MAT/LAD2 puede identificar a los pacientes con riesgo de reacciones graves a la picadura y, por tanto, puede ayudar a orientar las recomendaciones para la inmunoterapia con veneno y mejorar el tratamiento de los pacientes con HVA.

Palabras clave: Prueba de activación de basófilos no respondedores. Alergia al veneno de himenópteros. LAD2. Prueba de activación de mastocitos. Gravedad de la reacción.

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Summary box

- **What do we know about this topic?**

The MAT is a diagnostic tool that can help resolve the diagnosis of food and drug allergies. However, the use of MAT in Hymenoptera venom allergy (HVA) is lacking, especially in the case of BAT nonresponders.

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

This study showed that the LAD2 MAT can resolve diagnosis for most HVA patients with nonresponding basophils. The LAD2 MAT can also identify patients at risk of severe sting reactions and thus can improve the management of patients with HVA.

1. Introduction

Testing for serum IgE to recombinant venom component and the basophil activation test (BAT) can improve the diagnosis and monitoring in Hymenoptera venom allergy (HVA) [1–4]. The availability of venom allergen components has advanced our understanding of HVA and enabled molecular diagnosis of HVA, which can distinguish true allergy from cross-reactivity to honey bee and yellow jacket venoms, whereas BAT can confirm sensitization in case of negative skin and venom sIgE testing and evaluate venom-specific clinical reactivity in case of a dual honeybee and vespid sensitization and unknown culprit history [3–9]. However, BAT has two inherent weaknesses that hinder its clinical use [10]. Firstly it requires fresh blood for testing [11]. Secondly, approximately 10% of the patients have basophils that do not react to IgE/FcεRI-mediated positive control nor the allergen, but only non-IgE mediated stimulants and are designated »nonresponders« [1,10]. In this group of patients, the result of BAT is uninterpretable.

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We recently undertook initial validation and assessment of a novel *in vitro* diagnostic tool, the mast cell activation test (MAT), using primary human blood-derived mast cells (pMCs) generated from CD117+ peripheral blood precursors, which were passively sensitized with patients' sera and then incubated *in vitro* with an allergen [12]. Activation of mast cells (MCs) was assessed with flow cytometry analysis of expression of activation markers CD63 and CD107a, and the MAT potential was tested in patients with peanut and HVA and as a diagnostic tool for peanut allergy compared with existing diagnostic tests [12]. The practical limitation of our initial approach [12] was that pMCs generation is difficult to standardize for routine clinical laboratory use as pMCs generation is donor-dependent with distinct combinatorial phenotypic profiles [12] and as different culturing protocols can impact the result [14,15]. However, in comparison to the BAT, no fresh blood is required to perform the MAT, and MAT might be applicable in individuals with HVA who have uninterpretable venom BAT results caused by nonresponding basophils to both IgE-mediated positive controls (anti-IgE and anti-FcεRI) [11,16,17]. Routine maintenance of cell lines is more convenient than obtaining mast cells from the blood of multiple donors. LAD2 is a human MC line that is SCF-dependent and expresses FcεRI, and LAD2 was recently used in peanut LAD2 MAT studies [18,19]. However, hMCs are more susceptible to IgE-mediated degranulation than LAD2 cells [12].

The utility of venom MAT in diagnosing BAT nonresponders with HVA has not yet been broadly addressed. It should be further evaluated if venom LAD2 MAT is associated with the severity of Hymenoptera sting reaction, as recently shown for venom BAT [7]. To explore these diagnostics and clinical goals of venom LAD2 MAT in HVA, we first optimized the susceptibility of LAD2 cells to IgE-mediated degranulation [12] in a group of well-defined HVA allergic patients. We then i.) determined the diagnostic utility of venom LAD2 MAT in HVA, ii.) assessed if the LAD2 MAT is associated with the severity of sting reactions, and iii.) evaluated the utility of venom LAD2 MAT to diagnose BAT nonresponders.

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2. Methods

2.1 Patients and study design

We prospectively recruited 39 *Hymenoptera* allergic patients (64.1 % females, 21-77 years; median age 46 years) and 22 *Hymenoptera* non-sensitized non-allergic controls (81.8 % females, 25-57 years; median age 35 years). All patients were mono-allergic to honey bee venom (HBV) or yellow jacket venom (YJV) and had a single positive intradermal (ID) skin test, sIgE, and BAT results for the culprit allergen (HBV/YJV). All controls had double negative sIgE and BAT results for HBV and YJV. All patients were recruited prospectively between 2018 and 2019; BAT, sIgE, tIgE, and tryptase measurements were performed at recruitment. The patient's plasma and serum samples were stored at -20°C and MAT testing was conducted in 2020 and 2021. All LAD2 MAT optimization experiments were performed with up to 8 selected patients from this cohort. For evaluating the use of venom LAD2 MAT in patients with nonresponding basophils we retrospectively selected a group of 37 HVA patients with nonresponding basophils in BAT test. Those patients were selected from 1839 patients in which we routinely performed venom BAT tests between years 2013 and 2021. Plasma samples were collected from the same heparinized blood used for BAT. All specimens were collected at the University Clinic of Respiratory and Allergic Diseases, Golnik, Slovenia. The study was approved by the National Medical Ethics Committee of the Republic of Slovenia (N° 0120-188/2017/4). All patients provided written informed consent.

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2.2 LAD2 Mast cell activation test (LAD2 MAT)

LAD2 cells were cultured in StemPro-34 complete media with added 100 ng/ml SCF (STEMCELL Technologies, Vancouver, Canada) (CM) and sensitized with the participants' plasma overnight (1:10 dilution) in the final density of $2x10^5$ cells/ml. Cells were washed, and $2x10^4$ cells were stimulated with 0.01, 0.1, and 1 mg/mL HBV or YJV (both HalAllergie, Leiden, the Netherlands) for 15 minutes at 37°C. For the controls, the cells were exposed to media alone (negative control) or 10 µg/ml anti-IgE mAb (positive control) (Sigma-Aldrich, MO, USA). Degranulation was stopped by chilling on ice, after which CD63-APC, CD107a-PE, CD107b-Alexa 700 (all BioLegend, CA, USA), and IgE-FITC (Miltenyi Biotec, Germany) were added and incubated for 20 minutes. Finally, cell probes were washed twice and fixed before flow cytometry analysis using the FACSCanto II flow cytometer (BD Biosciences, NJ, USA). Degranulation was measured based on the surface expression of CD63 on LAD2 cells by flow cytometry as previously described [12,18,19].

To ensure quality control across testing, we performed immunophenotyping of LAD2 cells and determined FcεRI surface expression measurements weekly. In case of reduction of any observed markers, the new batch of LAD2 cells was thawed.

2.3 Optimization of susceptibility of LAD2 cells to IgE-mediated degranulation in MAT

IL-4 and human myeloma IgE

For the evaluation of the effect of IL-4 and IgE treatment on FcεRI LAD2 expression, LAD2 cells, were treated with 10-100 ng/ml of recombinant human IL-4 (Peprotech, NJ, USA) or 1 µg/ml of human myeloma IgE (Millipore, Burlington, Massachusetts, USA) for up to 9 days. We then evaluated FcεRI cell expression and anti-IgE mAb response.

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Stimulation media, IL-33 and IL-6

Stimulation media that were tested were StemPro-34 CM, tyrode buffer (TB) (Sigma-Aldrich), RPMI-1640 medium (Sigma-Aldrich) with 0.1% bovine serum albumin (BSA) (Gibco, MA, USA), and HBSS buffer (Sigma-Aldrich). For evaluation of the effect of IL-33 and IL-6 on LAD2 IgE-mediated degranulation, LAD2 cells were pre-stimulated with 1-100 ng/ml of IL-33 (Peprotech, NJ, USA) and 1-100 ng/ml of IL-6 (Peprotech, NJ, USA) dissolved in StemPro-34 CM, for 1 hour at 37°C and 5% CO_2 and then stimulated with the allergen/anti-IgE. The combined effect of IL-33 and IL-6 was observed at 1 ng/ml of IL-33 and IL-6.

2.4 Data analysis

The data distribution was determined using the D'Agostino and Pearson omnibus tests. Since most data was not normally distributed, we used the Mann- Whitney U test or Wilcoxon matched-pairs signed-rank test as appropriate, unless stated otherwise. We used the Spearman rank correlation test to analyse associations between variables. Flow cytometric analyses were performed using BD FACSDiva (version 8.0.1) (BD Biosciences) or FlowJo (version 10.7.2) (BD Biosciences) analysis software. Statistical analyses were done using GraphPad Prism 9 (GraphPad Software, CA, USA). A *P*-value below 0.05 was considered statistically significant. All reported *P*-values are two-tailed.

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3. Results

3.1 Prestimulation with IL-33 and IL-6 in tyrode buffer with SCF increases the susceptibility of LAD2 cells to IgE-mediated degranulation in MAT

We improved the LAD2 MAT by testing different stimulation media, allergen concentrations and cytokine prestimultions. Our findings are explained in detail in the Supplementary section of the manuscript. Briefly, the strongest specific response was obtained when using tyrode buffer (TB) with SCF and stimulation with 1 µg/ml HBV/YJV allergen. This effect was furthermore enhanced after prestimulation of LAD2 cells with IL-33 and IL-6 as previously demonstrated by Cop et al. [20]. Consequently, for all further MATs, including basophil nonresponders, we used TB with SCF and prestimulation with IL-33 and IL-6.

3.2 LAD2 MAT results correlated with BAT results

We performed LAD2 MATs in a cohort of 39 *Hymenoptera* venom allergic patients and 22 healthy controls and compared those results to BATs. Demographic and clinical features of the study population are shown in **Table S1**. The receiver operating characteristic curve (ROC) analysis yielded an area under the curve (AUC) of 0.67 when using TB with SCF and IL-33 and IL-6 prestimulation. Further, when using TB with SCF and IL-33 and IL-6 prestimulation, the optimal cut-off value for positive results was 5.5% of CD63+ LAD2 cells (established with the use of Youden index, **Table S2**). Other protocols used (StemPro-34 CM and StemPro-34 CM with IL-33 and IL-6) demonstrated lower AUCs (**Figure S8)**. The LAD2 response was allergen-specific, and there was no evidence of LAD2 activation when we used sera from healthy control patients, which were not sensitized to allergens that were used for stimulation

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(Figure 2). Thus, all healthy controls showed negative LAD2 response to HBV or YJV stimulation (**Table S2**).

The comparison between LAD2 MAT and BAT results showed that patients had significantly higher LAD2 MAT (at HBV or YJV of 1 µg/ml, median 3.6% vs 2.4%, P=0.03) and BAT (at HBV or YJV of 0.1 µg/ml, median 45.7% vs 1.8%, P<0.0001; at HBV or YJV of 1 µg/ml, median 78.0% vs 4.5%, P<0.0001) results than controls. Fifteen out of 39 patients with positive BAT also had positive MAT result for the culprit allergen. LAD2 MATs were correlated with BATs (R_s=0.48, P=0.002) (**Figure 2**). The correlation between LAD2 MAT and sIgE (R_s=0.34, P=0.03) was comparable with the correlation between BAT and sIgE results (R_s=0.39, P=0.01) (**Figure S9**).

The expression of CD63 in LAD2 MAT was strongly correlated with the expression of other lysosomal associated membrane proteins (LAMPs) CD107a (R_s=0.70, P=0.0001) and CD107b (R_s=0.89, P<0.0001) (**Figure S10).** There was a strong positive correlation between serum tIgE levels and IgE sensitisation rate (% of IgE+ LAD2 cells) (R_s=0.93, P<0.0001), FcεRI LAD2 expression (R_s=0.64, P<0.0001) and the CD63 LAD2 anti-IgE response (R_s=0.41, P=0.0009) (**Figure S11**).

3.3 Patients with severe reactions had greater proportions of activated LAD2 cells compared with patients with mild-to-moderate reactions

Twelve (31 %) patients experienced severe allergic reactions (7 Mueller grade IV and 5 grade III), whereas 27 (69 %) patients experienced nonsevere allergic reactions (11 Mueller grade II, 12 grade I and 4 large local reactions (LLR) (**Table S1**). Demographic, clinical and laboratory features are presented in **Table S3**. There were no significant differences in sex and

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age between patients with severe and nonsevere allergic reactions. There were also no differences in the BST, tIgE levels, levels of sIgE to HBV/YJV and basophil CD63 expression (at HBV or YJV of 1 µg/ml or 0.1 µg/ml) and basophil response to anti-FcεRI mAb positive control. Although statistically insignificant, a trend toward higher LAD2 response to anti-IgE mAb positive control was observed in patients with severe reactions (25.0% vs 19.1%, *P*=0.07). Patients with severe allergic reactions exhibited higher LAD2 CD63 expression (at HBV or YJV of 0.1 µg/ml, median, 3.5% vs 2.4%, *P*=0.04; at HBV or YJV of 1 µg/ml, median 7.8% vs 3.1%, *P*=0.02) (**Figure 3**).

3.4 LAD2 MAT provides conclusive results in more than half of patients with *Hymenoptera* venom allergy, but nonresponding basophils in BAT

From January 2013 to December 2021, 1839 BAT tests for *Hymenoptera* venoms were performed. 57 out of 1839 had a negative anti-FcεRI response and were designated as BAT nonresponders. For 37 out of 57 (65%), plasma samples were available, and those 37 (median age, 51 years; age range, 24-80; 22 female) were analysed further. Demographic and clinical features of 37 patients are shown in **Table S4**. Most patients (26.7%) had severe allergic reactions (Mueller grade III-IV). Thirteen out of 37 (35%) patients were allergic to HBV, 15/37 (41%) were allergic to YJV or hornet and in 9/37 (24%) of cases, the stinging insect was not identified. The median CD63 basophil response to anti-FcεRI mAb was 5.0% (IQR 3.5-6.9). Three out of 37 patients had paired BAT results (median time after initial BAT was 13 months); anti-FcεRI basophil response was reversed to positive in 2 patients. The median CD63 LAD2 mast cell response to anti-IgE mAb positive control was 16.4% (IQR 12.9-21.1). The median CD63 LAD2 mast cell response in the HBV-allergic subgroup (at 1 µg/ml HBV) was 8.3% (IQR 5.1-13.1), and in YJV/hornet-allergic subgroup (at 1 µg/ml YJV) was 3.3% (IQR 2.4-6.8)

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and in the subgroup where culprit insect was not identified, the median LAD2 mast cell response to 1 µg/ml HBV was 5.0% (IQR 3.3-9.7) and 2.85% (IQR 1.48-4.02) to 1 µg/ml YJV. Considering the previously defined cut-off value for positivity of 5.5% CD63+ cells, LAD2 MAT was positive in 20 out of 37 (54.1%) patients (**Figure 4**). In more than half of LAD2 MAT positive BAT non-responders (11 of 20; Patient ID: 1, 6, 8, 9, 10, 13, 19, 20, 21, 28, and 37 in Table S5), which were all double sensitized for HBV and YJV, the LAD2 MAT results were single positive.

The correlation between LAD2 MAT and sIgE results (R_s =0.33, P=0.02) (**Figure S12**) was similar to the correlation between LAD2 MAT and sIgE results in BAT responders (**Figure S9**). The sIgE levels to HBV and YJV were significantly higher in patients with positive LAD2 MAT compared to patients with negative LAD2 MAT results (median, 7.0 vs 1.4 kU/l, P=0.002) (**Figure S12**). Detailed clinical and laboratory data of 37 patients with nonresponding basophils are presented in **Table S5.**

4. Discussion

MAT is a new cellular assay, and recent studies have shown that MAT could be used as a diagnostic tool for food and drug allergies [12,18,12,22]. Herein we evaluated LAD2 MAT in HVA. We confirmed previous observations [12,18] that MAT results correlated with the BAT results. Moreover, LAD2 MAT provided conclusive results for more than half of the subjects with nonresponding basophils and thus uninterpretable BAT results. Additionally, our results may suggest that the patients with severe reactions had greater proportions of activated LAD2 cells compared to the patients with mild-to-moderate sting reactions.

In HVA, patients often have low venom-specific IgE levels [23,24], and it was previously shown that low sIgE can affect the results of MAT testing [12,18,19]. To overcome this sIgE

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threshold limitation of MAT, which is in HVA, due to lower sIgE levels, more critical than in peanut allergy [12], we first optimized the susceptibility of LAD2 cells to IgE-mediated degranulation. For this, we wanted to improve cellular FcεRI density and increase the magnitude and specificity of the LAD2 IgE response.

Studies show it is possible to increase FcεRI expression with IL-4 and IgE pretreatment [25,26]. Our results showed no increase in FcεRI surface expression after up to 9 days of treatment with IL-4 and an extensive rise in FcεRI expression after 1st day of treatment with IgE, which coincided with increased IgE activation capacity of LAD2 cells. On the other hand IL-33 potentiates IgE-mediated mast cell degranulation by increasing the number of responding cells and enhancing the responses of individual mast cells [27]. In the MAT, IL-33 and IL-6 synergistically enhance the degranulation of pMCs [20]. We confirmed that IL-33 and IL-6 treatment increased IgE responsiveness of LAD2 cells; this augmentation was allergen-specific and had no impact on basal LAD2 activation.

4.1 LAD2 MAT in *Hymenoptera* venom allergy

Our results showed that in HVA the LAD2 MAT correlated with the BAT result; similar results were also shown in previous studies. [12,18,19]. However, additional larger studies are necessary to evaluate this association. Further, we showed that the LAD2 response was allergen (venom)-specific and concentration-dependent and that there was no response when we used sera from healthy controls (not sensitized for venom). Importantly, it was previously shown that pMCs sensitized with sera from patients with positive skin test and basophil activation test to chlorhexidine showed drug-specific and concentration-dependent degranulation upon stimulation with chlorhexidine [21,22].

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Several factors could significantly impact the performance of MAT. Some already published studies used patients' plasma [18], while others used serum [12,21,22] for MAT. According to our results, the choice of serum or plasma does not impact the MAT result. The significant factor potentially influencing the MAT is the level of sIgE in plasma used for sensitization. In general, MAT sensitivity correlates with sIgE levels, meaning that the sensitivity of MAT increases with higher sIgE levels, which was observed in current and previous studies [12,18]. It is worth mentioning that our cohort of patients with HVA demonstrated notably lower levels of sIgE (median 4.1 kU/L) in comparison to patients with peanut allergy used in previous MAT studies (median 26 [12], 14 [18], and 151 [19] kU/L, respectively).

Our previous study also showed that the MAT response does not solely depend on sIgE levels [12] and that MAT response might also depend on the functional characteristics of IgE antibodies (t.i. clonality and affinity) [26]. In addition, individual differences in tIgE levels could also be a factor in MAT response. We demonstrated a positive correlation between tIgEs and the % of IgE+ LAD2, LAD2 FcεRI expression and higher CD63 anti-IgE response. The positive correlation between FcεRI expression and plasma IgE concentration is a well-known observation [28]. Since basophils and MCs share the FcεRI degranulation pathway, the dynamics in FcεRI expression and magnitude of CD63 activation concerning the patient's tIgE levels could also affect MAT results.

4.2 LAD2 MAT in HVA patients with nonresponding basophils in BAT

To our knowledge, this is the first study which assesses the performance of MAT in BAT nonresponders with HVA. So far, the utility of MAT in BAT nonresponders has only been reported by Santos et al. [18], but only in individual cases of two patients. Basophil

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unresponsiveness occurs in up to 10% of patients [1,2], and its cause has not yet been uniformly explained. MCs are believed to play a more significant role in the allergic reaction than basophils [29], which could be even increased in the case of unresponsive basophils. Evaluation of the activation of the patient's MCs could be an additional tool for diagnosing the IgE mechanism of allergic reactions. Still, the isolation of mature tissue-resident MCs is challenging. LAD2 MAT could therefore represent an alternative to confirm the diagnosis in case of BAT nonresponders as was shown with our data. LAD2 MAT managed to resolve diagnosis in 11 cases that had inconclusive results of the other tests. However, additional studies are needed to clarify the role of MAT testing in BAT non-responders on a routine clinical basis.

Previous studies suggested that MAT could identify patients at risk of severe peanut anaphylaxis [12,18] and that the MAT is a reliable diagnostic tool for chlorhexidine allergy [21,22]. Further, our study demonstrated that LAD2 MAT might be associated with the severity of the sting reaction. These findings are similar to our recent study, where we showed that BAT is predictive of severe allergic sting reactions in HBV allergy [7]. However, in the current study we could not confirm the association between age and BST [7,30,31] and the severity of sting reaction, which is probably due to the relatively low sample size compared to the previous studies.

In summary, the LAD2 MAT represents a new tool for sequential analysis if the HVA patient has nonresponding basophils in the BAT. Further, LAD2 MAT may identify patients at risk of severe sting reactions and thus can help guide recommendations for venom immunotherapy and improve the management of patients with venom allergy.

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CONFLICT OF INTEREST

The authors declare that the research was conducted without any commercial or financial relationships that could be construed as a potential conflict of interest.

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FIGURES

Figure 1. LAD2 degranulation capacity and susceptibility to IgE mediated venom stimulation before and after the optimisation of LAD2 MAT protocol. Experiments were performed with 8 *Hymenoptera* venom allergic patients (4 were allergic to honey bee venom and 4 were allergic to yellow jacket venom, respectively). Also see the figures S3 and S4 in the supplementary materials.

***P< .001 and **P< .01 for comparing groups using the paired t-test.

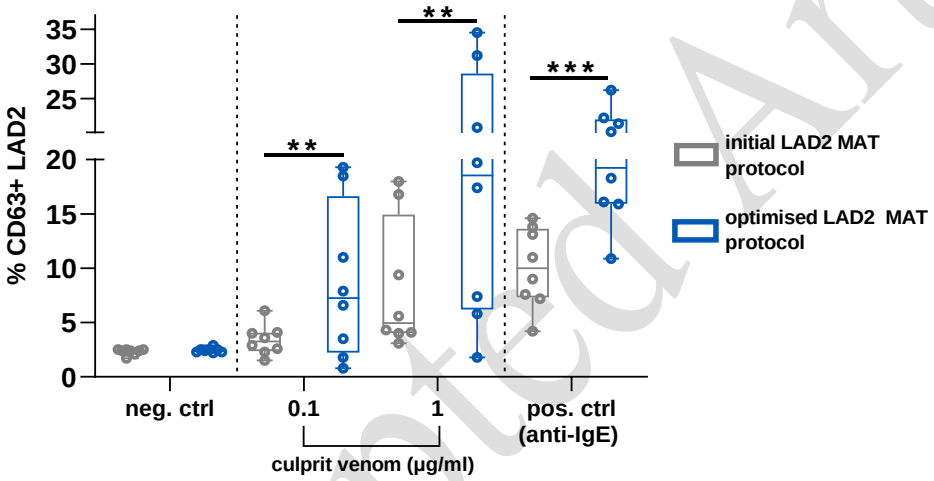

Figure 2. Dose-dependent **A)** basophil activation test (BAT) and **B)** mast cell activation test (MAT) in patients (P) with *Hymenoptera* venom allergy (HVA) and healthy controls (C) after stimulation with culprit venom (honey bee venom (HBV) or yellow jacket venom (YJV)) ranging from 0.001-1 µg/ml or positive control anti-IgE/anti-FcεRI. **C)** Spearman's coefficient correlation analysis between BAT and MAT results after the stimulation with 1 µg/ml culprit venom in HVA patients.

*****P*< .0001 and **P*< .05 for comparing groups using the Mann-Whitney U test. HBV: honey bee venom; YJV: yellow jacket venom.

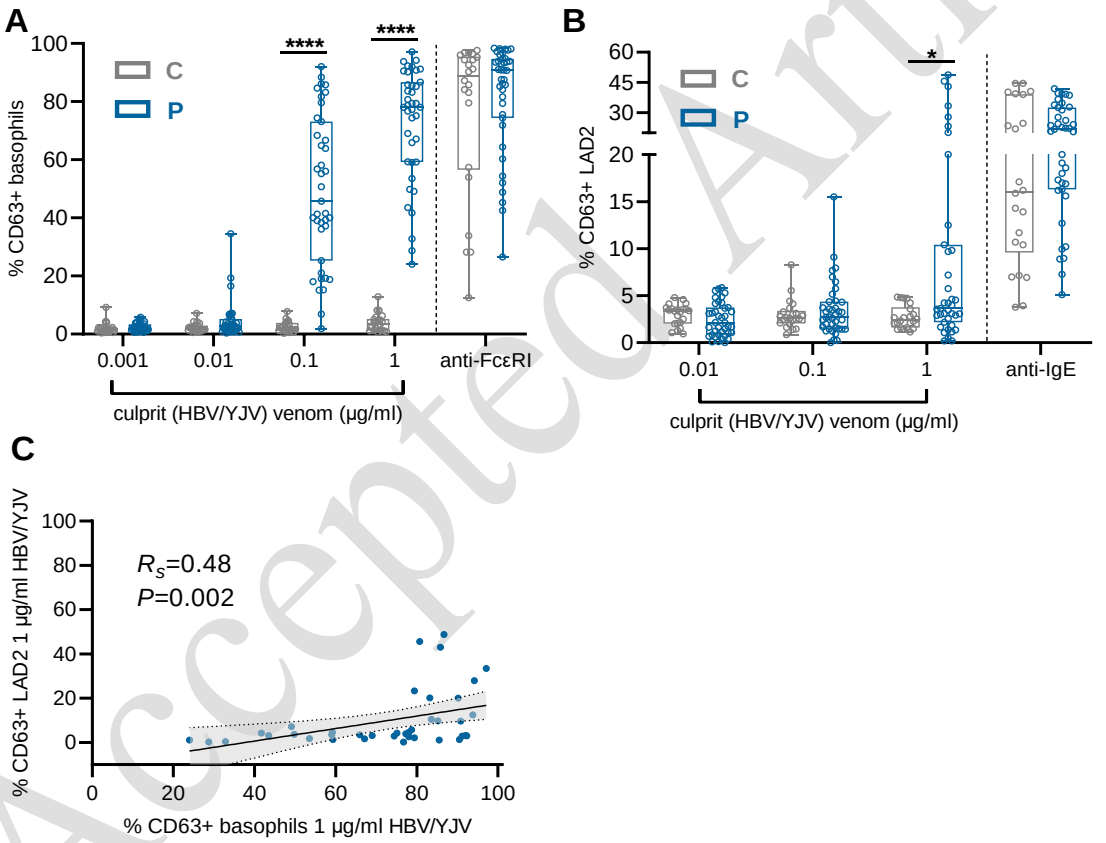

Figure 3. Activation of LAD2 mast cells in response to stimulation with culprit venom (0.01-1 µg/ml) in patients with nonsevere allergic sting reactions (n=27) vs those with severe allergic sting reactions (n=12). The severity was evaluated according to the Mueller scale. Large local and grade I-II reactions were considered nonsevere, while grade III and IV reactions were considered severe.

*P<.05 for comparing groups by using the Mann-Whitney U test. HBV: honey bee venom; YJV: yellow jacket venom

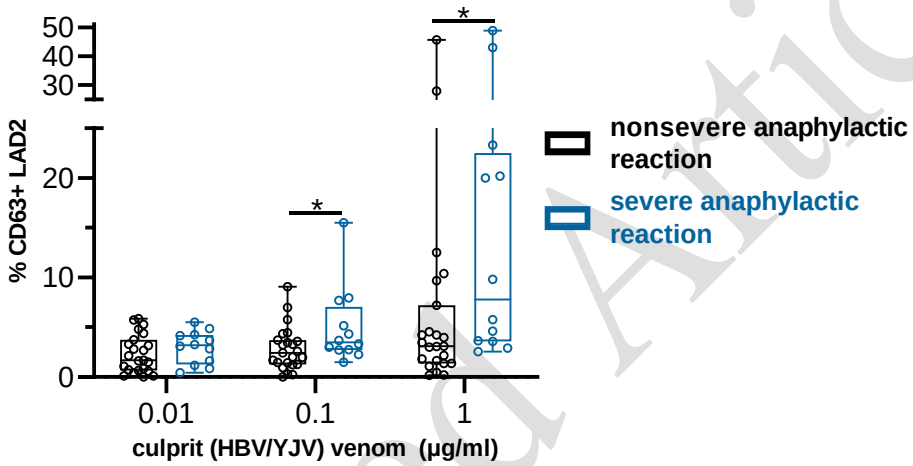

Figure 4. The results of A) BAT and B) LAD2 MAT in 37 patients with nonresponding basophils and thus uninterpretable BAT result. Red dotted line indicates the cut-off for the positivity of the test.

HBV: honey bee venom; YJV: yellow jacket venom.