UNIVERZA V LJUBLJANI
MEDICINSKA FAKULTETA
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Julij ŠELB

CELOSTNA OBRAVNAVA DIAGNOSTIKE BOLNIKA Z ALERGIJO PO PIKU KOŽEKRILCEV (Hymenoptera)

DOKTORSKA DISERTACIJA

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INTEGRATED DIAGNOSTIC EVALUATION OF HYMENOPTERA VENOM ALLERGIC PATIENT

DOCTORAL DISSERTATION

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Naslov CELOSTNA OBRAVNAVA DIAGNOSTIKE BOLNIKA Z ALERGIJO PO PIKU KOŽEKRILCEV (Hymenoptera)
INTEGRATED DIAGNOSTIC EVALUATON OF HYMENOPTERA VENOM ALLERGIC PATIENT

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Izjavljam, da je doktorska disertacija rezultat mojega raziskovalnega dela.

V Ljubljani, 3.3.2017

Julij Šelb

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List of abbreviations used

ACE – angiotensin converting enzyme

AUC – area under the curve

B - bee

BAT – basophil activation test

BAT-AUC – basophil activation test area under the curve

BAT-only –basophil activation test only diagnostic approach

BAT-ratio – basophil activation test results ratio based diagnostic approach

BAT+SSMA-ratio – basophil activation test and species specific major antigen results ratio based diagnostic approach

C.D. – correct diagnosis

CAP – ImmunoCAP assay

CCD – cross reactive carbohydrate determinants

CD34+ - cluster of differentiation 34 positive cell

CD64+ - cluster of differentiation 64 positive cell

CD203+ - cluster of differentiation 203 positive cell

CI – confidence interval

CRD – component resolved diagnosis

DA – diagnostic approach

FcRI – receptor for a Fc region of IgE

H - history

IgE – immunoglobulin E

IgG – immunoglobulin G

LITE – ImmuLITE assay

LLR – large local reaction

O.D. – over diagnosis

PAF – platelet activation factor

PB – patent basal response

rApi m 1 – recombinant honeybee allergen 1

rApi m 2 – recombinant honeybee allergen 2

rApi m 3 – recombinant honeybee allergen 3

nApi m 4 – recombinant honeybee allergen 4

rApi m 5 – recombinant honeybee allergen 5

rApi m 10 – recombinant honeybee allergen 10

RAST – radioallergosorbent test

ROC – receiver operator characteristic curve

ROC-AUC – receiver operator characteristic curve area under the curve

rVes v 1 – recombinant wasp allergen 1

rVes v 5 – recombinant wasp allergen 5

Sf9 – Spodoptera frugiperda 9 cells

SPT – skin prick test

SR – systemic reaction

SSMA – species specific major allergen

SSMA-only – species specific major allergen results only diagnostic approach

SSMA-ratio – species specific major allergen results ratio diagnostic approach

tIgE – total IgE

U.D. – under diagnosis

USA – united states of america

VIT – venom immunotherapy

W - wasp

WAO – world allergy organization

Povzetek

Doktorska naloga je sestavljena iz 4-ih ločenih raziskav, na katere se lahko gleda kot na samostojne enote, vendar pa so rezultati raziskav in na njih temelječi zaključki prepleteni ter skupaj znatno doprinesejo k bolj celoviti diagnostični obravnavi bolnika po piku kožekrilca, saj na tem področju ostaja še precej neznank (predvsem pri razlikovanju med senzibilizacijo [pozitivni rezultati testov, ki dokazujejo prisotnost IgE protiteles proti alergenu] in klinično izraženo alergijo).

Raziskava primerjave sistemov za določanje imunoglobulinov E v krvi

OZADJE: Število raziskav, ki bi primerjale rezultate IgE protiteles proti rekombinantnim proteinom strupov kožekrilcev, če so le-ti izmerjeni na obeh rutinsko uporabljenih sistemih za določanje IgE protiteles v krvi (Immulite [LITE] in ImmunoCAP [CAP]), je omejeno.

HIPOTEZA: Občutljivost rezultatov IgE protiteles proti rekombinantnem proteinu osjega strupa rVes v 5 se bo statistično značilno razlikovala med obema rutinsko uporabljenima sistemoma za določanje IgE protiteles v krvi, dočim bo specifičnost rezultatov med obema sistemoma primerljiva.

METODE: V raziskavo smo vključili 110 bolnikov alergičnih na osji pik (bolniki so imeli pozitivna [> 0.35 kU/L] IgE protitelesa proti nativnem osjem in negativna [≤ 0.35 kU/L] IgE protitelesa proti nativnem čebeljem strupu). Bolnikom smo izmerili IgE protitelesa proti rekombinantnem proteinu osjega strupa rVes v 5 (CAP, LITE) in rekombinantnem proteinu osjega strupa rVes v 1 (CAP). Na vzorcu bolnikov smo primerjali občutljivost rezultatov IgE protiteles proti rVes v 5 proteinu, med obema rutinsko uporabljenima sistemoma. Dodatno smo na vzorcu 49 zdravih kontrol pomerili IgE protitelesa proti rekombinantnem proteinu osjega strupa rVes v 5 z obema sistemoma (CAP, LITE) ter primerjali specifičnost dobljenih rezultatov med sistemoma.

REZULTATI: Občutljivost rezultatov IgE protiteles proti rekombinantnem proteinu osjega strupa rVes v 5 je bila statistično značilno večja (82 % proti 93 %, $p < 0.05$), če so bili le-ti izmerjeni z LITE, kot pa s CAP sistemom. Skupna občutljivost rezultatov IgE protiteles proti proteinu rVes v 5 merjenih na LITE sistemu ter rezultatov IgE protiteles proti proteinu rVes v 1 merjenih na CAP sistemu je skoraj dosegla občutljivost nativnega strupa (97 %), dočim pa je bila skupna občutljivost rezultatov IgE protiteles proti proteinu rVes v 5 in proteinu rVes v 1, če so bili le-ti merjeni na CAP sistemu, nižja od občutljivosti rezultatov IgE protiteles proti

proteinu rVes v 5 merjenih na LITE sistemu (90 % proti 93 %). Rezultati meritev IgE protiteles proti proteinu rVes v 5, so statistično značilno korelirali med obema sistemoma. Pri 12-ih bolnikih, katerih rezultati IgE protiteles proti proteinu rVes v 5 so bili negativni, če so bili izmerjeni s CAP sistemom, so bili le-ti pozitivni, če so bili izmerjeni z LITE sistemom. Po drugi strani pa pri nobenem od bolnikov, pri katerih so bili rezultati IgE protiteles proti proteinu rVes v 5 negativni, če so bili izmerjeni z LITE sistemom, le-ti niso bili pozitivni, če so bili izmerjeni s CAP sistemom. Specifičnost rezultatov je bila visoka in primerljiva na obeh sistemih (96 % za LITE sistem ter 98 % za CAP sistem).

ZAKLJUČKI: Občutljivost rezultatov IgE protiteles proti proteinu rVes v 5 je statistično značilno večja, če so protitelesa izmerjena z LITE sistemom (v primerjavi s CAP sistemom), dočim je specifičnost rezultatov primerljiva med obema sistemoma.

Raziskava izboljšanja diagnostičnega postopka diagnostike kožekrilcev

OZADJE: Ker ima do 60 % bolnikov, ki po piku kožekrilca doživijo anafilaktično reakcijo, pozitivne rezultate IgE protiteles proti nativnem strupu tako ose, kot tudi čebele, čeprav le manj kot 10 % bolnikov doživi klinično očitno reakcijo, če jih pičita osa in čebela, je diagnostični postopek določitve kožekrilca na katerega strup je bolnik alergičen, težak in nezanesljiv.

HIPOTEZA: Kombinirana uporaba IgE protiteles proti rekombinantnim sestavinam strupov (t.i. SSMA [angl. 'species specific major allergen'] protitelesa) in testa aktivacije bazofilcev (t.i. BAT [angl. 'basophil activation test']), v obliki stopenjskega procesa diagnostike oz. z uporabo algoritma razmerij bo statistično značilno izboljšala diagnostični postopek, saj bo na vzorcu bolnikov, ki imajo pozitivne rezultate IgE protiteles tako proti nativnem strupu ose, kot tudi proti nativnem strupu čebele, zmanjšala število prediagnosticiranih bolnikov.

METODE: V študijo smo vključili 177 zaporednih bolnikov, ki so po piku kožekrilca (družina Hymenoptera) doživeli anafilaktično reakcijo. Pri vseh bolnikih smo izmerili IgE protitelesa proti nativnem strupu ose in čebele, IgE protitelesa proti rekombinantnim proteinom strupov ose in čebele (rApi m 1 in rVes v 5) ter test aktivacije bazofilcev s 4-imi koncentracijami (0.001-1 μg/L) nativnega strupa ose in čebele.

REZULTATI: V končno analizo smo vključili 133 bolnikov, pri katerih je bila anamneza o kožekrilcu, ki je povzročil anafilaktično reakcijo, nedvoumna.

Na vzorcu bolnikov, ki so imeli pozitivne rezultate IgE protiteles proti nativnem strupu enega izmed kožekrilcev (proti nativnem strupu ose ali pa proti nativnem strupu čebele; tako imenovan IgE +/- vzorec [n = 73]), je bilo ujemanje rezultatov IgE protiteles proti nativnem strupu z anamnezo o kožekrilcu, ki je povzročil anafilaktično reakcijo, odlično (96 %). Prav tako je bilo zelo dobo ujemanje med rezultati IgE protiteles proti nativnem strupu in rezultati IgE protiteles proti rekombinantnim proteinom strupov, kot tudi ujemanje med rezultati IgE protiteles proti nativnem strupu in rezultati testa aktivacije bazofilcev (> 90 %).

Na vzorcu bolnikov, ki so imeli pozitivne rezultate IgE protiteles proti nativnima strupoma obeh kožekrilcev (proti nativnem strupu ose in tudi nativnem strupu čebele; tako imenovan IgE +/+ vzorec [n = 56]), smo sestavili in ovrednotili algoritme, ki so temeljili na razmerjih rezultatov IgE protiteles proti rekombinantnim sestavinam strupov čebele in ose in/ali razmerjih rezultatov testa aktivacije bazofilcev, izraženega kot površina pod krivuljo aktivacije (t.i. BAT-AUC), z nativnim strupom čebele in ose. Vsi diagnostični postopki, ki so temeljili na algoritmih razmerij, so bili statistično značilno boljši (statistično značilno višji delež pravilnih diagnoz) od

diagnostičnih postopkov, ki so temeljili na standardni interpretaciji diagnostičnih testov (statistično značilno izboljšanje je bilo opazno tudi nasproti, če se je diagnostični postopek izvajal stopenjsko). Od vseh diagnostičnih postopkov, ki so temeljili na algoritmih razmerij, je bil najboljši SSMA+BAT-AUC diagnostični algoritem. S pomočjo tega algoritma je bil delež pravilnih diagnoz več kot 80 % (statistično značilno izboljšanje v primerjavi z diagnostičnim postopkom, ki je uporabljal samo standardno interpretacijo rezultatov testa aktivacije bazofilcev oziroma standardno interpretacijo rezultatov IgE protiteles proti rekombinantnim sestavinam strupov [delež pravilnih diagnoz pri teh dveh postopkih je bil okoli 50 %]; prav tako statistično značilno izboljšanje v primerjavi s stopenjskim diagnostičnim postopkom standardne interpretacije obeh testov [60 % delež pravilnih diagnoz]). Statistično značilno izboljšanje diagnostičnih postopkov, ki so temeljili na algoritmih razmerij, smo potrdili z analizo ROC-krivulje.

Na vzorcu bolnikov, ki so imeli negativne rezultate IgE protiteles proti nativnima strupoma obeh kožekrilcev (proti nativnem strupu ose in tudi nativnem strupu čebele; tako imenovan IgE -/- vzorec [n = 4]), smo s testom aktivacije bazofilcev dokazali senzibilizacijo pri vseh 4-ih bolnikih, dočim smo, z uporabo IgE protiteles proti rekombinantnim sestavinam strupov, to lahko storili le pri enem bolniku.

ZAKLJUČKI: S to raziskavo smo: i.) potrdili, da je enojno pozitiven rezultat IgE protiteles proti nativnem strupu zanesljiv pokazatelj kožekrilca, ki je pri bolniku povzročil anafilaktično reakcijo, zaradi česar nadaljnji diagnostični testi niso potrebni. ii.) pokazali, da je pri dvojno pozitivnem rezultatu IgE protiteles proti nativnima strupoma ose in čebele potrebno nadaljnje testiranje z IgE protitelesi proti rekombinantnim sestavinam strupov in testiranje s testom aktivacije bazofilcev, ter interpretacija rezultatov teh testov v obliki razmerij. iii.) pokazali, da je v skupini bolnikov z dvojno negativnimi rezultati IgE protiteles proti nativnima strupoma ose in čebele, nujno testiranje s testom aktivacije bazofilcev (za dokaz senzibilizacije).

Raziskava razlikovanja med različnimi stopnjami alergijske reakcije

OZADJE: Diagnostični postopki, ki se trenutno uporabljajo pri diagnostiki alergije na kožekrilce, ne omogočajo niti razlikovanja med bolniki z velikimi lokalnimi reakcijami (VLR) in sistemskimi reakcijami (SR), kot tudi ne razlikovanja med različnimi stopnjami sistemih reakcij.

HIPOTEZA: Rezultati različnih 'in-vitro' diagnostičnih testov pri bolnikih, ki so alergični na pik kožekrilcev, bodo korelirali s stopnjo alergične reakcije po piku.

METODE: Sto-sedmim bolnikom, ki bili sprejeti zaradi alergije na pik kožekrilca in so imeli pozitivne rezultate IgE protiteles proti nativnem strupu enega izmed kožekrilcev (ali proti nativnem strupu ose [rezultati IgE protiteles proti nativnem strupu ose > 0.35 kU/L in rezultati IgE protiteles proti nativnem strupu čebele ≤ 0.35 kU/L], ali pa proti nativnem strupu čebele [rezultati IgE protiteles proti nativnem strupu čebele > 0.35 kU/L in rezultati protiteles proti nativnem strupu ose ≤ 0.35 kU/L]), smo izmerili IgE in IgG protitelesa proti ustreznem nativnem strupu, IgE protitelesa proti rekombinantnim proteinom ustreznega nativnega strupa (rApi m 1 ali rVes v 5), test aktivacije bazofilcev izmerjen s 4-mi koncentracijami (0.001-1 µg/L) ustreznega nativnega strupa in ocenjen kot BAT-AUC, ter bazalno triptazo. Izmerjene imunološke faktorje smo nato primerjali med skupinami bolnikov z VLR in SR ter znotraj različnih stopenj SR, ter jih nadalje uporabili v modelu penalizirane logistične regresije za napoved stopnje alergične reakcije (VLR proti SR).

REZULTATI: Rezultati IgE protiteles proti rekombinantnim sestavinam strupov in rezultati testa aktivacije bazofilcev, ocenjenega kot BAT-AUC, so bili edini neodvisni statistično značilni napovedni dejavniki SR. Noben izmed faktorjev ni zanesljivo ločeval med različnimi stopnjami SR, vendar pa, ko smo rezultate različnih imunoloških faktorjev korelirali s številsko spremenjenimi stopnjami resnosti alergične reakcije po piku kožekrilcev (VLR = 1, Mueller I = 2, … Mueller IV = 5), je bila pri testu aktivacije bazofilcev, ocenjenem kot BAT-AUC in bazalni triptazi ta korelacija statistično značilna.

ZEKLJUČKI: Rezultati IgE protiteles proti rekombinantnim sestavinam strupov in rezultati testa aktivacije bazofilcev, ocenjenega kot BAT-AUC, lahko ločijo med bolniki z VLR in SR. Noben izmed preizkušenih faktorjev ne more zanesljivo ločevati med različnimi stopnjami SR.

Raziskava diskriminacijske moči kožnih vbodnih testov

OZADJE: Diskriminacijska moč kožnih vbodnih testov ni znana.

HIPOTEZA: Diskriminacijska moč kožnih vbodnih testov je nizka saj je delež dvojno pozitivnih oziroma dvojno negativnih rezultatov (proti nativnem strupu ose in čebele) visok.

METODE: V bolnišničnem informacijskem sistemu smo pregledali bolnike, ki so imeli tekom rutinske diagnostične obravnave po piku kožekrilcev opravljene kožne vbodne teste z 10 in 100 μg/ml čebeljega in osjega strupa. Te rezultate smo nato korelirali z anamnezo o kožekrilcu, ki naj bi povzročil alergijsko reakcijo, in izračunali delež pravilnih diagnoz ter diskriminacijsko moč kožnih vbodnih testov.

REZULTATI: Pri 133-ih (39.7 %) bolnikih je prišlo do ujemanja med rezultati kožnih vbodnih testov in anamnezo. Pri 127-ih (37.9 %) bolnikih so bili rezultatov kožnih vbodnih testov dvojno negativni, kljub prepričljivi anamnezi o SR po piku kožekrilca. Pri 64-ih (19.1 %) bolnikih so bili rezultati kožnih vbodnih testov dvojno pozitivni, kljub dejstvu, da je imela večina bolnikov (62 [96.9 %]) anamnezo pika le s strani enega kožekrilca (le ose oz. le čebele). Pri 144-ih bolnikih (43.0 %) so bili rezultati kožnih vbodnih testov enojno pozitivni in pri večini (131 [91.0 %]), skladni z anamnezo o kožekrilcu, katerega pik je povzročil anafilaktično reakcijo. Pri 13-ih bolnikih (9 %), se enojna pozitivnost kožnih vbodnih testov ni skladala z anamnezo o povzročitelju reakcije.

Ker je 7 bolnikov navajalo alergijo tako po piku tako ose, kot tudi po piku čebele, so bili izvzeti iz končne analize, zaradi česar je bila diskriminacijska moč kožnih vbodnih testov ocenjena na 328 (97.9 %) bolnikih.

Pri 143-ih (43.6 %) bolnikih so bili kožni vbodni testi diskriminativni (enojno pozitiven rezultat za ali oso ali za čebelo) in nediskriminativni pri 185 (56.4 %) bolnikih. Nediskriminativnost kožnih vbodnih testov je bila v večini posledica dvojne negativnosti le-teh, saj so bili dvojno negativni pri 123-ih (66.5 %) bolnikih.

Moč diskriminacije kožnih vbodnih testov je torej znašala 143/328 (43.6 %) z 95 % binominalnim intervalom zaupanja med 38.2 % in 49.2 %.

ZAKLJUČKI: Kožni vbodni testi so nediskriminativni pri 56.4 % bolnikov, kar je najpogosteje posledica dvojne negativnosti le-teh (negativen rezultat s strupom ose in čebele).

S pomočjo vseh 4-ih raziskav smo pokazali, da je za zaznavo vseh bolnikov, ki so alergični na pik kožekrilca bolje meriti IgE protitelesa proti rekombinantnim sestavinam strupov na LITE-sistemu. Pokazali smo tudi, da je potrebno rezultate IgE protiteles in testa aktivacije bazofilcev interpretirati v obliki razmerij, saj na ta način povečmo delež pravilnih diagnoz. Večji delež pravilnih diagnoz ob interpretaciji rezultatov v obliki razmerij temelji na dejstvu absolutne razlike med vrednostmi IgE protiteles proti rekombinantnim sestavinam strupov ter vrednostmi testa aktivacije bazofilcev med različnimi skupinami alergijske reakcije (VLR proti SR) – obstoj te razlike smo dokazali v 3 raziskavi doktorskega dela. Pri interpretaciji rezultatov v obliki razmerij je ta razlika postavljena v kontekst ene osebe (ponavadi ima bolnik SR le po piku enega kožekrilca; po piku drugega kožekrilca pa ima VLR ali pa reakcije nima [asimptomatska senzibilizacija]), zaradi česar pri postavljanju mejnih vrednosti ni potrebno upoštevati razlik med posamezniki in so tako določene meje še bolj točne. Pokazali smo tudi, da je diskriminacijska moč kožnih vbodnih testov nizka (manj kot 50 %), kar je predvsem posledica pogoste dvojne negativnosti teh testov.

Summary

The PhD study is composed out of 4 separate parts, which can be looked at as individual units, however the results and the conclusions of the 4 studies are interconnected, and together significantly contribute to an improvement of a diagnostic evaluation of a Hymenoptera venom allergic individual. This improvement is especially obvious in the case, when one has to distinguish between sensitization (presence of positive results of IgE detecting tests) and clinically apparent allergy.

Assay comparison study

BACKGROUND: There is a lack of studies that compare the results of IgE antibodies against recombinant venom allergens between the two routinely used assays (ImmuLite [LITE] and ImmunoCAP [CAP]).

HYPOTHESIS: The sensitivities of measurements with LITE and CAP assay will be different for r Ves v 5 antigen. Specificities of the two assays will be comparable.

METHODS: One-hundred and ten wasp sting allergic patients (measurements of IgEs to native wasp venom > 0.35 kU/L and measurements of IgEs to native honey-bee venom ≤ 0.35 kU/L) were included in the study. IgE antibodies to rVes v 5 (LITE, CAP), rVes v 1 (CAP) were measured and sensitivities between the two assays were compared. Additionally, 49 healthy controls were included, IgE antibodies against rVes v 5 were measured on them and the results between the two assays were compared, to establish the differences in the specificities between the two.

RESULTS: The diagnostic sensitivity of rVes v 5 results was significantly higher (82 % vs. 93 %, $p < 0.05$) with the LITE than with the CAP assay. The combination of rVes v 5 (LITE) and rVes v 1 (CAP) results almost reached the diagnostic sensitivity of native venom (97%), whilst the diagnostic sensitivity of the combination of rVes v 5 (CAP) and rVes v 1 (CAP) results, did not reach the sensitivity of just rVes v 5 (LITE) results (90% vs. 93%). The results of rVes v 5 measured with both system correlated significantly. The results of rVes v 5 measurements were on average 2.3 times higher when they were measured with the LITE system than when they were measured with the CAP system. Twelve patients, who had negative results of rVes v 5 when measured with the CAP system, had positive results when they were measured with the LITE system. None of the patients who had negative results of rVes v 5 when measured with the LITE system, had positive results when they were measured with the CAP system. The

specificities of the two systems were comparable (96 % for the LITE and 98 % for the CAP system).

CONCLUSIONS: The use of rVes v 5 on LITE system significantly enhances diagnostic sensitivity while the specificity is comparable to the CAP system.

Diagnostic procedure improvement study

BACKGROUND: Diagnosis of insect sting allergy is often difficult, since up to 60 % of patients have positive IgE antibodies to native venoms from bee and wasp insect family, despite the fact that only less than 10 % of patients develop an SR after being stung by insects from both families.

HYPOTHESIS: The combined use of the results of IgE antibodies against species specific major antigen (SSMA-Ab) and the basophil activation test (the BAT) measurements in a stepwise manner and even more so as results of test pair ratio multiplication will significantly decrease the number of over-diagnosed patients in a diagnostically challenging subsample of patients that have positive results of IgE antibodies against venoms from both insect families (results of IgE antibodies against native bee venom > 0.35 kU/L and results of IgE antibodies against native wasp venom > 0.35 kU/L).

METHODS: Hundred and seventy-seven consecutive Hymenoptera sting allergic patients were included. Testings for IgE antibodies against native venoms [venom-IgEs], IgE antibodies against SSMAs (rApi m 1 and rVes v 5) and the BAT were performed for each patient.

RESULTS: One hundred and thirty-three patients with unequivocal culprit history were analysed.

In the venom-IgE single positive group (n=73), the agreement between venom-IgE results and culprit histories was excellent (96%). Very good agreement was also observed between venom-IgE results and SSMA-Ab results (> 90 %) and between venom-IgE results and the results of the BAT measurements (> 90 %).

In the venom-IgE double positive group (n=56), we constructed diagnostic algorithms that relied on the ratio of bee/wasp SSMA-Ab and/or BAT area under the curve (BAT-AUC) test pair results. All of the ratio-algorithms outperformed the standard cut-off tests when used separately or in a stepwise manner. The best ratio-algorithm was the SSMA and BAT-AUC algorithm, which correctly diagnosed more than 80% of the patients in comparison to the diagnostic approaches that used standard cut-offs which correctly diagnosed only around 50 % (BAT-only and SSMA-only) or just around 60 % (both of the stepwise approaches) of patients ($p < 0.05$). The significant improvement of correct diagnosis of the ratio-algorithm based diagnostic approaches, was confirmed with ROC curve analyses.

In the venom-IgE double negative group, sensitization was showed in all 4 patients with the BAT, but in only 1 patient with SSMA-Ab measurements.

CONCLUSIONS: This study: i.) confirmed that venom-IgE single positivity is a reliable indicator of the culprit insect and that, when such a result is encountered, no additional testing is necessary ii.) showed that additional testing in the venom-IgE double positive group is imperative with superior performance when interpreting the results in a ratio-based algorithm manner. iii.) confirmed that the BAT is essential to prove sensitization in the venom-IgE double negative group.

Reaction severity study

BACKGROUND: Current diagnostic procedures in Hymenoptera venom allergy can neither distinguish between patients with systemic reactions (SRs) and large local reactions (LLRs) nor determine the severity of SRs.

HYPOTHESIS: The results of different in-vitro diagnostic tests correlate to the severity of the reaction after a Hymenoptera sting in a Hymenoptera venom allergic individual.

METHODS: In 107 Hymenoptera venom allergic patients with single positive venom-IgE results, the levels of the IgE and IgG antibodies against the appropriate (the one the patient was single positive to) native bee or wasp venom, IgE antibodies against rApi m 1 or rVes v 5 (as appropriate), the basal tryptase and the BAT with 4 bee or wasp venom concentrations (as appropriate), evaluated as BAT-AUC, were measured. The factors were compared between LLR and SR patients and between varying SR severity groups, and used as predictors in a penalized logistic regression analysis to try to predict LLR vs. SR patients.

RESULTS: The recombinant IgEs and the BAT-AUC were the only independent statistically significant predictors of SR. No factor reliably differentiated between SR severities, however the BAT AUC and the basal tryptase were significantly correlated to the reaction severity if the reaction severity groups were numerically transformed (LLR = 1; Mueller I = 2; Mueller II = 3, Mueller III = 4, Mueller IV = 5).

CONCLUSIONS: Recombinant IgEs and the BAT-AUC discriminate between LLRs and SRs, however there is no test that could reliably distinguish between SR severities (a statistically significant trend can be observed in the BAT-AUC).

Skin prick test discrimination power study

BACKGROUND: There is not much information about the discrimination power (the ability to discriminate between bee and wasp culprits) of the skin prick tests (SPTs).

HYPOTHESIS: The discrimination power of skin prick tests is low, since the number of double positive or double negative results is high

METHODS: In hospital's information system, we looked up all patients in whom the SPTs with 10 and 100 μg/ml of honey-bee and wasp venom were performed during the course of diagnosis. The results of SPTs were correlated to the sting history and the proportion of correct diagnoses and also the discrimination power of SPTs was calculated.

RESULTS: One hundred and thirty three patients (39.7 %) had SPT diagnosis in accordance with history. In 127 patients (37.9 %) the results of SPT were double negative, despite a convincing history of an SR after a sting from at least one of the Hymenoptera species insects. In 64 (19.1 %) patients, the results of SPT were double positive, despite the fact that the majority (62 [96.9 %]) of patients had single positive culprit history.

In 144 (43.0 %) patients the results of SPT were single positive either to wasps or to honey bees. In majority (131 [91.0 %]) of patients the result of single positive result was in accordance with their sting history, however in 13 (9 %) patients the single positive SPT was discordant with culprit history.

Since 7 patients were allergic to honey bee and wasp stings, they were excluded and the final analysis of the discrimination power of SPTs, which was done on 328 (97.9 %) patients.

Out of 328 patients, SPTs were discriminative (either single positive for wasps or single positive for bees) in 143 (43.6 %) and indiscriminative (either double positive or double negative) in 185 (56.4 %) patients. Indiscrimination was mainly due to double negative results of SPTs, since the double negative results represented 123 (66.5 %) of all indiscriminative cases.

The combined discrimination power of SPT was therefore 143/328 (43.6 %) with binominal 95 % confidence interval for this sample proportion being between 38.2 % and 49.2 %.

CONCLUSIONS: SPT are indiscriminative in 56.4 % of patients, mainly due to double negativity of the results.

In the current work, we have shown that in order to detect patients allergic to Hymenoptera venom one should measure the recombinant IgEs with the LITE assay, since it has higher diagnostic sensitivity than the CAP assay, however the specificities of the two assays are comparable. We have also shown that in order to get a higher proportion of correct diagnoses in a diagnostically challenging subsample of patients who have positive results of IgE antibodies to native bee and wasp venom, it is advisable to interpret the results of recombinant IgEs and the BAT in a ratio based manner. The ratio based interpretation of recombinant-IgEs and the BAT results capitalizes on the absolute differences of those factors between different groups of allergic reaction levels (LLR vs. SR) – this absolute difference was proven in the 3-rd part of the work. In the ratio based interpretation approach, the absolute differences between the allergic reaction levels are put inside a context of one person (which usually has an SR after a sting from insects of only one insect family [either wasps or bees], and if stung by an insect from another insect family, the patient does not react to that sting with an SR [has just a LLR or no reaction at all]), therefore the ratio based interpretation does not have to take into account inter-individual differences when setting the cut-offs and is for that reason more precise. We have also shown that SPTs lack the discrimination power to distinguish between the two insect families, this manly being due to the inadequate sensitivity of the SPTs.

Introduction

Prevalence

Insect stings are, together with different food sources and medications, one of the three most common triggers of an anaphylactic attack, and represent around 20% of all causes of anaphylaxis (Wood et al. 2014). It is estimated that life-threatening systemic reactions (SRs) to insect stings occur in 0.4 % to 0.8 % of children and in approximately 3% of adults (Golden et al. 2011).

On the other hand, large local reactions (LLRs) were reported to occur in the range from around 2.5 % to up-to around 25 % of the population (Fernandez et al. 1999; Incorvaia et al. 1997) after a sting.

Taxonomy

Most authors use Chinery classification (Figure 1) of Hymenoptera species (Chinery 1984). Clinically relevant in Europe are stings from insect families Apidae and Vespidae. The family Apidae consists of the honeybees (Apis mellifera) and bumblebees (Genus Bombus). Bumblebees are bigger and more harry than honeybees and have distinct yellow or white bands on their abdomen. (Biló et al. 2005)

Insects from Vespidae family are almost hairless with black and yellow stripes on their abdomen. The Vespidae family consists of Vespinae and Polistinae subfamilies. Main differences between Vespinae and Polistinae subfamilies exist at the junction of the thorax and abdomen with Vespinae having a truncated thoraco-abdominal junction while the junction in insects of Polistinae subfamily is more oval in shape (Biló et al. 2005).

Vespula, Dolichovespula and Vespa make up the three genera of the Vespinae. The Vespula spp. (V. germanica and V. vulgaris), called also yellow-jackets in the USA and wasps in Europe, are the most important species in Europe, and can be easily distinguished from Vespa spp.(hornets) by their smaller size, but much harder from those of the genus Dolichovespula (shorter distance between their eyes and upper jaws(Biló et al. 2005)). In the genus Vespa, Vespa crabro (European hornet) is the most prevalent in Europe.

Polistinae subfamily (called wasps in Europe and the USA) are widespread especially in the Mediterranean areas.

In central and northern Europe the stings of Vespinae subfamily (mainly Vespula genus) and Apinae subfamily (Apis mellifera [honey-bee]) are the most prevalent (Biló et al. 2005). Because of the structural similarities of venoms of the European hornet and yellow-jacket (both belonging to the subfamily of Vespinae), the main emphasis in Hymenoptera venom allergy diagnosis lies in distinguishing between the allergy to venom from Vespinae and Apinae subfamily insects.

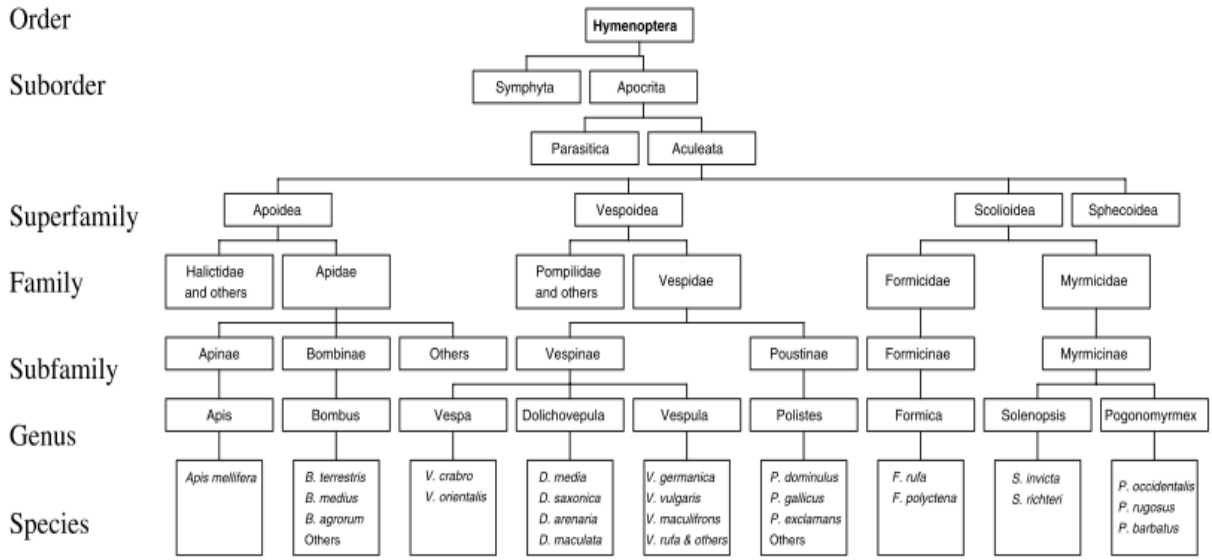

Figure 1: Taxonomy of Hymenoptera species.

Venom allergens

Most venom allergens are glycoproteins of 10–50 kDa with 100–400 amino acid residues (King and Spangfort 2000). The amount of venom which is released during a sting varies from species to species and even within the same species. The major allergen in honeybee venom is phospholipase A2 (Api m 1) that comprises 12–15 % of the dry weight of bee venom (Habermann 1972). Important component of honey-bee venom is also hyaluronidase (Api m 2), an allergen that shares a 50 % sequence identity with vespid venom hyaluronidase (Marković-Housley et al. 2000). Also present in honey-bee venom are acid-phosphatase (Api m 3 (Grunwald et al. 2006)), mellitin (Api m 4), dipeptidylpeptidase IV (Api m 5 (Blank et al. 2010)), Api m 6 (Michel et al. 2012), major royal jelly proteins 8 and 9 (Api m 11.0101 and Api m 11.0201 (Blank et al. 2012)), icarapin (Api m 10 (Blank et al. 2011)) and vitellogenin (Api m 12 (Blank et al. 2013)).

The major allergens in vespid venoms are antigen 5 (Ves v 5), phospholipase A1 (Ves v 1) and hyaluronidase (Ves v 2) (Biló et al. 2005).

Clinical features

Swelling, itching, redness and transient pain are characteristic for every insect sting, however when patients are allergic to insect venoms, they develop more severe local reactions or generalized systemic reactions.

LLRs

Those are the more severe forms of local reactions that are characterized by extensive oedema and swelling around the insect sting site, can persist for several days and are accompanied by pruritus and pain or both. They exceed a diameter of 10 cm, usually occur within 24 to 48 hours after a sting and last longer than 24 h (Biló et al. 2005).

SRs

The main characteristic of SRs is that signs and symptoms appear away from the sting site, since there has been a systemic dispersion of the causative allergen. They can range in severity from only cutaneous involvement to life threatening reactions with involvement of the respiratory and cardiovascular system. The onset of reactions is generally within 10 to 30 minutes of the sting. Onset of symptoms 1 to 4 hours after a sting has been reported in a small number of cases (Lockey et al. 1988). Usually the slower the onset of the signs and symptoms of anaphylaxis the less likely it is for the reaction to proceed to life threatening event (Lockey et al. 1988).

SRs can also have a biphasic time course. A biphasic reaction is a reaction in which there is a recurrence of SR symptoms after an initial remission of those symptoms. However biphasic reactions seem to be rare with insect stings, but when they do occur, they are usually associated with the most severe SRs (Bilò and Bonifazi 2009).

Classification of SR severity

Various classification schemes have been proposed, however two clinically most often used are those proposed by Mueller (Mueller 1966) and by Ring and Messmer (Table 1; (Ring and Messmer 1977)).

Mueller classification	
I	Generalized urticaria, itching, malaise, and anxiety
II	Any of the above plus two or more of the following: angioedema, chest constriction, nausea, vomiting, diarrhea, abdominal pain, dizziness
III	Any of the above plus two or more of the following: dyspnea, wheezing, stridor, dysarthria, hoarseness, weakness, confusion, feeling of impending disaster
IV	Any of the above plus two or more of the following: fall in blood pressure, collapse, loss of consciousness, incontinence, cyanosis
Ring and Messmer classification	
I	Generalized skin symptoms (e.g. flush, generalized urticaria, angioedema)
II	Mild to moderate pulmonary, cardiovascular, and/or gastrointestinal symptoms
III	Anaphylactic shock, loss of consciousness
IV	Cardiac arrest, apnea

Table 1: Mueller and Ring and Messmer classification of systemic reaction severity after a Hymenoptera sting.

Risk factors for allergic reactions after a Hymenoptera sting

Severity of the preceding reaction

The risk of a subsequent SR differs according to the severity of previous allergic reactions after Hymenoptera stings. The risk of developing an SR in up to 10 years after a previous sting is 10%, if the last reaction was a LLR, 10 %/20 % (children/adults) if a previous reaction was an SR with cutaneous involvement, and 40 %/60 % (children/adults) if a previous reaction was an SR more severe than one with just cutaneous involvement (Golden 2015).

Age

In children about 60% of systemic sting reactions are mild, whereas in adults respiratory or cardiovascular symptoms occur in about 70% (Biló et al. 2005). Elderly patients more often develop particularly severe sting reactions, and the fatality rate is higher than in children and young adults (Biló et al. 2005). Age has also been an independent statistically significant

predictor of severe SR in a study on adult Hymenoptera venom allergic patients (Ruëff et al. 2009).

Medication

Cardiovascular medications, namely the ACE-inhibitors and B-blockers have been shown to be predictors of a more severe SR (Ruëff et al. 2009).

Elevated baseline tryptase

Elevated serum baseline tryptase has been linked to higher chances of developing a more severe SR (Ludolph-Hauser et al. 2001; Ruëff et al. 2009).

Insects

Bee venom-allergic patients are at a greater risk of an SR on re-stings than those with vespid venom allergy (Bonadonna et al. 2012), however a vespid species sting has been linked to higher odds for developing more severe SR (Ruëff et al. 2009).

Sex

In the vast majority of investigations of Hymenoptera venom allergy, men are more often affected than women (Bilò and Bonifazi 2009), and also male sex was found to be a statistically significant risk factor for developing a more severe SR (Ruëff et al. 2009).

Other factors

One study has shown that the levels of total IgE antibodies (tIgE) were higher in a group of patients with milder SRs, as compared to the group of patients with more severe SRs (Sturm et al. 2007). In another study, it was also shown that that the serum platelet activating factor (PAF) levels were directly correlated and serum PAF acetylhydrolase activity was inversely correlated with the severity of anaphylaxis (Vadas et al. 2008). The PAF study was conducted on all types of anaphylaxis (food, drug and venom), thereby indicating a role of PAF in anaphylaxis whatever its underlying trigger.

Diagnosis of Hymenoptera venom allergy

Diagnostic testing should be performed when the history is consistent with the indications for venom immunotherapy (VIT; please see below) and is recommended based on the clinical history, even when the systemic reaction was many years or decades earlier, because the risk of reaction can persist for long periods of time (Golden et al. 2011). The presence of IgE

antibodies against Hymenoptera venom allergens must be confirmed with one of the following diagnostic tests.

Skin testing

Skin testing should be performed at least 2 weeks after the reaction to a sting, to avoid the possibility of false negative results during the refractory period. If negative after 2 weeks they should be repeated after 1 to 2 months (Biló et al. 2005). Skin tests are performed by means of prick or intradermal testing. If the patient has a conclusive reaction at a set concentration the test can be stopped. For skin prick tests, venom concentrations of 0.01–100 μg/ ml are usually used. Intradermally a 0.02 ml venom concentration ranging from 0.001 to 1 μg/ml is injected into the volar surface of the forearm. Even at 100 μg the sensitivity of skin prick tests is definitely lower than that of the intradermal test (Biló et al. 2005). The specificity of skin tests and also of other diagnostic tests with Hymenoptera venoms is difficult to define without prospective sting challenges, because exposed patients who never developed a systemic reaction may have been sensitized following their last sting (Biló et al. 2005), and will develop an SR after a subsequent sting.

IgE antibodies to native Hymenoptera venoms

In vitro laboratory tests that determine serum allergen-specific IgE antibodies, and are in one way or another derived from radioallergosorbent (RAST) test, have been routinely used and developed in the last 40 years in the clinical diagnosis of allergic diseases, such as asthma, allergic rhino conjunctivitis, atopic dermatitis and also venom allergy (Lee et al. 2009).

One of the basic steps in diagnosing Hymenoptera venom allergy is the measurement of IgE antibodies against native Hymenoptera venoms (the so-called venom-IgEs). However, one of the main problems of IgE antibodies directed against native venoms is the lack of specificity, since it has been shown that positive results to Hymenoptera venoms are common among general population, but SRs after deliberate sting challenges among those patients are very rare (Sturm et al. 2014).

Due to their lack of specificity, positive results of venom-IgEs to bee and vespid venoms (double positivity), in patients with a history of an SR after a Hymenoptera sting, are frequently observed, despite the fact that patients are usually allergic to an insect sting from only one insect family (Eberlein et al. 2012; Peternelj et al. 2009). This false double positivity is primarily due

to either antibodies against cross-reactive carbohydrate determinants (so-called CCD-antibodies) that are present in venom from both families or antibodies against epitopes of homologous proteins of both venoms, such as hyaluronidase or dipeptidylpeptidase (Seismann et al. 2010). Another possible explanation for double sensitization could be interidividual differences in tIgE levels, where double sensitization would represent the binding of the unspecific IgE antibodies to venom allergens, as it has been shown that the levels of tIgE antibodies were significantly higher in a group of double sensitized Hymenoptera venom allergic individuals, as opposed to the group of mono sensitized, allergic individuals (Sturm, et al. 2011).

Basophil activation test

Basophils are granulocytes that develop from CD34+ pluripotent progenitor cells and represent less than 1% of the peripheral blood leukocytes. Their excretory granules contain, among other, histamine and heparin and are thought to be involved in allergic reactions. The activation of basophils (release of mediator containing granules), can be roughly divided into FcεRI (high affinity receptor for IgE antibodies) dependent (allergens, autoantibodies ...) and FcεRI-independent activation (cytokines, anaphylatoxines ...). The ability of a substance to activate basophils can be measured through the basophil activation test (the BAT). The BAT is a flow cytometric technique that measures the presence of different activation markers (CD63, CD203) on the surface of the cells and by doing so, quantifies the response (activation) of the cell to a specific concentration of the allergen. The BAT is becoming an increasingly valuable tool in diagnosing different types of allergy (from medication and food allergy to insect sting allergy (Uyttebroek et al. 2014)).

In Hymenoptera venom allergy the BAT has been used in the harder to diagnose cases, with either negative venom specific IgEs and skin prick test results (Korosec et al. 2009) or to better distinguish between culprits in patients with double positive venom IgEs (Eberlein et al. 2012). The BAT has also been shown, to be connected to the increased risk of side effects after the start of VIT(Korošec et al. 2015; Kosnik et al. 2005), however these results were not consistently reproducible (Eberlein-König et al. 2006). But in food allergy model, the connection of the BAT to the more severe clinical phenotype was again shown in a study by Santos et al. (Santos et al. 2015) that demonstrated the BAT to be the only independent statistically significant predictor of the reaction severity in peanut allergic children.

Recombinant IgE antibodies

As pointed out previously, one of the basic steps in Hymenoptera venom allergy diagnosis is to measure IgE antibodies against native venoms. But, as mentioned above, native-venom IgE antibodies can be double positive in up to 60 % (Hofmann et al. 2011; Müller et al. 2009; Stoevesandt et al. 2013; Sturm et al. 2011) of patients, despite the fact that the majority of patients are allergic to venom from only one insect family (Eberlein et al. 2012; Peternelj et al. 2009).

Therefore, in the last decade, preparations with recombinant venom components that are devoid of CCD's have begun to gain importance, since it was thought that they will solve the problem of clinically irrelevant sensitization.

There are currently two systems for the quantitative detection of recombinant venom IgE antibodies used in routine practice: the ImmunoCAP (CAP) solid phase assay (Thermo Fisher Scientific, Waltham, MA USA) and ImmuLite (LITE) liquid allergen testing system (Siemens AG, Erlangen Germany).

In the CAP system, the allergen is covalently bound to a solid phase consisting of an activated hydrophilic polymer. The secondary anti-IgE antibodies are bound to the enzyme B-galactosidase, which transforms added methylumbelliferyl-B-D-galactoside into a fluorescent product.

The LITE system uses allergens bound covalently to soluble biotinylated polylysine polymers in a fluid phase that in turn binds to a streptavidin-covered polystyrene ball through a streptavidin-biotin complex. The secondary anti-IgE antibody is conjugated to alkaline phosphatase acting on an adamantly-dioxetane phosphate ester substrate to emit a chemiluminescent signal.

The first routinely used major honeybee venom recombinant allergen rApi m 1 (phospholipase A2; i208) became available for the CAP assay in 2009. It is a substitute for the previously used natural allergen Api m 1. The main limitation of this particular commercially available recombinant Api m 1 allergen is its low sensitivity (approximately 70% (Hofmann et al. 2011; Köhler et al. 2014; Korošec et al. 2011; Sturm et al. 2011)). This value is much lower than the sensitivities of native or custom recombinant Api m 1 allergen preparations, which have been reported to reach more than 90 % (Mittermann et al. 2010; Müller et al. 1997; Müller et al. 1995).

Because of the aforementioned limitations in the sensitivities of recombinant proteins in the CAP system, which were confirmed by recent studies (Müller et al. 2009), additional recombinant allergens (rApi m 2, rApi m 3, nApi m 4, rApi m 5, rApi m 10) have been produced by the CAP system manufacturer, to enhance sensitivity (Köhler et al. 2014).

The first routinely used recombinant allergen from wasp venom rVes v 5 (antigen 5; i209) became available in 2010 for the CAP system. It has higher sensitivity than commercially used rApi m 1, however the sensitivity does not match the sensitivity level of the native venom (Korošec et al. 2012). Therefore, another recombinant allergen of yellow jacket venom, the rVes v 1 (phospholipase A2; i211) allergen was produced for CAP. The combined use of rVes v 5 and rVes v 1 had slightly increased sensitivity, but it still did not match the sensitivity of the native venom (Korošec et al. 2012).

In 2013, routine recombinant Api m 1 (a45), Api m 2 (a46) and Ves v 5 (a670) allergen preparations also became available for the LITE system. There has been a comparison study between the two assays for native venom extracts (Watanabe et al. 2012) whose results showed that the sensitivities between the 2 assays might differ. However, to date, no study has compared the results of diagnostic sensitivity of the rVes v 5 allergen between the two assays.

Despite the fact that the recombinant allergens lack CCDs, a lot of studies have shown that their use in patients who have positive values of IgE antibodies against native venoms will also have positive values of at least one antigen in the extended spectrum of recombinant antigens of that venom in component resolved diagnosis (CRD). In a study by Eberlin et al. it was shown that all but one (21/22) patients that had double positive results of IgE antibodies to native venoms had also double positive results with at least one of the extended spectrum of recombinant antigens of both insects (Eberlein et al. 2012). Also in a study by Sturm et al (Sturm et al. 2014), 76 % of patients who had positive IgE antibodies against native wasp venom and a negative result of a sting challenge after a wasp sting, had positive measurements of IgE antibodies against rVes v 5 and/or rVes v 1. In the same study it was however shown that the proportion of rApi m 1 positive patients that were honey-bee sting challenge negative and had had positive measurements against native honey-bee venom was only 16 %, but the authors argued that this was probably due to the lack of sensitivity of rApi m 1 on ImmunoCAP assay (Korošec et al. 2011), not to its superior specificity. Importantly, they only measured the response to rApi m 1 allergen and not to other honeybee recombinant allergens. On the other hand in a study by Kohler et al (Köhler et al. 2014), they measured an extended spectrum of recombinant honey-bee allergens (rApi m 1, rApi m 2, rApi m 3, nApi m 4, rApi m 5 and rApi m 10), and showed

that in 6 bee non-allergic controls that had positive IgE antibodies to native honey bee venom, 3 shoved positivity to rApi m 1, 2 to rApi m 5 and 1 to rApi m 10. So again the majority of sensitized non –allergic controls had at least one positive recombinant allergen (50-100%).

Treatment of allergic reactions

Acute treatment

LLRs

Cold compresses might help to reduce local pain and swelling. Oral antihistamines and analgesics help reduce the itching or pain associated with cutaneous reactions. Although there are no controlled studies, use of oral corticosteroids is effective to limit swelling in patients with a history of large local reactions (Golden et al. 2011).

SRs

Acute treatment of anaphylaxis from insect stings is the same as the treatment of anaphylaxis from any other cause.

Epinephrine

Epinephrine is the medication of choice, for treatment of anaphylaxis, recommended by World Allergy Organization's (WAO) anaphylaxis guidelines (Simons et al. 2011). There is no contraindication to the use of epinephrine in a life-threatening situation, such as anaphylaxis. Delayed use of epinephrine might be ineffective and reports of fatal and near-fatal anaphylaxis show that fatal outcome is associated with delay or lack of administration of epinephrine (Golden et al. 2011). Epinephrine, through its action on alpha-1 adrenergic receptors, exhibits a vasoconstrictor effect that prevents swelling and oedema and relieves hypotension and shock (Simons and Simons 2010). It also acts on beta-1 adrenergic receptors and augments inotropic and chronotropic properties of the heart leading to an increase in force and rate of cardiac contractions, and on beta-2 adrenergic receptors through which it decreases mediator release and causes bronchodilator.

It should be injected by the intramuscular route in the mid-anterolateral thigh as soon as anaphylaxis is diagnosed or strongly suspected, in a dose of 0.01 mg/kg of a 1:1,000 (1 mg/mL) solution, to a maximum of 0.5 mg in adults (0.3 mg in children (Muraro et al. 2007, 2014;

Simons et al. 2011)). Depending on the severity of the episode and the response to the initial injection, the dose can be repeated every 5-15 minutes, as needed (Simons et al. 2011).

H1-Antihistamines.

In anaphylaxis, H1-antihistamines relieve itching, flushing, urticaria, angioedema, and nasal and eye symptoms (Gaeta et al. 2007). There are concerns about potential harmful central nervous system effects, for example, somnolence and impairment of cognitive function caused by first-generation H1-antihistamines.

Beta-2 Adrenergic Agonists.

Selective beta-2 adrenergic agonists such as salbutamol (albuterol) are sometimes given in anaphylaxis as additional treatment for wheezing, coughing, and shortness of breath not relieved by epinephrine (Simons et al. 2011).

Glucocorticoids.

Glucocorticoids switch off transcription of a multitude of activated genes that encode proinflammatory proteins (Krishnan et al. 2009). The onset of action of systemic glucocorticoids takes several hours, so they can hypothetically relieve protracted anaphylaxis symptoms and prevent biphasic reactions.

Other therapy

Patients should be placed on the back with their lower extremities elevated or, if they are experiencing respiratory distress or vomiting, they should be placed in a position of comfort with their lower extremities elevated (Simons et al. 2011). If in respiratory distress or are receiving repeated doses of epinephrine, patients should receive supplemental oxygen by facemask or by oropharyngeal airway at a flowrate of 6-8 L/min (Simons et al. 2011) and if they are hypotonic or in shock, patients should receive rapid intravenous infusion of 0.9% saline (eg. 5-10 mL/kg in the first 5-10 minutes to an adult; or 10 mL/kg to a child (Simons et al. 2011)).

It is important to note that other therapy (antihistamines, glukocorticoids, beta-2 adrenergic agonists, elevation of the extremities …) should not be considered as substitutes for epinephrine in acute treatment (Golden et al. 2011).

Disease modifying treatment

Venom immunotherapy

Hymenoptera venom anaphylaxis can be treated with venom immunotherapy. VIT should be recommended to all patients that have experienced an SR to an insect sting and have detectable IgE antibodies to venom allergens by means of *in vitro* or *in vivo* testing (Golden et al. 2011). However the following considerations should apply (Golden et al. 2011):

- VIT is generally not necessary in children 16 years of age and younger who have experienced cutaneous systemic reactions without other systemic manifestations after an insect sting.
- Adults who have experienced only cutaneous manifestations to an insect sting are generally considered candidates for VIT, although the need for immunotherapy in this group of patients is controversial.
- VIT is generally not necessary in patients who have experienced only large local reactions to stings but might be considered in those who have frequent unavoidable exposure (i.e. occupations and/ or hobbies where the risk of re-exposure is high) or in patients where psychological factors arising from anxiety can seriously impair patient quality of life.

The risk of re-experiencing an SR after VIT is reduced to as low as 5% (Golden et al. 2011), while the proportion of patients that can re-experience an SR after a sting, if they have not been treated with VIT can reach up to 60% (Golden 2015).

Selection of venom for VIT

This is based on the identification of the species of Hymenoptera involved and cross-reactivity between venoms (Bonifazi et al. 2005):

- Honey-bee and bumblebee venoms show marked cross-reactivity. Venom immunotherapy with honey- bee venom alone will be sufficient in non-professionally exposed bumblebee-allergic patients who most likely react on the basis of a cross-reactivity in the presence of primary sensitization to bee venom.
- Pronounced cross-reactivity exists between the major venom components of several vespids, particularly between Vespula, Dolichovespula and Vespa venoms, but less so between Vespula and Polistes venoms (Biló et al. 2005). In view of the relatively limited clinical importance of Polistes in temperate European climates, treatment with Vespula

venom alone is usually sufficient in these areas. In the Mediterranean area, due to the difficulty in distinguishing among Vespula and Polistes, patients with positive diagnostic tests to both venoms would seem to warrant treatment with both venoms.

- In the case of double positive diagnostic tests, that show presence of IgE antibodies to venoms from Vespidae and Aptidae spp. families there is no consensus, as to which venom to use for VIT. Some authors argue to use only one venoms, while others argue to use both (Golden et al. 2011).

VIT protocols

Protocols of various durations have been devised in an effort to maximize protection, minimize side-effects and optimize patient convenience. The time required to reach the generally adequate maintenance dose of 100 μg with each venom, with slow protocols(Bonifazi et al. 2005; Golden et al. 1980) is several weeks to months, whilst rush (Bonifazi et al. 2005; Yunginger et al. 1979) and ultra-rush protocols (Bonifazi et al. 2005; Michils et al. 2000) take several days or only a few hours respectively. The physician and patient might consider a variety of factors, such as the characteristics and circumstances of the sting reaction and the patient's lifestyle and preferences, in choosing a schedule.

The interval between maintenance dose injections is usually increased to 4 weeks during the first year and possibly to every 6 to 8 weeks during subsequent years (Bonifazi et al. 2005; D. Golden et al. 1981). Experts in the field support the regimen of a 4-week maintenance interval for 12 to 18 months followed by a 6-week interval for 12 to 18 months and then 8-week intervals (Golden et al. 2011).

The major risk of VIT, as with other types of allergen immunotherapy, is anaphylaxis. There have been reports of patients who had serum sickness–like reactions from VIT (De Bandt et al. 1997). Premedication with antihistamines during build-up VIT has been shown to reduce the incidence of local reactions and mild systemic reactions (Brockow et al. 1997), and was also shown to improve the efficacy of VIT (Müller et al. 2008).

Patients who are taking β-adrenergic blocking agents might not respond readily to epinephrine treatment if they experience an allergic reaction, so they should not be taking β-adrenergic blocking agents unless absolutely necessary (Golden et al. 2011). However if a patient cannot stop taking β-adrenergic blocking agents, VIT should still be given, but with greater caution. The same applies for angiotensin converting enzyme inhibitors (Golden et al. 2011).

Duration of VIT

VIT should usually be continued for at least 3 to 5 years. Although most patients can then safely discontinue VIT, some patients might need to continue VIT for an extended period of time or indefinitely (Golden et al. 2011). The special considerations include (Golden et al. 2011):

- high risk factors (near-fatal reaction before VIT, systemic reaction during VIT, honeybee allergy, increased baseline serum tryptase levels, underlying medical conditions and concomitant medications, and frequent exposure).
- quality of life (eg, limitation of activity and anxiety about unexpected stings).

Major issues in Hymenoptera venom allergy management and outlooks

Because VIT is, as already mentioned, on the one hand very effective (treating up to around 95% (Wood et al. 2014) of patients that had had a SR after a Hymenoptera sting), but on the other hand also a long lasting (Bilò 2011; Golden et al. 2011) and expensive (Stoevesandt et al. 2013) procedure, the choice of the correct venom for VIT (correct diagnosis) is of paramount importance in Hymenoptera venom allergy, since under-diagnosis leads to insufficient treatment and hence the risk of reaction reoccurrence, however, over-diagnosis is associated with unnecessary medical expenditures.

However, correct diagnosis of Hymenoptera venom allergy is hard, especially in cases when the patient did not see, or recognize the stinging insect (being stung in the back, or felt into an anaphylactic shock right immediately after the sting), because then the diagnosis and hence the choice of VIT is based solely on different diagnostic tests. But, as already mentioned, evaluations of routinely used diagnostic tests (e.g., skin tests and measurement of IgE to native venom extract) are double positive in 40 to 60 % of patients with a history of an SR after a Hymenoptera sting (Hofmann et al. 2011;Müller et al. 2009; Stoevesandt et al. 2013; Sturm et al. 2011), despite the fact that the minority (Eberlein et al. 2012; Peternelj et al. 2009) of patients actually have an SR when they are stung by insects from both families.

It was thought that the problem of this clinically irrelevant double sensitization will be overcome by the introduction of IgE antibodies against recombinant venom components, since these components lack cross-reactive carbohydrate determinants (CCDs) and are therefore unable to bind IgE antibodies against those determinants, which were thought to represent the major cause of irrelevant sensitization. However, as more and more recombinant venom components are being introduced to the market, it is becoming obvious that the use of

recombinants with simple cut-off based interpretation of the results (cut-off dividing the results to positive/negative values), will not solve the problem of clinically irrelevant sensitization, since there is growing body of evidence showing that patients sensitized to native-venom(s) are also sensitized (have positive results of IgE antibody measurements) to at least one of the recombinant components of the venom that they are sensitized to (Eberlein et al. 2012; Frick et al. 2015; Köhler et al. 2014; J. Michel et al. 2016; Sturm et al. 2014).

Therefore the need for different interpretation of the results of recombinant IgEs is necessary. One interesting concept of test result interpretation was presented in a study by Eberlin et al.(Eberlein et al. 2012) where it was shown, on double sensitized Hymenoptera venom allergic patients, that the bee/wasp ratio based interpretation (the ratio results indicating predominant culprit sensitivity) of the BAT results was better in culprit discrimination than the interpretation of the untransformed BAT results (Eberlein et al. 2012). This better discrimination inside a ratio was probably due to the property of the BAT, which was not jet established in Hymenoptera allergy model, however it was proven in food allergy models, and that is that the BAT can distinguish between asymptomatic sensitization (AS) and clinically apparent allergy (Ford et al. 2013; Santos et al. 2014; 2015), since the case of clinically irrelevant sensitization for one venom and clinically relevant sensitization for the other represent a similar problem, however, here, different allergy level groups (AS vs. clinical allergy) are present inside the same person.

Purpose of the work and working hypotheses

As described above, the main hurdles in Hymenoptera venom allergy diagnosis are a lack of sensitivity of recombinant allergens, a lack of discrimination power of the routinely used tests to distinguish between the culprit whose sting has caused an allergic reaction and the culprit whose sting has not caused an allergic reaction and also a lack of a test that could reliably distinguish between different SR reaction severity groups. In the framework of the PhD thesis we have posed the following hypotheses:

1. The sensitivities of rVes v 5 measurements with ImmuLite and ImmunoCAP assays will be different. Specificities of the two assays will be comparable.

2. The combined usage of the results of SSMA-Ab measurements and the BAT in a stepwise manner and even more so as results of test pair ratio multiplication will significantly decrease the number of over-diagnosed patients in the diagnostically challenging venom-IgE double positive subsample of patients.

3. The level of the allergic reaction after a Hymenoptera sting is dependent of the factors that can be measured with in-vitro diagnostic tests.

4. Discrimination power of skin prick tests is low, since the proportion of double positive or double negative results with skin tests is high.

Materials and Methods

Assay comparison study

Patients

One hundred and ten (50.9 % males; median age = 40.5 years, IQR = 21.75 years) patients allergic to wasp stings with different levels of allergic reaction severity after stings were included in the study.

Reaction severity

Patients were divided into 3 groups according to their reaction severity after a sting:

- LLR group: This group consisted of 12 patients who had an LLR without any systemic signs after an insect sting.
- Mild-SR group: This group consisted of 36 patients who had a mild SR without any signs of cardiorespiratory involvement (Mueller grade 1 and 2) after an insect sting.
- Severe-SR group: This group consisted of 62 patients who had a severe SR with signs of cardiorespiratory involvement (Mueller grade 3 and 4) after an insect sting.

Measurements of IgE antibodies on different assays

All patients had positive measurements of IgE antibodies to wasp venom (i3) and negative IgE measurements to bee venom (i1). The measurements of IgE antibodies against native Hymenoptera venoms were done using the CAP technique.

A battery of different diagnostic tests was performed on each patient:

- Measurements of IgE antibodies against recombinant Ves v 5 allergen with CAP (rVes v 5 CAP [i209])
- Measurements of IgE antibodies against recombinant Ves v 5 allergen with LITE (rVes v 5 LITE [a670])
- Measurements of IgE antibodies against recombinant Ves v 1 allergen with CAP (rVes v 1 CAP [i211]). The measurements were performed only on patients who had negative measurements for rVes v 5 CAP.

We also tested 49 healthy controls, with rVes v 5 measurements of both LITE and CAP systems.

Calculations of the sensitivities of different diagnostic tests

The sensitivities of different assays were calculated as follows:

- The number of patients positive with a particular test in a wasp-allergic sample / number of all patients in a wasp-allergic sample

The sensitivities of rVes v 5 with both techniques were also calculated for each of the reaction severity group subsamples.

Correlations between different diagnostic methods

Correlation coefficients between the results of the CAP and LITE techniques (rVes v 5 CAP vs. rVes v 5 LITE) were calculated.

The status of the result (positivity/negativity) using the LITE technique was calculated on a subsample of patients who were CAP-negative (rVes v 5 LITE status on a subsample of rVes v 5 CAP-negative patients) or vice versa.

The linear regression between CAP and LITE results was performed on a subsample of patients who had positive results for measurements with both techniques and the values of measurements with both techniques were below 100 kU/L.

The combined use of the BAT and measurements of IgEs against SSMA to diagnose Hymenoptera venom allergic patients

Patients

All patients (n=177; 55.4 % males, median age = 46 years, IQR = 23 years) with a history of insect venom allergy that were appointed to the University Clinic of Golnik in in the summer 2013 were included in the study.

Because of the ethical limitations of diagnostic sting tests, the main endpoint for analysis was a systemic-reaction after a Hymenoptera sting. To obtain a reliable culprit history collection, an experienced senior alergologist, in collaboration with the patient, took a detailed approach in identifying the insect by focusing not only on the stinging insect (such as its size and whether it left behind a stinger) but also on the circumstances regarding the sting (such as what the patient was doing at the time of the sting and whether a honey-bee hive was present in the vicinity). The severity of the reaction was also determined. All previous reactions after Hymenoptera stings were likewise evaluated. Only patients in which we unequivocally

identified the culprit(s) responsible for the systemic reaction(s) were utilized in evaluating the diagnostic approaches. In the majority of patients (83.6 %) the reactions happened within 1 year of the examination.

Mistakes in diagnosing a Hymenoptera sting allergy

When diagnosing a Hymenoptera sting allergic patient, 2 types of mistakes can emerge, by comparing the results of a culprit history to the result of a diagnostic approach (Table 2):

- Under-diagnosis: Under-diagnosed patients can have:
    - Double positive culprit history (wasp [W] and bee [B]) and single positive or double negative result of a diagnostic approach.
    - Single positive culprit history (just W or just B) and double negative or non-corresponding single positive result of a diagnostic approach.
- Over-diagnosis: Over-diagnosed patients have:
    - Single positive culprit history and double positive result of a diagnostic approach.

In correctly diagnosed patients the result of a diagnostic approach matches the culprit history.

History / Diagnostic approach	DA –/-	DA +/-	DA+/+
H +/+	U.D.	U.D.	C.D.
H +/-	U.D	C.D./U.D (if oncoressponding)	O.D.

Table 2: The relationship between the result of the diagnostic approach (DA) and history (H) in terms of correct diagnosis (C.D.), over diagnosis (O.D.) and under-diagnosis (U.D.).

Diagnostic algorithm construction, evaluation and comparison

Because the most diagnostically challenging cases are those that have double positive or double negative results of IgE measurements against native Hymenoptera venoms, patients were

divided into 3 groups according to the results of their native venom-IgE measurements (Figure 2).

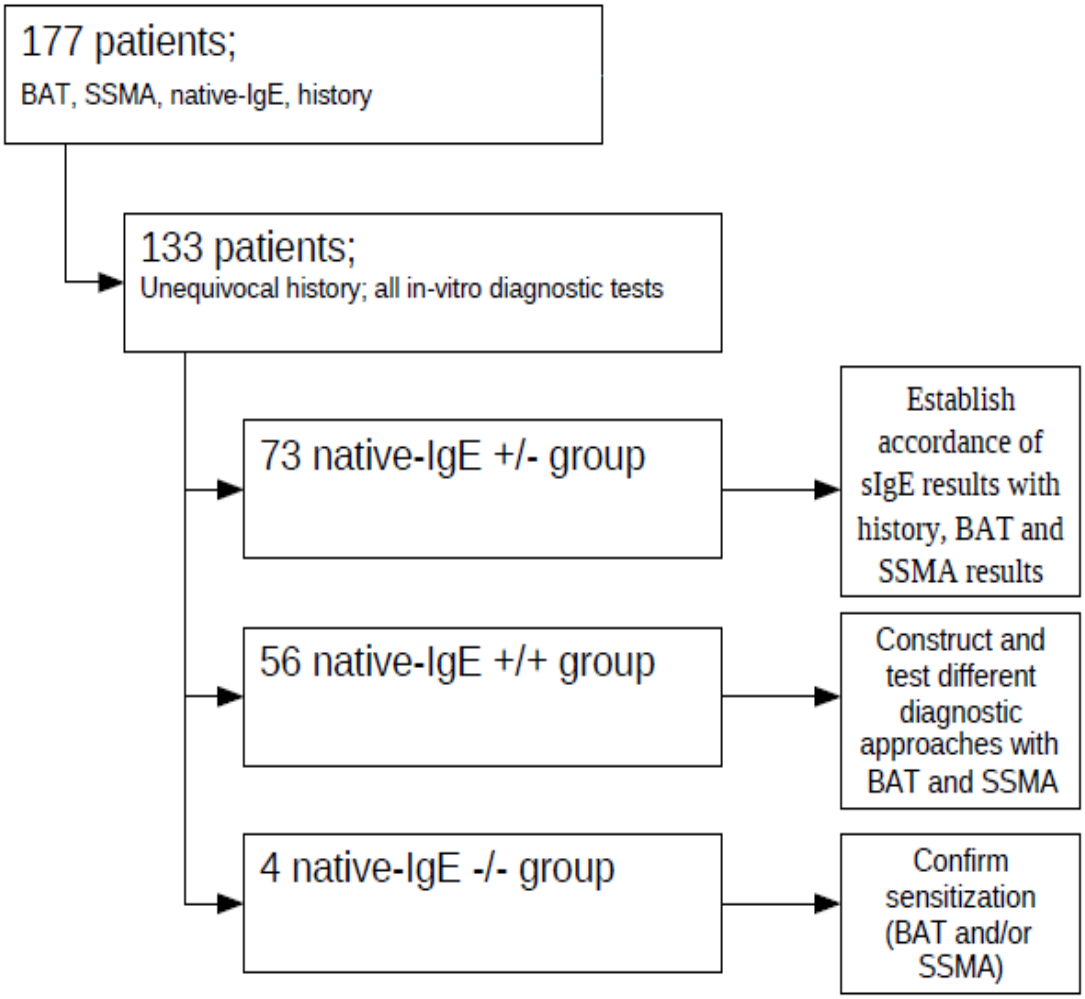

Figure 2: Flowchart of the study.

Venom-IgE single positive group (venom-IgE +/-): This group consisted of patients who had positive results of IgE measurements against only one native Hymenoptera venom. Here, culprit histories of patients were correlated to the results of venom-IgE measurements to establish the number of correctly diagnosed patients and to validate history collection. The results of the BAT and SSMA-Ab measurements were also correlated to the results of venom-IgE measurements to establish the agreement between the tests.

Venom-IgE double positive group (venom-IgE +/+): This group consisted of patients who had positive results of IgE measurements against both native venoms (bee and wasp). Here, two different groups of diagnostic approaches were constructed:

- The stepwise diagnostic approaches: The basic principle in constructing the stepwise diagnostic approaches was to consider the first single positive result in diagnostic test measurement evaluations as diagnostic. Two distinct stepwise approaches were considered:
    - Stepwise 1: Here, we first evaluated the results of SSMA antibody measurements for both insects and, if one was positive and the other negative, the procedure was terminated. In cases in which the recombinant antibody results were either double positive (SSMA +/+) or double negative (SSMA-/-) the results of the BAT measurements were evaluated.
    - Stepwise 2: This approach was similar to stepwise 1, except that we evaluated the results of the BAT measurements in the first step, and if they were either double positive (BAT +/+) or double negative (BAT -/-), we then, in the second step, evaluated the results of SSMA antibody measurements. Otherwise (BAT +/-), the procedure was stopped.

  The number of correctly/over/under-diagnosed patients by each of the stepwise approaches was calculated by correlating the results of the approach to the patient's culprit history.
- The algorithm-based diagnostic approaches: The basic idea behind the construction of the algorithm-based diagnostic approaches was to take a bee test pair result and divide it by a wasp test pair result (i.e., BAT AUC for bee venom divided by BAT AUC for wasp venom). Three different algorithms were constructed as follows: i.) BAT-algorithm: included the results of BAT AUC for bee/ BAT AUC for wasp ratio; ii.) SSMA algorithm: included the results of the rApi m 1/rVes v 5 ratio and iii.)

SSMA±BAT algorithm: the results of a BAT-algorithm and SSMA-algorithm were multiplied together.

The results of different algorithms were then logged (decimal logarithm), and the continuum of result values divided by two cut-offs into 3 intervals: i.) wasp interval (from negative infinity to the negative cut-off); ii.) wasp and bee interval (from negative cut-off to positive cut-off); iii.) bee interval (from positive cut-off to positive infinity). The cut-offs were set in such a way as to obtain as few possible over-diagnosed patients while keeping the number of under-diagnosed patients at less than 10% (Table 3).

Different diagnostic approaches were then compared to each other and also to the results of diagnostic approaches that used single diagnostic tests with simple cut-offs (SSMA-only, BAT-only) in terms of correct, over, and under-diagnosis.

Name of a diagnostic alg.	**Lower cut-off**	**Upper cut-off**
BAT-ratio	0	1.3
SSMA-ratio	-1.4	0.1
BAT+SSMA-ratio	-2.5	0

Table 3: Cut-offs for different ratio based algorithms.

Venom-IgE double negative group (venom-IgE -/-): This group consisted of patients who had negative results of IgE measurements against both native venoms. Here, we wanted to establish in how many patients we would be able to confirm sensitization with the BAT and SSMA antibody measurements.

Validation of ratio-based diagnostic algorithm values

Receiver operator characteristic (ROC) curves were drawn for all the single test results and the corresponding single test algorithms (i.e., SSMA results and SSMA algorithm results). The AUCs of ROC curves of single test results were compared to the AUCs of ROC curves of the corresponding single test algorithm results.

ROC curves were also drawn with BAT+SSMA algorithm values, and AUCs of those ROC curves were compared to AUCs of ROC curves of single test algorithm values.

Predictors of the severity of the allergic reaction after a sting in a Hymenoptera venom allergic patient

Patients

One hundred and seven patients (51.6 % males; median age = 41 years; IQR = 27 years) allergic to Hymenoptera stings with single positive venom-IgE results to either of the Hymenoptera venoms (positive results to honey-bee venom and negative results to wasp venom or negative results to honey-bee venom and positive results to wasp venom) that were the BAT responders (anti-FcRI response was above 15% of activated basophils) were included in the study. In all patients the following diagnostic tests were performed: i.) IgE antibodies against native venoms, .ii.) IgE antibodies against recombinant SSMAs iii.) the BAT with 4 [1 μg/ml, 0.1 μg/ml, 0.01 μg/ml, 0.0001 μg/ml] concentrations of bee/wasp venom iv.) IgG antibodies against native bee/wasp venom v.) the basal tryptase,.

Study design

The results of all tests (BAT-AUC, venom IgEs, recombinant IgEs and venom IgGs) administered using the appropriate venom (single positive venom of each individual), the basophil response to anti-FcRI positive control, baseline activation levels (patient background, PB) and the basal tryptase levels were compared across different levels of allergic reaction (LLRs and Muller I – Mueller IV). The levels were additionally transformed into numerical values of ascending order (LLR = 1, Mueller I = 2 … Mueller IV = 5) and correlated with the levels of different immunological factors. Afterwards the Mueller I-IV reactors were pooled together to create a single group of systemic reactors and the factors were compared among the LLR group and the group of pooled SRs. Two models of a penalized logistic regression analysis were additionally constructed (LLR vs. SR and LLR+Mueller I vs. Mueller II,III and IV) to establish an independent statistically significant correlation of the factors to different levels of allergic reaction.

Skin prick tests – discrimination power

Patients

We retrospectively included into analysis all patients (58.5 % males; median age = 48 years and IQR = 24 years) in whom skin prick tests with bee and wasp venom were performed as part of a routine diagnostic workup for Hymenoptera venom allergy.

Determination of skin prick test discrimination power and calculation of CIs

The results of positivity/negativity of skin prick tests to bee/wasp allergens were evaluated and the numbers of single positive test results to bee venom (bee venom positive response, wasp venom negative response), single positive test results to wasp venom (bee venom negative response, wasp venom positive response), double positive test results (bee venom positive response, wasp venom positive response) and of double negative test results (bee venom negative response, wasp venom negative response) were evaluated.

Double positive test results and double negative test results were considered as non-discriminative; so the discriminative power of skin prick tests was therefore calculated as 1 – (the number of double positive + the number of double negative test results)/ (the number of all tests [wasp single positive + bee single positive + double positive + double negative]).

Confidence interval of the discriminative power of skin prick tests was additionally calculated as a binominal confidence interval for sample proportions.

Diagnostic tests

Laboratory tests

- Specific IgE antibodies against native venoms: concentrations of IgEs against native bee/wasp venoms were measured using the Immulite 2000 test (Siemens, Tarrytown, NY, USA). If indicated explicitly, the values were measured using ImmunoCAP (Thermo Fisher Scientific, Waltham, MA USA) test. Values > 0.35 kU/L were considered positive.
- IgE antibodies against SSMA (rApi m 1, rVes v 5): concentrations of IgE antibodies against SSMAs were measured using the Immulite 2000 test (Siemens). If indicated

explicitly the values were measured using ImmunoCAP (Thermo Fisher Scientific, Waltham, MA USA) test .Values > 0.35 kU/L were considered positive.

- The BAT was administered according to methods described previously (Eržen et al. 2012; Kosnik et al. 2005). Briefly, whole blood with heparin anticoagulant was preincubated with basophil stimulation buffer containing IL-3 (patient background), honey bee or wasp venom (Hal Allergy) with a final concentration of 0.001 µg/ml to 1 µg/ml of and 0.55 µg/ml anti-Fc RI mAb (Buehlmann Laboratories, Basel, Switzerland) at 37°C for 15 min. Degranulation was prevented by chilling the mixture on ice. Next, FITC-conjugated anti-CD63 mAb, PE-conjugated anti-CD123 mAb, and PerCP-conjugated anti-HLA-DR mAb (BD Biosciences, Franklin Lakes, NJ, USA) were added and the sample was incubated for 20 min on ice. Finally, the samples were lysed, washed, fixed and analysed within 2 h on a FACSCanto II flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

  To represent the BAT as a single number, we calculated the area under the curve (AUC) as described earlier (Korošec et al. 2015). To validate the analysis, the BAT was also used conventionally; the cut-off for positive results was 15 % of CD63-positive basophils evaluated at every venom concentration.

Skin prick tests

Bee and wasp venoms (Venomenhal, HAL, Haarlem, The Netherlands) were used in skin prick tests at concentrations of 10 and 100 µg/ml. In accordance with national guidelines, end point titration was used in standardized skin prick venom testing in our clinical practice. All patients were also tested with a positive histamine control and a negative diluent (saline) control test. Diameter of the weal to bee/wasp allergens were read at 15 minutes after starting the test. A weal of 3 mm or more in diameter was considered to represent a positive response.

Data analysis

All statistical analyses including the penalized log-regression analysis were performed using the R statistical software package (Team R Core 2013) and its affiliated software packages (Heinze and Ploner 2013; Wickham 2009; Xavier et al. 2011). The normality of the data was evaluated using Shapiro-Wilk test. The correlation coefficients were calculated as Pearsons's or Sperman's correlation coefficients as appropriate. The ROC curve AUCs were compared to

each other using the bootstrap method. The sample proportions were compared against each other using the Fisher's exact test. A p-value < 0.05 was considered statistically significant.

Results

Assay comparison study

Calculations of sensitivities of different diagnostic tests

The results of sensitivities of different diagnostic tests (and their combinations) are presented in Figure 3. The sensitivity of rVes v 5 using the LITE system was significantly higher when compared to the CAP system (93 % [LITE] vs. 82 % [CAP], p = 0.026). The addition of another recombinant allergen (rVes v 1) increased the sensitivity even further but this increase in sensitivity was not statistically significant. The combined sensitivity of both recombinant allergens (rVes v 5 and rVes v 1) using the CAP system was lower than the sensitivity of just rVes v 5 using the LITE system (90 % vs. 93 %). However, these differences were not statistically significant. It is also interesting to note that the combined sensitivity of rVes v 5 LITE and rVes v 1 CAP almost reached the sensitivity of native wasp venom (97 %; 95 % CI = 92 % - 99 %). The combination of recombinant allergens using different systems (rVes v 5 [LITE] and rVes v 1 [CAP]) were calculated, because and rVes v 1 allergen is not available for LITE technique.

The results of the diagnostic sensitivities of rVes v 5 with both techniques on subsamples of patients with different groups of reaction severities are presented in Table 4. It can be seen that the difference in sensitivities was higher in patients with more severe reactions (severe SR + mild SR).

Two out of 49 healthy controls were positive on LITE and one on CAP system (specificities of 96 % and 98 % respectively).

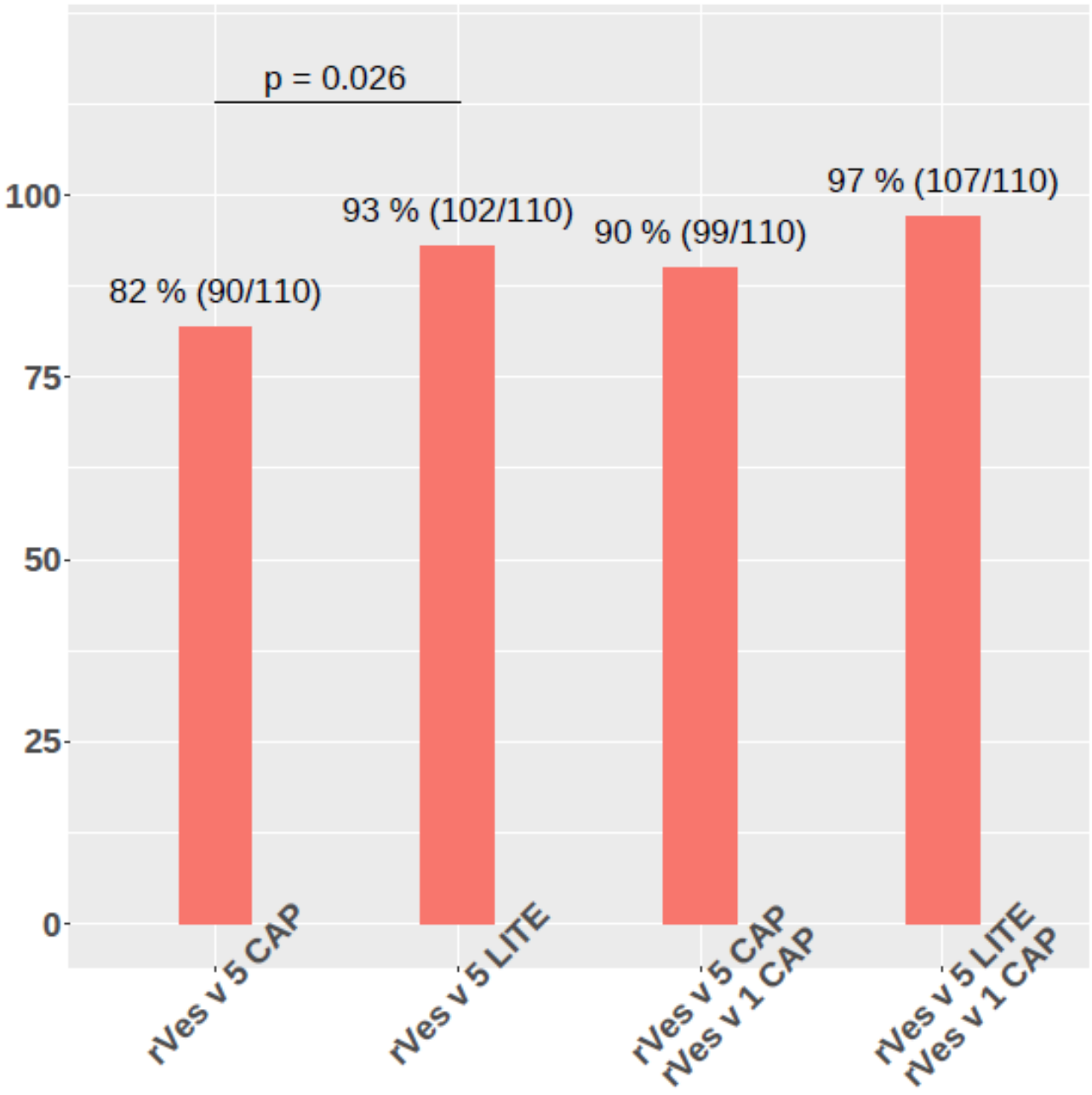

Figure 3: Sensitivities of different diagnostic tests for wasp venom allergy.

Reaction severity	Sensitivity (CAP)	Sensitivity (LITE)	Difference in sensitivity	p - value
wasp venom allergic patients (n = 110)				
LLR	10/12 (83%)	11/12 (92%)	9%	1
Mild-SR	31/36 (86%)	35/36 (97%)	11%	0.2
Severe-SR	49/62 (79%)	56/62 (90%)	11%	0.13

Table 4: Sensitivity of both techniques (CAP/LITE) with rVes v 5 allergen in subsamples of patients with different reaction severities.

Correlations between different diagnostic methods

The scatterplots of log values measured with CAP and LITE (on a subsample of patients who had positive results using both techniques [CAP and LITE], and whose results using both techniques were < 100 kU/L) are presented in Figure 4 (please note that log results are presented only in the Figure 4 for better visual representation of the results that are clustered at lower values; when discussing linear regression coefficients they are referring to the non-logged results]).

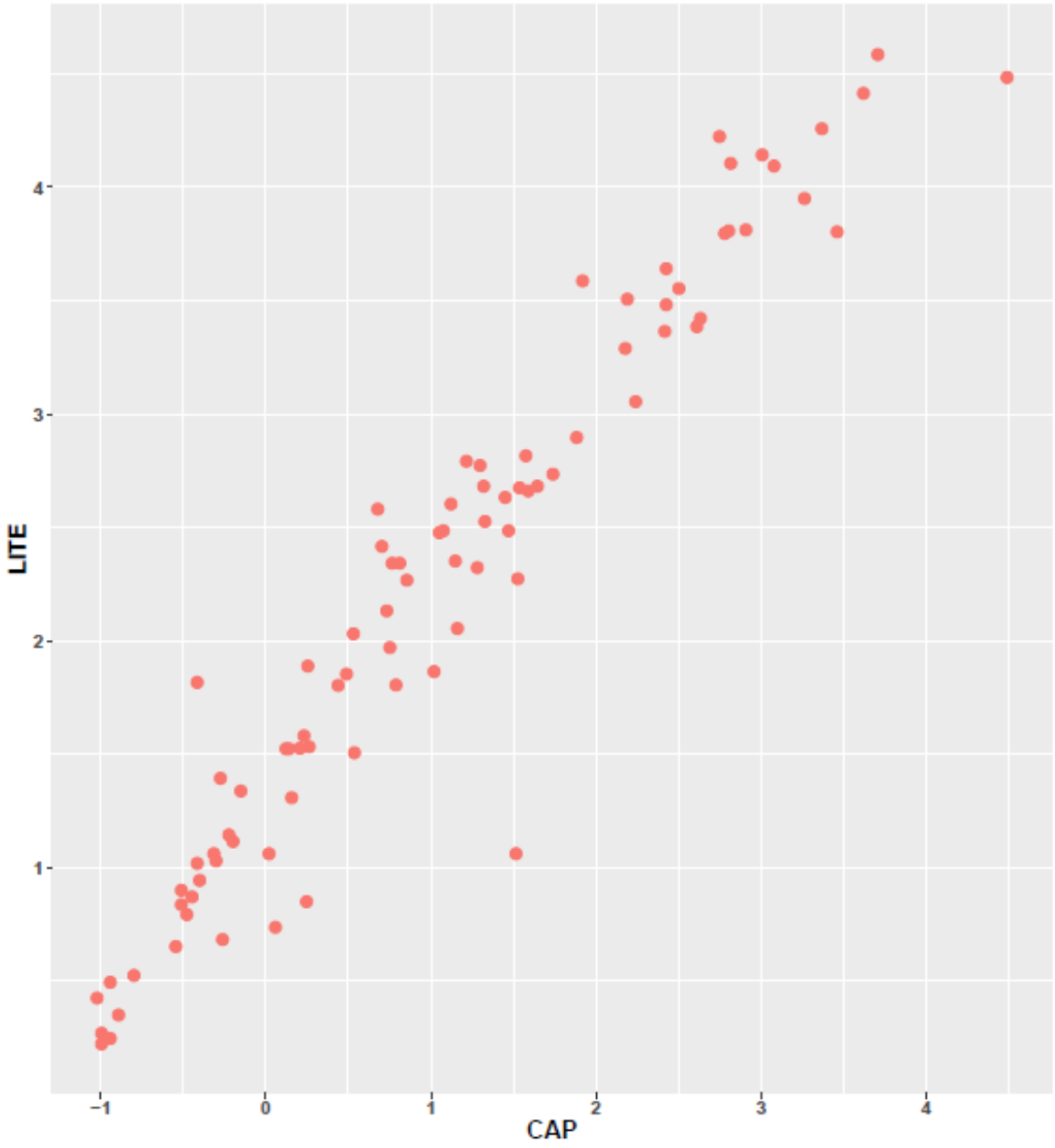

Figure 4: Scatterplot of log rVes v 5 values measured with CAP and LITE technique.

The Spearman's correlation coefficient rho (as the data were not normally distributed) between CAP and LITE measurements was higher than 0.98 ($p < 0.001$), suggesting a very strong positive correlation. Also, the results of regression coefficient (2.3 for rVes v 5) and R^2 for linear regression indicate a very strong and positive dependency between both variables, as more than 85% of one variable can be explained by the variability of the other. The numbers of patients with positive/negative results for IgE measurements against recombinant Ves v 5 with the CAP/LITE technique are presented in Table 5. Interestingly, approximately 60% of patients who had negative results for rVes v 5 measurements with the CAP technique had simultaneously positive results for rVes v 5 measurements with LITE; by contrast, none of the patients who had negative results for rVes v 5 measurements with LITE had positive results for rVes v 5 measurements with CAP.

Since both assays measure down to values of 0.1 kU/L the agreement between the two assays were separately measured on patients whose CAP measurements were between 0.1 kU/L and 0.35 kU/L (despite the fact that both manufacturers state that the cut-off for positivity is > 0.35 kU/L). The correlation coefficient rho was again high (0.94) and the linear regression coefficient (LITE measurements as dependent variable CAP measurements as independent variable) was 3.6. All of the coefficients (rho and linear regression coefficients) were statistically significant.

	LITE positive	LITE negative
wasp venom allergic patients (n= 110)		
CAP positive	90	0
CAP negative	12	8

Table 5: Patients positive/negative for rVes v 5 allergen with each of the techniques (CAP/LITE).

The combined use of the BAT and measurements of IgEs against SSMA to diagnose Hymenoptera venom allergic patients

Patients

Out of the 177 patients, 133 (75.1 %) were included in the statistical analysis and 44 (24.9%) were excluded. The reasons for exclusion were that in 26/44 the culprit history was not clear, in another 9/44 subjects the BAT was not performed (random), and the remaining 9/44 were BAT non-responders. According to culprit history, 42/133 (31.6 %) were bee allergic, 84/133 (63.2 %) were wasp allergic, and 7/133 (5.3 %) were bee and wasp venom allergic. Twenty-two patients were positive that they have been stung by wasps and did not react with a systemic reaction to a wasp sting and further 51 were likewise certain to have been stung by bees and did not react to a bee sting with a systemic reaction.

Out of 133 patients, 73 (54.9 %) had venom-IgE +/- measurements, 56 (42.1 %) had venom-IgE +/+, and 4 (3.0 %) had venom-IgE -/- measurements.

Evaluation and comparison of diagnostic approaches

Venom-IgE ±/- group: In the venom-IgE +/- group, the correlation between the culprit history and the results of the venom-IgE measurements was 70/73 (95.8 %). In 3 patients whose venom-IgE measurements did not correlate to the culprit history, 1 had a bee and wasp positive history, and the results of the venom-IgE measurements indicated that he was just wasp allergic. In the other 2 patients, the results of the venom-IgE measurements were discordant with culprit history (1 for wasp and 1 for bee). The concordance between the venom-IgE and SSMA-Ab test results, and also between the venom-IgE and BAT results, was 66/73 (90.4 %). In all 7 venom-IgE and SSMA-Ab discordant results, the venom-IgE results indicated a bee culprit. In 5 out of those 7 cases, the SSMA-Ab results indicated a bee and wasp (in 4/5 the BAT indicated the culprit that was concordant with the venom-IgE results and also with the patient history). In 1 out of the remaining 2, the results of the SSMA-Ab tests were double negative (but the BAT results were concordant with the venom-IgE results and history), and in the last one the results of SSMA-Ab and venom-IgE measurements were discordant (BAT double positive), and SSMA-Ab indicated the culprit that was concordant with the history. In 6 out of 7 BAT and venom-IgE discordant cases, the BAT was double positive, and in 5 out of those 6 BAT double positive cases, the SSMA-Ab measurements were single positive and concordant with the history.

Venom-IgE ±/± group: The results of different diagnostic approaches with respect to correct, over- and under- diagnosis, and the comparison between them in the venom-IgE +/+ group are presented in Figure 5, the histogram of the algorithm based diagnostic approach in Figure 6 and the stepwise diagnostic approaches are presented in Figure 7.

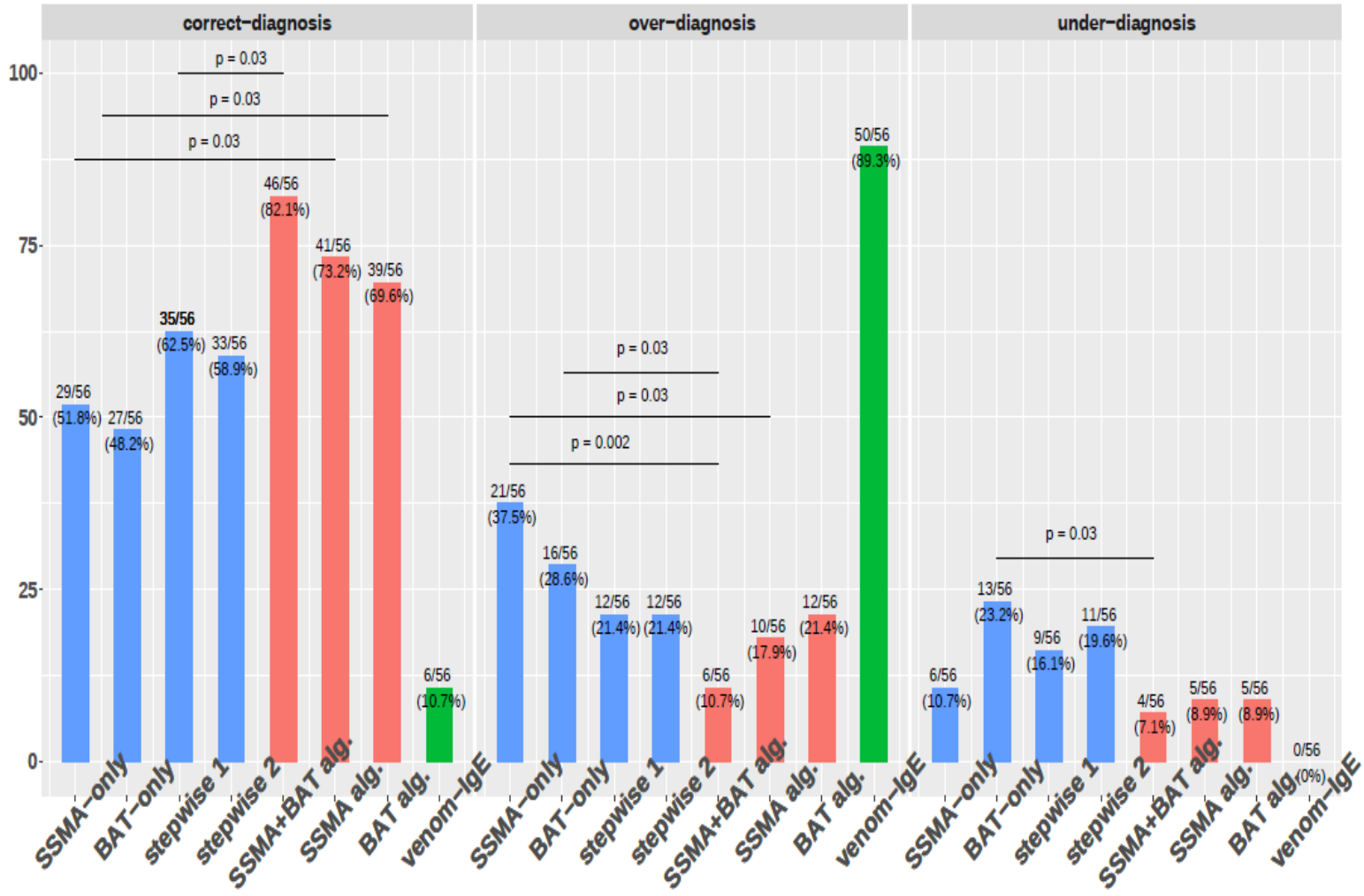

Figure 5: Bar plots representing the number of correct over/under diagnosed patients with different diagnostic approaches. Simple cut-off based diagnostic approaches are coloured blue, algorithm based diagnostic approaches are coloured red and IgE-only diagnostic approach is coloured green. P-values are written if the two approaches that are being compared are using equal amounts of diagnostic tests and if a p-value was significant.

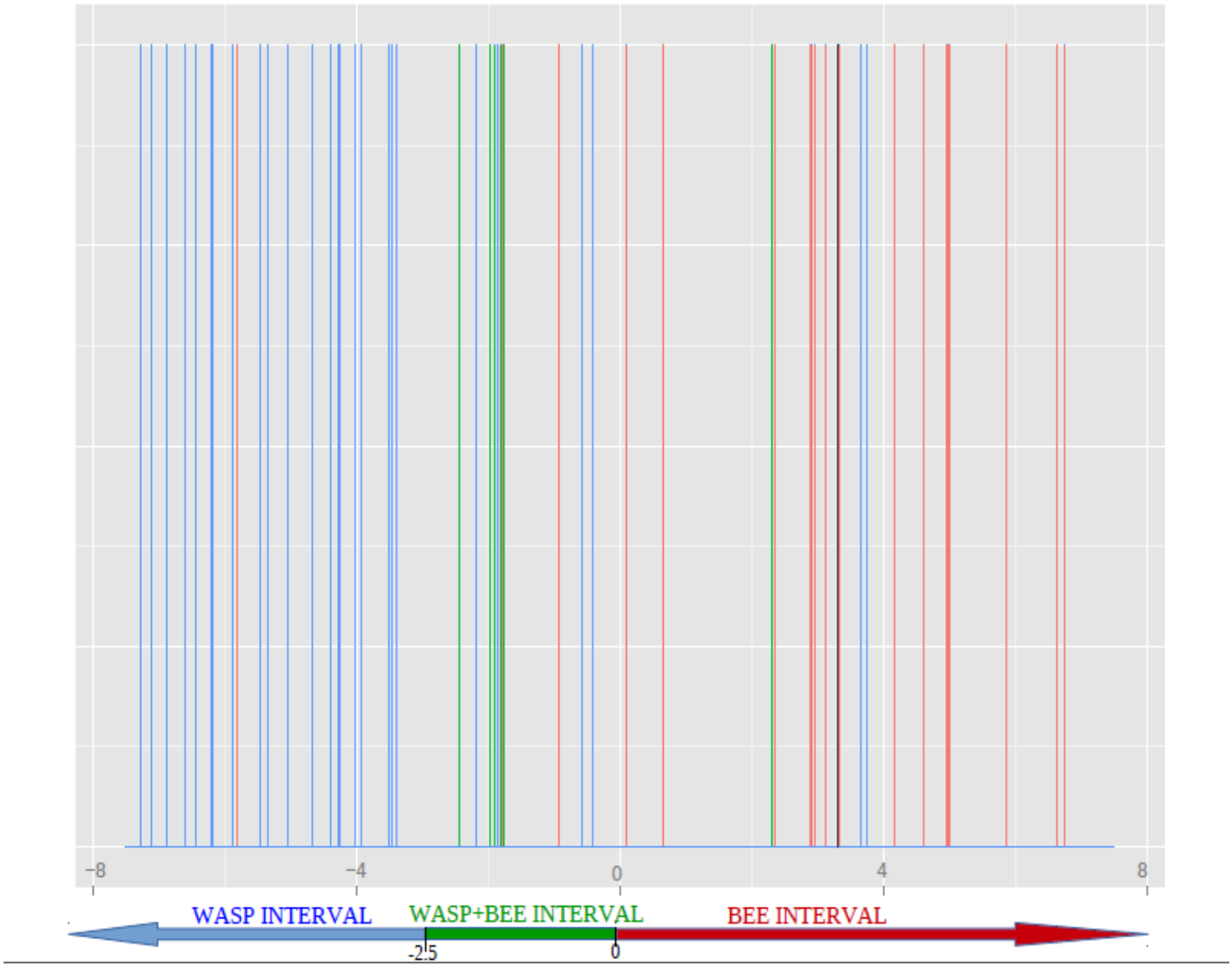

Figure 6: Histogram of SSMA+BAT algorithm values in a venom-IgE+/+ subsample, with arrows showing the cut-offs of each interval. Wasp allergic patients are coloured blue, bee allergic patients are coloured red and bee and was allergic patients are green. Almost all the patients allergic to bees and wasps and all the colour mixing is clustered in the middle.

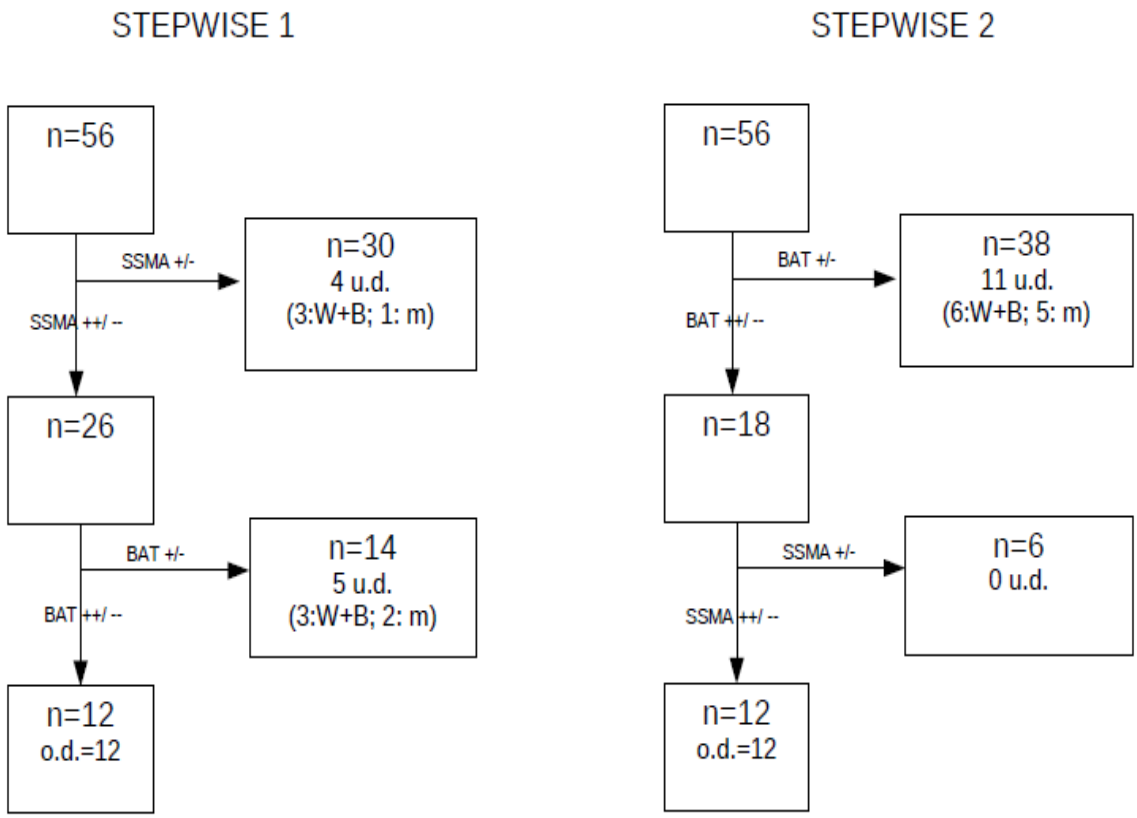

Figure 7: Stepwise diagnostic approaches; W - wasp, B - bee, u.d. = under diagnosed, o.d. – over diagnosed.

The rate of correct diagnosis in the venom-IgE+/+ group, when using only venom-IgE measurements was 6/56 (10.7 %). All other patients (50/56, 89.3 %) were over-diagnosed. The best-performing diagnostic approach in terms of correct diagnosis was the BAT+SSMA-algorithm. It correctly diagnosed 46/56 (82.1 %) patients, which was significantly more than all of the other diagnostic approaches using the simple cut-offs of diagnostic tests (SSMA-only [29/56 {51.8 %}; p=0.002]; BAT-only [27/56 {48.2 %}; p < 0.001]; stepwise 2 [33/56 {58.9 %}; p = 0.01]; stepwise 1 [35/56 {62.5 %}; p=0.03]). It likewise performed better than the other two single test algorithms (BAT-algorithm [39/56 {69.6 %}] and SSMA-algorithm [41/56 {73.2 %}]), but this improved performance was not statistically significant. It is also worth noting the significantly better performance of the single test algorithms compared to the single tests (41/56 [73.2 %] vs. 29/56 [51.8%]; p=0.03 for SSMA-algorithm vs. SSMA-only approach and 39/56 [69.6 %] vs. 27/56 [48.2%]; p=0.03 for BAT-algorithm vs. BAT-only approach).

The best performing of the diagnostic approaches in terms of over-diagnosis was also the SSMA+BAT- algorithm. It over-diagnosed 6/56 [10.7 %], which was approximately 2 times better than the BAT-algorithm, stepwise 1, stepwise 2 (all over-diagnosing 12/56 [21.4 %]) and SSMA-algorithm (10/56 [17.9 %]), but these differences were not statistically significant. It performed significantly better when compared to both of the single-test simple cut-off diagnostic approaches (SSMA-only [21/56 {37.5 %}; p=0.002] and BAT-only [16/56 {28.6 %}, p = 0.03]). Again, it is worth noting the significantly improved performance of over-diagnosed patients when comparing the SSMA- algorithm to the SSMA-only diagnostic approach (10/56 [17.9 %] vs. 21/56 [37.5 %]; p=0.03).

In terms of under-diagnosis, all of the algorithm-based approaches performed equally because they were calibrated to under-diagnose less than 10 % of patients (4/56 [7.1 %] for SSMA+BAT algorithm and 5/56 [8.9 %] for SSMA-algorithm and BAT-algorithm). The BAT-only diagnostic approach under-diagnosed 13/56 (23.3 %) patients, the SSMA-only under-diagnosed 6/56 (10.7 %), the stepwise-1 under-diagnosed 9/56 [16.1 %] and the stepwise-2 under-diagnosed 11/56 [19.6 %] patients. Again, it is important to note the almost significant performance improvement of the BAT algorithm compared to the BAT-only approach (5/56 [8.9 %] vs. 13/56 [23.2 %]; p = 0.07).

Venom-IgE -/- group: With the use of the BAT, we were able to show sensitization in all 4 venom-IgE -/- patients (in 3 patients, the BAT was concordant with the culprit history, and in 1 patient the BAT was double positive). With SSMA-Ab measurements, we were able to show sensitization in only 1 patient out of 4.

Validation of algorithm values

The ROC curves of single tests and single test algorithms are shown in Figure 8 and Figure 9. It can be observed that all single test algorithms outperformed single tests in diagnosing bee/wasp allergy (they have higher AUCs). This better performance was, except in the case of comparing rVes v 5 ROC AUC to the SSMA-algorithm ROC AUC (0.9 vs. 0.94 [p=0.3]), significant or borderline significant (BAT 1 % bee venom [0.82, p < 0.001], BAT 0.1 % bee venom [0.91, p = 0.09], bee BAT AUC [0.89, p= 0.03], compared to BAT-algorithm in diagnosing bee allergy [0.94] or BAT 1 % wasp venom [0.83, p = 0.1], BAT 0.1 % wasp venom [0.78; p = 0.005], wasp BAT AUC [0.8; p= 0.02] compared to BAT-algorithm in diagnosing wasp allergy [0.93] or rApi m 1 compared to SSMA-algorithm in diagnosing bee allergy [0.85 vs. 0.93; p=0.005]).

When we compared the algorithm-based approaches to each other, the best performing was always the BAT+SSMA algorithm, but this improved performance was not significant.

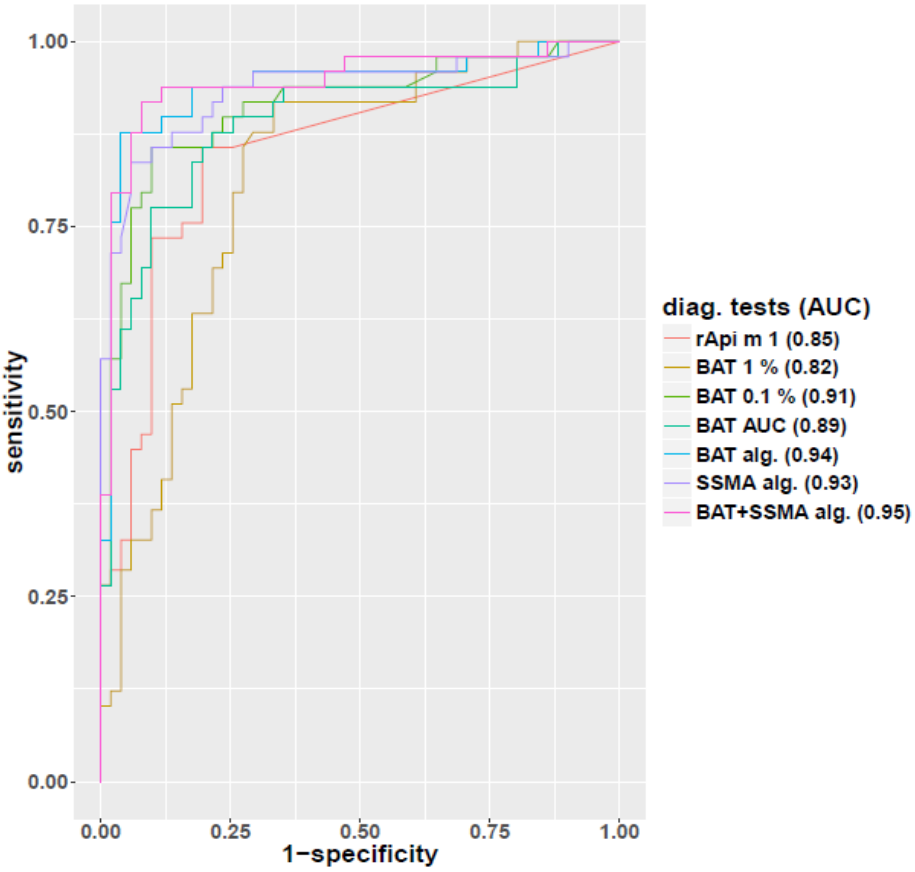

Figure 8: ROC curve analysis of different diagnostic approaches for diagnosing bee allergy; the AUC of each diagnostic approach is written beside the name of that diagnostic approach.

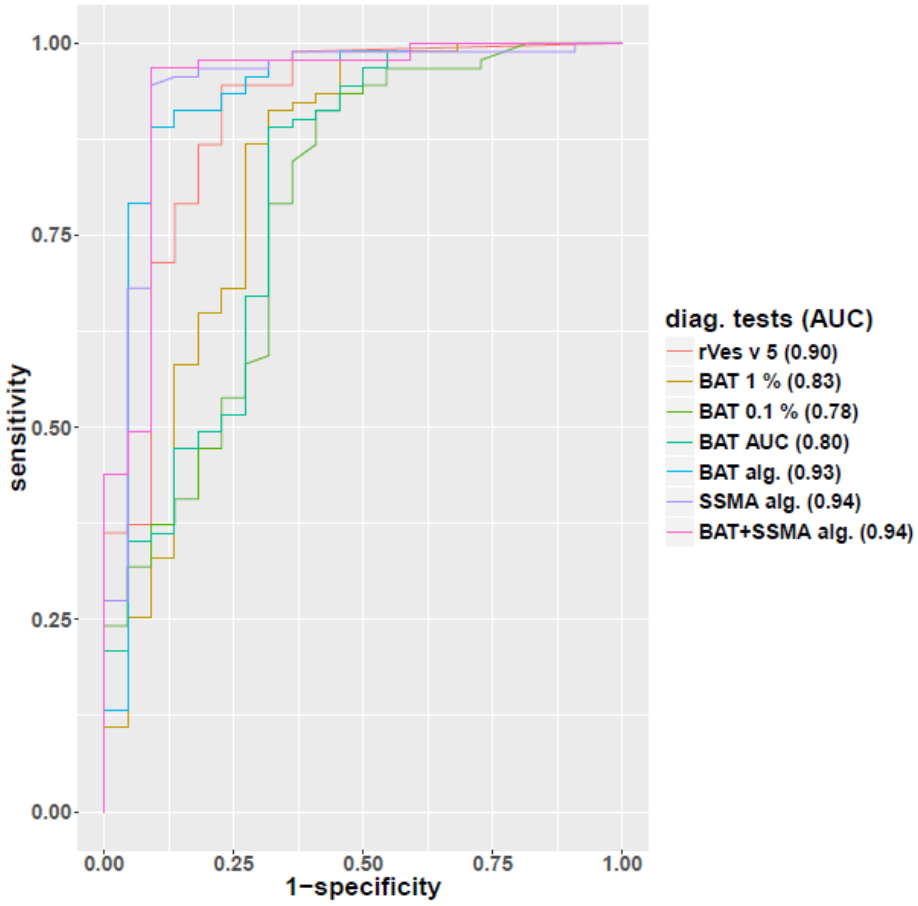

Figure 9: ROC curve analysis of different diagnostic approaches for diagnosing wasp allergy; the AUC of each diagnostic approach is written beside the name of that diagnostic approach.

Predictors of the severity of the allergic reaction after a sting in a Hymenoptera venom allergic patient

Patients

Twenty-eight patients were bee allergic and 79 were wasp allergic. Seventeen patients had a history of LLR, 11 had a Mueller grade I reaction, 16 had a Mueller grade II, 25 had a Mueller grade III and 38 had a Mueller grade IV SR.

Severity discriminating factors in single positive patients

The most significant between-group differences were observed in the recombinant IgE levels and the BAT-AUC (Figure 10). When comparing the values of the recombinant IgEs, the

patients with Mueller I, II, III, and IV reaction severities had significantly higher values than patients with LLRs. However, there were no differences between different Mueller groups. Similarly, the BAT-AUC was significantly higher in patients with Mueller II, III and IV reaction severities when compared to those of the patients with LLRs. Again, the values of the BAT-AUC did not differ significantly between different Mueller groups.

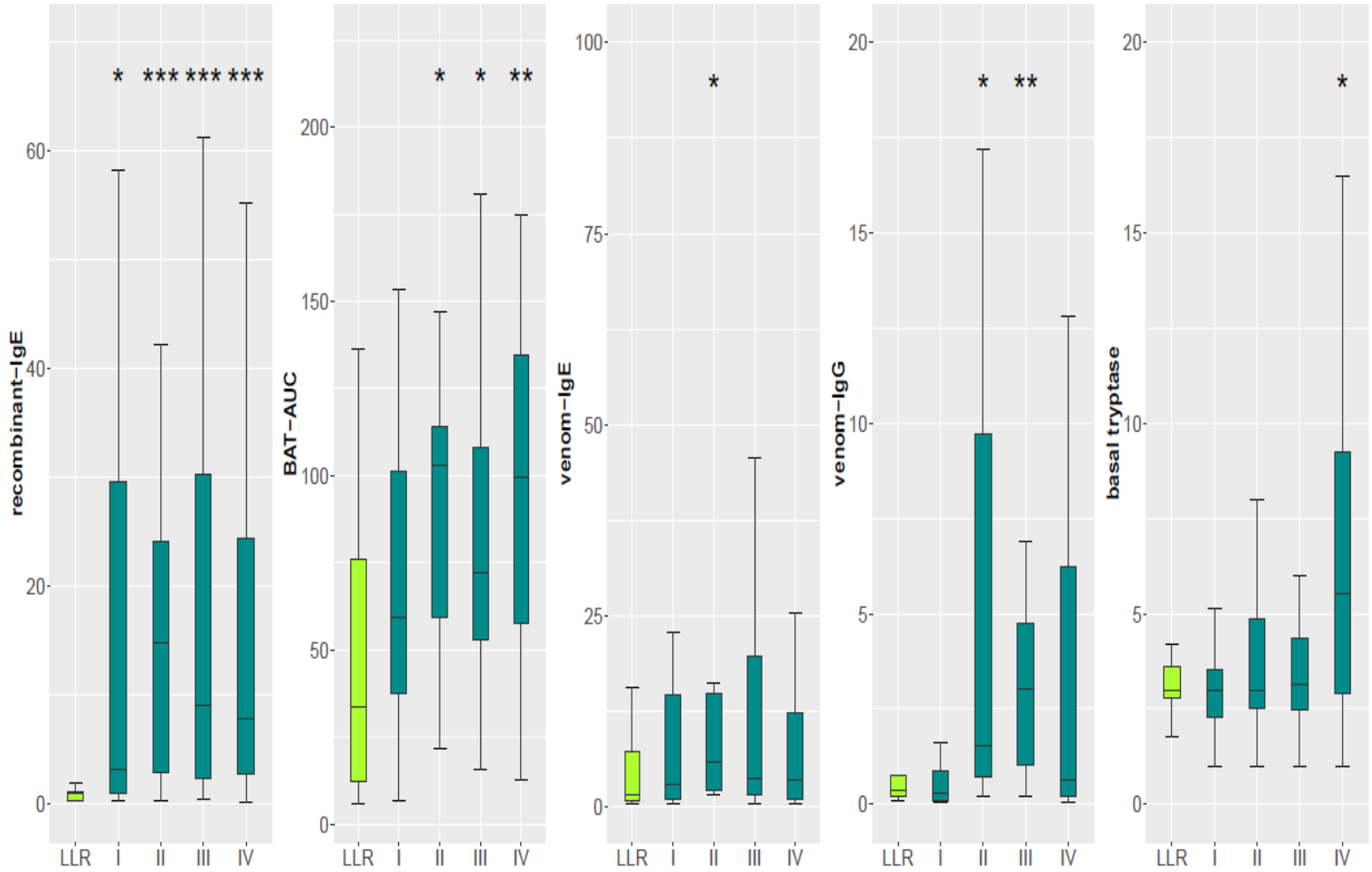

Figure 10: The association between different immunological factors and allergic reaction groups; * 0.05 > p ≥ 0.01; ** 0.01 > p ≥ 0.001; *** p < 0.001 (stars [*] are indicating the statistically significant difference between the group of interest and the LLR-group [except in the case of the basal tryptase, where they represent the difference between the group Mueller IV and all other groups]).

Levels of venom specific IgEs were higher only in the Mueller II group when compared to the patients with LLRs; the IgG levels were substantially higher in the Mueller II and III groups when compared to the Mueller I group and to patients with LLRs. The basal tryptase levels

were higher in the Mueller IV group when compared to all other groups. There were no other statistically significant differences.

Likewise there were no statistically significant differences between different allergic reaction levels in the anti – FcRI response and also in the patient basal (PB) response (Figure 11).

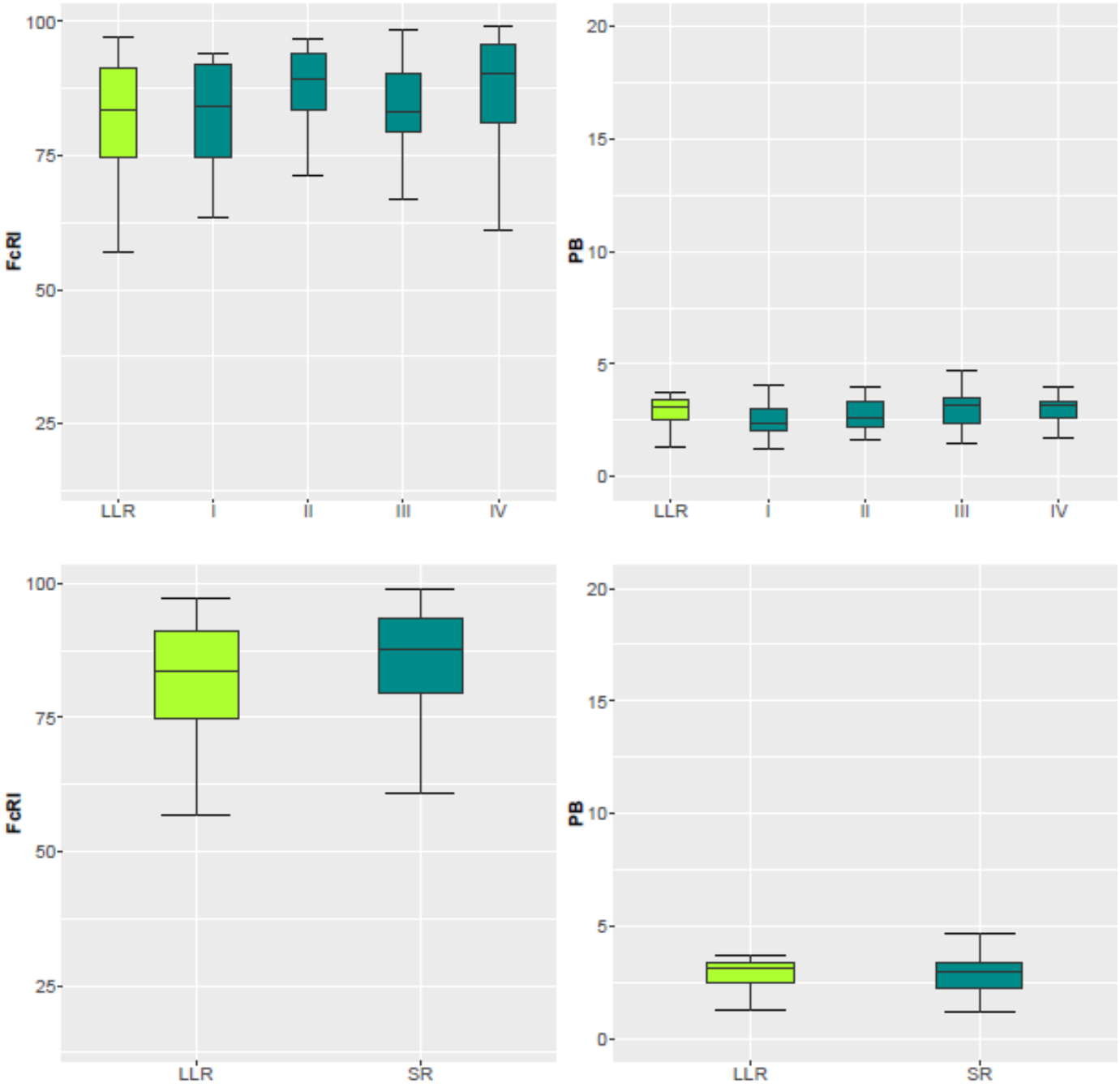

Figure 11: The anti FcRI and the PB response with respect to different allergic reaction groups.

The BAT-AUC and basal tryptase levels were significantly correlated (r = 0.3; p = 0.002 and r = 0.17; p = 0.04) with the numerically transformed levels of allergic reaction. There were no other statistically significant correlations (Figure 12).

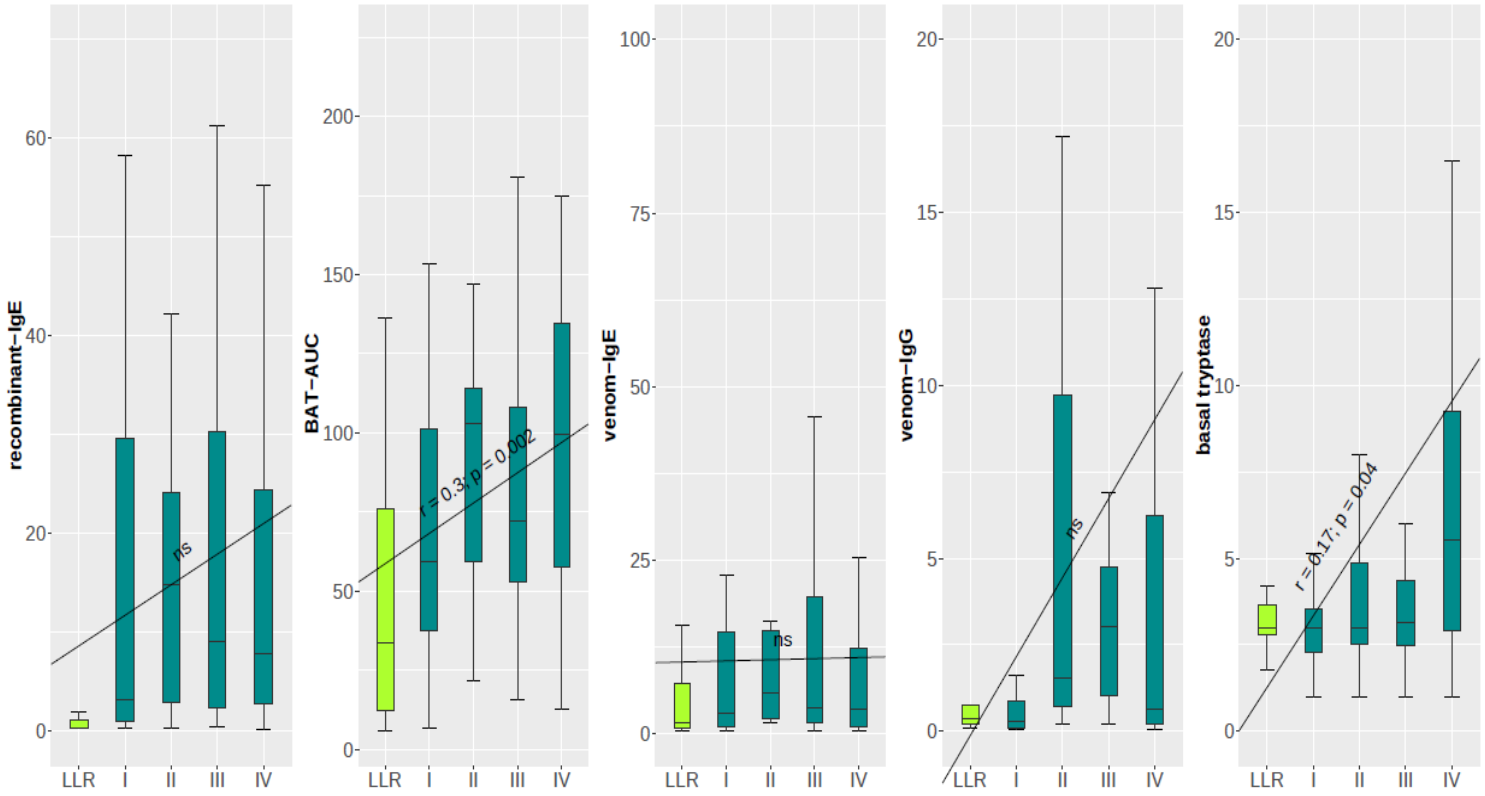

Figure 12: Correlation of the results of diagnostic tests to the numerically transformed levels of allergic reaction.

When all SR patients across all Mueller reaction grades were combined into a single group, only the recombinant IgEs and BAT-AUC varied significantly between this group and the patients with LLRs (Figure 13).

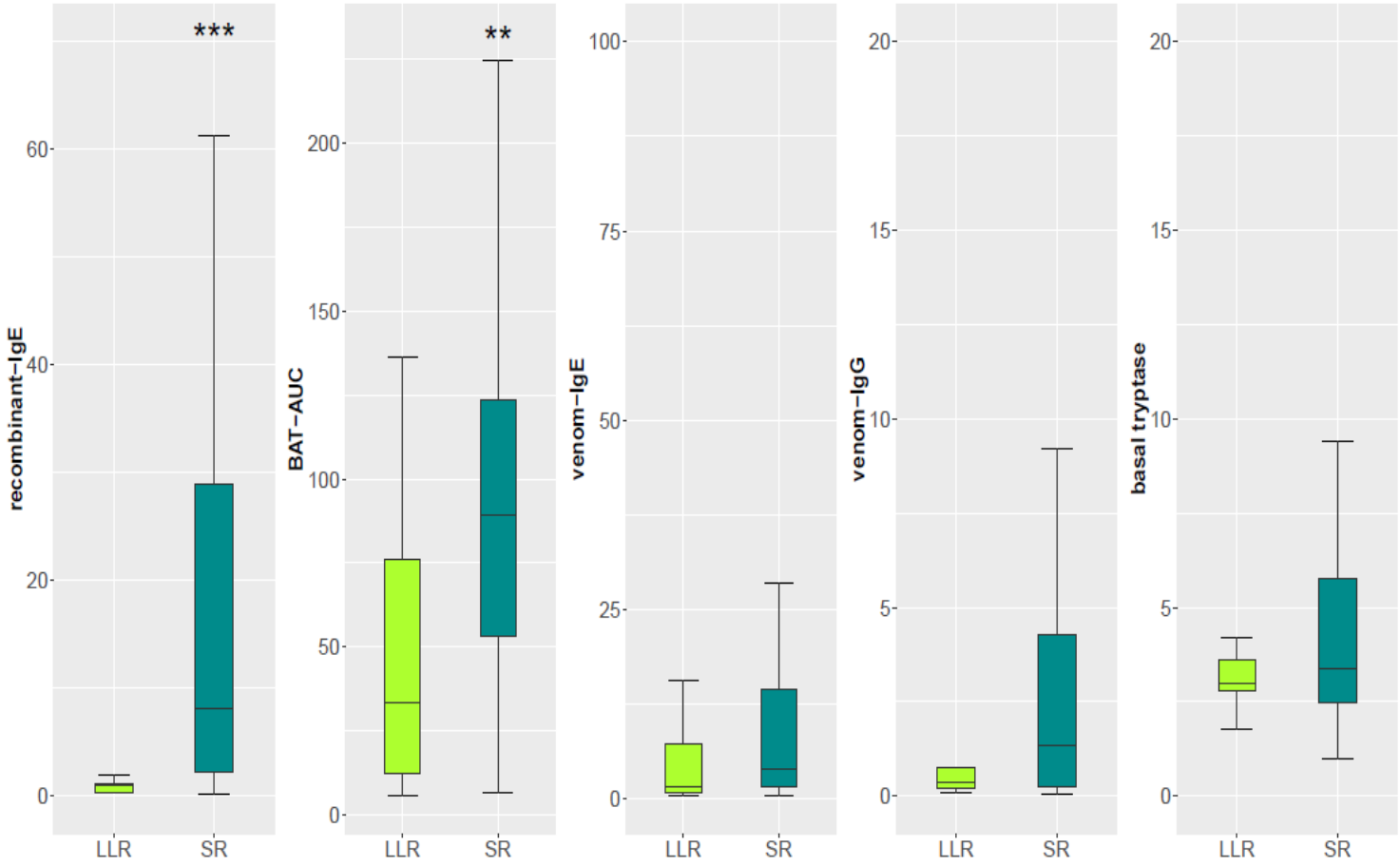

Figure 13: Differences between the groups of LLRs and SRs (all Mueller groups joined together; ** 0.01 > p ≥ 0.001; *** p < 0.001) in different immunological factors.

The results of the 2 penalized log-regression models are presented in Table 6. In the first model (LLRs vs. all the others) the recombinant IgEs and the BAT-AUC were the only independent statistically significant predictors of SRs, however in the second model (LLRs and Mueller I vs. all the others) none of the factors was able to predict the severity of the reaction with statistical significance.

	OR	Lower 95% CI for OR	Upper 95% CI for OR	p - value
LLR vs. SR				
BAT AUC	**1.019**	**1.004**	**1.037**	**0.01**
Recomb. IgEs	**1.495**	**1.183**	**2.314**	**<0.001**
FcRI	1.001	0.970	1.030	0.92
PB	0.924	0.406	2.033	0.84
Venom IgE	0.970	0.934	1.013	0.13
Venom IgG	0.999	0.988	1.024	0.83
Tryptase	1.002	0.979	1.235	0.87
Age	1.040	0.997	1.091	0.65
Sex (female)	0.319	0.072	1.182	0.09
LLR and Mueller I vs. Mueller II, Mueller III and Mueller IV				
BAT AUC	1.025	0.997	1.028	0.13
Recomb. IgEs	1.023	0.998	1.052	0.07
FcRI	1.028	0.999	1.046	0.06
PB	1.358	0.771	2.434	0.29
Venom IgE	0.994	0.965	1.028	0.67
Venom IgG	1.004	0.994	2.003	0.49
Tryptase	1.008	0.983	1.723	0.64
Age	1.013	0.985	1.042	0.37
Sex (female)	0.914	0.368	2.271	0.84

Table 6: Results of 2 penalized logistic regression models; the upper model is LLR vs. SR and in the lower model LLR and Mueller I vs. Mueller II, III and IV.

Skin prick tests – discrimination power

Patients

Three hundred and thirty five patients were included in the study. One hundred and twenty eight (38.2 %) patients were stung by bees, 125 (37.3 %) by wasps, 71 (21.2 %) by hornets, 4 (1.2 %) by wasps and hornets and 7 (2.1 %) were stung by wasps and bees according to history.

Determination of skin prick test discrimination power and calculation of CI

The distribution of positivity/negativity of SPT according to culprit history is presented in Table 7.

SPT/Culprit	Bee	Wasp	Hornet	Wasp and bee	Wasp and hornet
Double negative	50 (14.9 %)	51 (15.2 %)	21 (6.2 %)	4 (1.2 %)	1 (0.3 %)
Double positive	22 (6.6 %)	23 (6.9 %)	17 (5.1 %)	**2 (0.6 %)**	0 (0 %)
Single positive bee	**52 (15.5 %)**	4 (1.2 %)	4 (1.2 %)	0 (0 %)	0 (0 %)
Single positive wasp	4 (1.2 %)	**47 (14.0 %)**	**29 (8.7 %)**	1 (0.3 %)	**3 (0.9 %)**

Table 7: Distribution of SPT according to culprit history in absolute and in relative numbers. Bolded are table entries where SPT results and history results were concordant.

One hundred and thirty three patients (39.7 %) had SPT diagnosis in accordance with history (results that are bolded in Table 7). In 127 patients (37.9 %) the results of SPT were double negative, despite a convincing history of an SR after a sting from at least one of the Hymenoptera species insect. In 64 (19.1 %) patients, the results of SPT were double positive, despite the fact that the majority (62 [96.9 %]) of patients had single positive culprit history.

In 144 (43.0 %) patients the results of SPT were single positive either to wasps or to honey bees. In majority (131 [91.0 %]) of patients the result of single positive result was in accordance with their sting history, however in 13 (9 %) patients the single positive SPT was discordant with culprit history.

Since 7 patients were allergic to honey bee and wasp stings, they were excluded and final analysis of the discrimination power of SPTs was done on 328 (97.9 %) patients. Out of 328

patients SPTs were discriminative (either single positive for wasps or single positive for honey-bees) in 143 (43.6 %) and indiscriminative (either double positive or double negative) in 185 (56.4 %) patients. Indiscrimination was mainly due to double negative results of SPTs, since the double negative results represented 123 (66.5 %) of all indiscriminative cases.

Therefore the combined discrimination power of SPT was 143/328 (43.6 %) with binominal confidence interval for this sample proportion being between 38.2 % and 49.2 %.

Discussion

PhD work was composed out of four studies whose main objective was to improve diagnostic process of a Hymenoptera venom allergic individual.

Assay comparison study

In the assay comparison study, we showed that the measurements of IgE antibodies against rVes v 5 venom allergen with LITE assay have a significantly higher sensitivity for diagnosing wasp venom allergy than the measurements of the same antibodies with the CAP assay, while the specificity of both assays are comparable. Moreover, the combination of rVes v 5 with LITE assay and rVes v 1 with CAP assay almost reached (97 %) the sensitivity of native venom preparations.

The use of rApi m 1 and rVes v 5 was an advance in the diagnosis of Hymenoptera venom hypersensitivity because the preparations are devoid of CCD epitopes, and therefore the double positivity observed due to the response of IgE antibodies against those epitopes in native Hymenoptera venom preparations was resolved, thereby improving the diagnostic process. However, the question of insufficient sensitivity of the preparations arose because the sensitization pattern with respect to various venom components differs between Hymenoptera-allergic patients. The problem of inadequate sensitivity of SSMA-Abs is particularly concerning in honeybee venom allergy, since it was shown (Korošec et al. 2011) that as few as 57 % of patients who had an allergic reaction after a bee sting and positive IgE measurements against native bee venom also had positive measurements of IgE antibodies against rApi m 1. Because of the inadequate sensitivity of recombinant SSMA-Abs, other recombinant allergens (rApi m 2 and rVes v 1) were added to the standard battery of recombinant allergens for use in routine clinical practice.

Since the difference in the diagnostic sensitivity between the two routinely used assays (ImmunoCAP and Immulite 2000) with native venom extracts has shown (Watanabe et al. 2012), that the diagnostic sensitivities of the native extracts might differ between the two assays, we sought to evaluate the diagnostic utility of rVes v 5 allergen with both routinely used systems.

The sensitivities of IgE antibodies against rVes v 5 with CAP and LITE were calculated first. The sensitivity was significantly improved (93 %; $p = 0.026$) with LITE compared to the CAP technique. Surprisingly, the combined sensitivity of two routinely produced reagents (rVes v 5

and rVes v 1) for ImmunoCAP was measured and calculated, and it did not reach the sensitivity of only rVes v5 with LITE (90 % vs. 93 %). However this difference was not statistically significant (p=0.63).

The observation for the rVes v 5 were similar to the observations for rApi m 1 in a study conducted by our (Šelb et al. 2016) and also by an Austrian group (Schrautzer et al. 2016) that showed that the sensitivity of rApi m 1 with CAP was lower than with the LITE technique and low per se. This was also shown by other studies (Hofmann et al. 2011; Korošec et al. 2011; Sturm et al. 2011) that demonstrated that the detection of IgE against rApi m 1 with CAP is a valuable but not sufficient diagnostic tool in diagnosing Hymenoptera venom allergy. In a study conducted by our group (Šelb et al. 2016), the LITE rApi m 1 allowed unambiguous identification of all (100 %) honeybee-allergic patients in an anaphylactic reaction subsample in comparison to the sensitivity of 83 % with the CAP rApi m 1 allergen (p = 0.009). These high sensitivities of the LITE technique are comparable with previous in vivo skin tests (U R Müller et al. 1995) or in vitro evaluation of rApi m 1 IgE reactivity either with custom IgE immunoblotting and/or ELISA (Mittermann et al. 2010; Müller et al. 1997) or the discontinued ADVIA system (Siemens AG). Interestingly, the inconsistency in diagnostic sensitivity started with the reporting of rApi m 1 ImmunoCAP IgE testing (Hofmann et al. 2011; Köhler et al. 2014; Korošec et al. 2011; Sturm et al. 2011) and currently there is no clear explanation for such a discrepancy.

Importantly, the specificities of both assays (LITE and CAP) for rVes v 5 were high (from 96 to 98 %), what suggests that better sensitivity of the LITE system, was not due to non-specific bounding of IgE antibodies.

The correlation of the results of measurements of rVes v 5 using LITE and CAP was also calculated. As the data were not normally distributed, Spearman's correlation coefficient was used. The correlation coefficient was, as expected, statistically significant ($p < 0.001$) very high and positive (rho = 0.98). We also fitted a linear regression line and calculated R^2 for measurements of the rVes v 5 with CAP and LITE technique, using one as the predictor and the other as the outcome variable (CAP measurements as the predictor and LITE measurements as the outcome variable) on a subsample of patients who had positive values for the test using both techniques. The R^2 was around 0.85 thus concluding that 15% of variability of measurements with LITE technique can't be explained by the variability of measurements with CAP technique. It is also important to note the coefficient of linear regression line which was 2.3, indicating that the results of rVes v 5 LITE in an average patient were 2.3-times higher

than the results of that patient's rVes v 5 CAP measurements. These observations and the increased sensitivity of LITE may be due to differences in the detection (calibration/interpolation algorithm), differences in the measurement technique used or differences in allergen preparations used by both systems to bind available IgE antibodies.

For ImmunoCAP preparations, the manufacturer states that they are a CCD-free component, but it does not reveal in which expression system the preparations are produced. For ImmuLite preparations, the manufacturer states that they are cloned and expressed using Sf9 insect cells infected by a recombinant baculovirus.

Because the production system of the ImmunoCAP preparations is not known, one can speculate that the difference between the sensitivities and also the higher measurements in the LITE system might be related to differences in the activity of recombinant venom allergens (Soldatova et al. 1998), which can be the result of their production either in bacterial expression systems as non-glycosylated allergens or in different eukaryotic systems as glycosylated allergens (Seismann et al. 2010; Soldatova et al. 1998). Nevertheless, we are more prone to the idea that there are differences in the interpolation calibration algorithm between the two assays since the higher values were apparent also in controls. The on average higher measurement values of the LITE system were even more apparent at lower concentrations of IgE antibodies. In patients whose CAP measurements were between 0.1 and 0.35 kU/L, on average, the value of rVes v 5 measured with LITE was 3.6 times higher than the value of rVes v 5 measured with CAP.

Since just 1 control had values of rVes V5 CAP higher than 0.1, we were not able to draw the receiver operator characteristic curves to choose the best cut-off value, and also to compare the values of areas under the curve for those ROC curves.

Because the LITE system measures on average higher values, the trend of which was also apparent in controls, the calibration interpolation differences between the two assays can be the source of the discrepancy. But nevertheless, since the cut-off is set as it is, by the manufacturers of the assays, the specificities of both assays are comparable, but the sensitivity of the CAP is significantly lower.

The combined use of the BAT and measurements of IgEs against SSMA to diagnose Hymenoptera venom allergic patients

In the combined use of novel diagnostic tests study we showed that the BAT and SSMA ratio based diagnostic approach has superior culprit discriminating capabilities compared to the established approaches that use simple cut-offs of diagnostic tests.

The study included 177 consecutive Hymenoptera venom allergic patients, of which 133, that had unequivocal culprit history, were divided to 3 groups according to their venom-IgE status. The division based on venom-IgE status was done, because native venom double positive and double negative patients represent the diagnostically most challenging groups.

Native venom single positive group (venom-IgE +/-)

Here, correlation between the results of venom-IgE measurements and the sting history was excellent (70/73; [96%]). On the one hand, this shows the robustness of history acquisition of the included patients and, on the other hand, the robustness of single positive venom-IgE results. The agreement of different diagnostic tests with the venom-IgE measurements was also high, approximately 90% for both the BAT and the SSMA-Ab measurements. In cases of test measurement discordance, the results of the discordant test were mostly double positive, and the results of the other available tests were usually concordant with the venom-IgE single positive measurements and also with the history.

It seems that single positivity of IgE antibodies to whole venom extracts is a very reliable indicator of the culprit, and that those results need no additional testing. This is not a novel observation because other studies have also shown venom-IgE single positivity to be a strong indicator of the culprit insect (Ebo et al. 2007; Hofmann et al. 2011). The same has been shown for intradermal skin tests (Ebo et al. 2007). These results clearly suggest that a diagnostic process should start with measurements of venom-IgE antibodies or intra dermal skin tests (Ebo et al. 2007), and if a result is single positive, then the diagnostic process should be finished. Conducting a venom-IgE test in the first step of a diagnostic process is sufficient to diagnose approximately 60% of patients.

Native venom double positive group (venom-IgE +/+)

Indications of stepwise approaches have already been shown in previous studies (Eberlein et al. 2012; Sturm et al. 2011) in which the authors demonstrated that some of the venom-IgE double

positive cases could be resolved with a single venom positivity in at least one of the additional tests (BAT or SSMA-antibodies), but none of the studies evaluated their results in terms of correct, over- and under-diagnosis. In the current study, we used two different stepwise approaches with whom we correctly diagnosed around 60 % of patients. The stepwise approaches over-diagnosed only approximately 20 % of patients, demonstrating that a stepwise approach that includes IgE to SSMA and the BAT is a major advance in increasing the diagnostic specificity of Hymenoptera allergy testing.

Likewise, the rate of under-diagnosis with the stepwise approaches, was also around 20 %, that being mainly due to patients with a double positive insect sting history, all of whom had at least one test pair show a single positive result. All of those patients were sufficiently recognized solely with the venom-IgE-only approach, which, conversely, recognized just 1 true double positive patient at the expense of recognizing 8 false double positive patients, and, as such, the bias was simply too high.

The performance of the ratio algorithm based diagnostic approaches was even better than the performance of the stepwise approaches. The motivation for constructing ratio based algorithms has been described in a study conducted by Eberlein et al (Eberlein et al. 2012), which showed that calculating a ratio of C50 for bees and C50 for wasps for BAT measurements, with a grey zone of ratio results in the middle (0.1 and 10) indicating both insects, is superior in culprit discrimination to the use of BAT measurements with un-transformed (standard) cut-offs. It has recently been shown that distinct parameters of the basophil CD63 activation test reflect the severity and threshold of allergic reactions to peanuts (Santos et al. 2015) and that basophil allergen reactivity is a major factor associated with severe adverse systemic reactions during the build-up phase of honeybee VIT, with BAT AUC being the best (of all tested) indicator of that reactivity (P. Korošec et al. 2015). These 2 studies indicate that a higher BAT reactivity is a predictor of a more serious clinical outcome and support the idea that the BAT measurements should be interpreted as a ratio to better discriminate between the 2 culprits because a ratio abolishes all of the inter-individual variation that cannot be incorporated into simple cut-offs (i.e., a high overall response in BAT to different allergens in a particular individual may render that individual's BAT result positive to wasp and bee, even if the individual is allergic just to 1 insect [a ratio based approach would eliminate that shortcoming of a standard cut-off based approach]).

The study by Eberlin et al. revealed yet another flaw in Hymenoptera venom allergy diagnosis that this work tries to overcome, and that is a flaw of standard cut-off result interpretation for

component resolved diagnosis (CRD). In that study it was shown that all but one patients that had double positive results of IgEs to native venoms had also double positive results with at least one of the extended spectrum of recombinant antigens of both insects (Eberlein et al. 2012). So CRD did not resolve anything in those patients, in terms of clinically apparent allergy vs. sensitization (one of the big expectations in time of recombinant venom introduction was, that, since they lack CCDs and thus represent only the disease relevant part of the IgE response they would be able to differ between sensitization and clinically apparent allergy). The body of evidence supporting the inability of simple cut-off based interpretation of the recombinant IgEs to distinguish between sensitization and clinically apparent allergy, has been growing since the publication by Eberlin et al. It was also shown by Sturm et al (Sturm et al. 2014) that 76 % of patients who were sensitized to native wasp venom and had a negative result of a wasp sting challenge, had positive measurements of IgE antibodies against rVes v 5 and/or rVes v 1. In the same study the authors demonstrated that the proportion of patients with positive results of IgE antibodies to rApi m 1 and a negative honey-bee sting challenge was only 16.2 % (Sturm et al. 2014). However authors argued that this was probably due to inadequate sensitivity of rApi m 1 on ImmunoCAP assay, an observation confirmed also by other studies (Korošec et al. 2011; Schrautzer et al. 2016; Šelb et al. 2016), not to its superior specificity. Moreover the authors used only rApi m 1, and none of the other recombinant antigens of honey-bee venom. However, in a study by Kohler et al (Köhler et al. 2014), they measured an extended spectrum of recombinant honey-bee allergens (rApi m 1, rApi m 2, rApi m 3, nApi m 4, rApi m 5 and rApi m 10), and showed that in 6 bee non-allergic controls that had positive IgE antibodies to native honey bee venom, 3 showed positivity to rApi m 1, 2 to rApi m 5 and 1 to rApi m 10. Furthermore in a study by Frick et al. a group of 126 patients with positive IgE to native wasp and bee venom without clear sting history, 75 % (94/126) were double positive with at least one of the extended spectrum of recombinant allergens from both families (Frick et al. 2015). Aditionnaly, Michel et al. demonstrated that 75-90 % of double sensitized patients (depending on the cut-off used) were, again double positive with at least one of the extended spectrum of recombinant allergens for both insect families (Michel et al. 2016). In light of the above mentioned studies, standard cut-offs with CRD do not suffice in native venom double sensitized patients, since the majority of patients that are sensitized to native venoms are also sensitized to at least one of the extended spectrum of recombinant components of the native venom that they are sensitized to.

That is why we used the ratio based result interpretation approach, and took it one step further than the study by Eberlin et al. did, as we did a ratio based interpretation of the recombinant IgE antibodies and also multiplied the ratios by each other (when constructing the BAT+SSMA algorithm), according to the rationale that if two or more test pair ratios point to the same culprit, then the combined result reinforces the indication of that culprit. However, if the test pair result ratios are discordant or weakly indicate one culprit, then the combined result is pushed towards the grey zone and thus a double diagnosis. Three different algorithms were constructed (BAT-algorithm, SSMA- algorithm and BAT+SSMA algorithm); the best performing was the BAT+SSMA algorithm, which correctly diagnosed 46/56 [82.1 %] patients. The performances of the other 2 algorithms (SSMA-algorithm and BAT-algorithm) were also good and roughly equal. It is necessary to note that the proportion of correct diagnoses was significantly higher with all of the algorithm-based approaches compared to the diagnostic approaches that used equal amounts of diagnostic tests with standard cut-offs (i.e., SSMA-only vs. SSMA- algorithm, stepwise 1 vs. SSMA+BAT algorithm, etc).

Native venom double negative group (venom-IgE -/-)

In the venom-IgE -/- group the goal was to confirm sensitization. We were able to confirm sensitization with the BAT in all 4 patients, but with the IgE to SSMA measurements we could confirm sensitization in only 1/4 patients.

It seems that the BAT is essential in venom-IgE -/- patients, as has been shown previously (Korosec et al. 2009) and if it is not routinely available, the patient has to be appointed to a specialized centre to perform the BAT measurements.

Algorithm verification

The verification of the algorithm results was performed by comparing the AUCs of ROC curves from different diagnostic approaches. Because the cut-offs for the algorithm-based approaches were calibrated on our (venom-IgE +/+) group of patients, and because we compared the algorithm-based approaches which had cut-offs calibrated on our sample to the stepwise approaches (that used the tests with already established cut-offs), possible interpretation of the improvement in performance of the algorithm-based approaches versus the stepwise diagnostic approaches could be over-fitting. To evaluate the performance of a diagnostic test, one can use the AUC of the corresponding test ROC curve (because it represents the trade-off between

sensitivity and specificity of the test). Because the ROC curve could not be plotted for the stepwise approaches (since they use 2 diagnostic tests), we were not able to compare the AUC of the BAT+SSMA algorithm to the AUC of the stepwise approaches. Instead we compared the AUCs of the single test simple values to the AUCs of the corresponding single test algorithm values to show that the use of the results of a test as a ratio has better culprit discriminating capabilities than the use of un-transformed results. In comparing different AUCs, we were able to show that the use of test results in the form of a ratio always produced greater AUCs than the corresponding single un-transformed tests. The differences were in almost all cases significantly or borderline significantly higher. Therefore, the better performance of the BAT+SSMA algorithm was also likely not due to over-fitting the cut-offs to the sample.

Predictors of the severity of the allergic reaction after a sting in a Hymenoptera venom allergic patient.

In a predictors of the severity of the allergic reaction study, we have shown that the recombinant IgEs and the BAT-AUC are independent statistically significant predictors of an SR after a sting in a model of a penalized logistic regression analysis and that the BAT-AUC shows a monotonous, statistically significant trend of higher values, when the results of the BAT-AUC are correlated to numerically transformed levels of the reaction severity (LLR = 1, Mueller I = 2, … Mueller IV = 5).

A recent study on Hymenoptera venom allergies showed that the available diagnostic tests (recombinant IgEs, the BAT, skin tests) cannot distinguish between asymptomatic sensitization, LLRs and SRs (Sturm et al. 2014). However, the results of the tests were reported in terms of positivity or negativity, not in terms of the absolute value of the test results.

However, in peanut (Santos et al. 2014; 2015) and also in milk (Ford et al. 2013) allergy models, comparison of the absolute values of different immunological factors demonstrated that the median values of the recombinant-IgEs and the BAT were higher in patients with SRs when compared to patients who were sensitized but did not develop an SR after allergen ingestion. Furthermore, distinct BAT parameters were also shown to be independent statistically significant predictors of severe SRs (Santos et al. 2015). Therefore, we aimed to determine if this is also the case in Hymenoptera venom allergy. The results of our study were concordant with the results of previous studies examining peanut and milk allergies, in that, that the recombinant IgEs and the BAT (represented as BAT-AUC) were able to differentiate between

patients with SRs and patients with LLRs after a sting. More specifically, these two measures served as independent statistically significant predictors of an SR (higher values of each factor correlated with increased SR risk) in a penalized logistic regression analysis model. However, use of the BAT and recombinant IgE measurements was not able to discriminate between different levels of SRs, as it was demonstrated for the BAT in peanut allergy model (Santos et al. 2015). Nevertheless, when the reaction severity groups were numerically transformed (LLR = 1, Mueller I = 2, ... Mueller IV = 5) and correlated with the results of different immunological factors, the BAT-AUC were significantly positively correlated with the reaction severities while other allergen specific factors were not.

Our study showed that IgG-antibody levels were not elevated in patients with less severe reactions, suggesting that these antibodies do not play an inhibitory role in the pathogenesis of Hymenoptera allergy, unlike what had been suggested by the previous peanut allergy study (Santos et al. 2015). However, the latter result was a surprising one and has not been confirmed by other studies (Ford et al. 2013). Thus, it seems that IgGs do not play an important role in the pathogenesis of Hymenoptera venom allergy. These results are consistent with results obtained using the Hymenoptera venom immunotherapy model, where it has been shown that blocking IgG antibodies have little effect (González-de-Olano et al. 2011; Lambert et al. 2003).

Basal tryptase was not able to discriminate between LLRs and SRs. However, it was significantly higher in the Mueller IV patient group, which drove the significance of the correlation with reaction severity. Our observations were concordant with the results of a study conducted by Ruef et al, which showed that higher levels of basal tryptase were associated with an increased risk for the severe anaphylactic reactions (our Mueller IV was similar to their Ring and Messmer 3 or 4: anaphylactic shock, loss of consciousness, cardiac arrest (Ruëff et al. 2009)).

The BAT-AUC and the recombinant-IgEs were the only two independent predictors of SR evaluated in the venom-IgE single positive patients. When we did a post-hock analysis we also found out that the recombinant IgEs and the BAT-AUC were significantly higher with the SR causing venom as compared to the non-SR causing venom on the 50/56 (6 patients had double positive culprit history) venom IgE double positive patients with single positive culprit history, in the second part of the work (the combined use of the BAT and measurements of IgEs against SSMA to diagnose Hymenoptera venom allergic patients) thereby explaining the idea that the ratio based interpretation is better in culprit discrimination than the results of untransformed tests.

Skin prick tests – discrimination power

Major problems, that were encountered regarding skin prick tests for other allergy models, that is its lack of sensitivity (Heinzerling et al. 2013), and consequently also lack of discrimination power, were also encountered in our retrospective analysis of SPT performance for Hymenoptera venom allergy. The most frequent modality of test result interpretation was the double negative result, as compared to the single positive and double positive result and consequently also the discrimination power of the tests was hindered. However, because the SPTs are minimally invasive, safe (Reid et al. 1993), have limited side effects (Hug et al. 2003), are inexpensive, the results are available in 15-20 minutes after application, they can be performed and interpreted in an outpatient setting, they are an ideal solution for clear-cut cases, when a patient saw the stinging insect and a clinician needs a test just to prove IgE sensitization. Yet, when a patient did not see or recognize the stinging insect, in vitro tests interpreted in a ratio based manner should be used to indicate an appropriate venom to use in VIT.

Conclusions

All in all, in the current work we have shown that to get better diagnostic sensitivity in Hymenoptera venom allergy one should measure the levels of IgE antibodies against the recombinant venom components with the LITE assay. Despite the improved sensitivity the question inadequate specificity, in patients that have double positive results of IgE antibodies to both venoms, remains unanswered, even though one uses the recombinants and/or the BAT in the diagnostic procedure. However, the problem of inadequate specificity can be, as we have also shown in the current work, solved with the ratio based interpretation of the diagnostic test results. Furthermore, we have shown that the BAT and the recombinants are significantly higher in a group of patients with SRs as compared to the group of patients with LLRs and that the two factors are independent statistically significant predictors of an SR. This is concordant with the observation that the ratio based interpretation of the recombinants and the BAT is better in culprit discrimination than the interpretation of the untransformed test results, since a Hymenoptera venom allergic patient represents a specific case of different groups of allergic reaction levels, however these different level groups are present inside a single patient (for instance having an SR after a bee sting and an LLR/no reaction after a wasp sting). This fact is exploited by the ratio based interpretation of the test results, since the results of the tests with the venom the patient is allergic to are higher than the results of the tests with the venom the patient is not allergic to and therefore push the ratio result to the side of a more likely culprit. And because a ratio is a unit-less number, and because bee test results are always in the numerator, and wasp test results always in the denominator, one can multiply the ratio results of different tests together, and by doing so, the combined ratio result is based on more data and is therefore more precise (if both ratios [the BAT and the one of the recombinant IgEs] are concordant the combined result is pushed towards the culprit the tests are concordant of; however if the tests are discordant or are just weakly concordant of the culprit insect, the combined ratio result is pushed towards the grey zone and thus double diagnosis). Furthermore, because the ratio results are conducted inside a single person, all the interindividual variability that has to be taken into account when setting the cut-offs for the absolute values of the diagnostic tests, does not have to be considered when setting the cut-offs for the ratio based algorithms.

We have also shown that the SPTs do not have good discriminating power, i.e. the majority of their results are double negative (when compared to other modalities of test results [single

positive, double positive]), mainly because of inadequate sensitivity. However, because the tests are cheap and easy to perform, since they can be interpreted in 20 minutes after application, and do not need a specialized laboratory facility to perform the measurements of the tests, they should be used as a first step to confirm diagnosis in non-complicated, clear-cut cases of allergy, when the patient recognized the stinging insect and the test is only necessary to prove sensitization, not to establish an unknown diagnosis.

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Appendices

Appendix 1 – alphabetically ordered appliances used in doctoral dissertation

Appliance	**Manufacturer**
ImmunoCAP solid phase assay	Thermo Fisher Scientific, USA
ImmuLite liquid allergen testing system	Siemens AG, Erlangen Germany
FACS Canto	BD Biosciences, USA

Appendix 2 – alphabetically ordered reagents used in doctoral dissertation

Reagent	**Manufacturer**
fMLP	Sigma, USA
Honey bee venom	Hal Allergie, Netherlands
IL-3	Buhlmann Laboratories, Switzerland
Wasp venom	Hal Allergie, Netherlands

Appendix 3 –alphabetically ordered antibodies used in doctoral dissertation

Antibody	**Manufacturer**
Anti-CD123	BD Biosciences, USA
Anti-CD63	BD Biosciences, USA
Anti-HLA-DR	BD Biosciences, USA

Appendix 4 –alphabetically ordered allergens used in doctoral dissertation

Allergen	**Manufacturer**
Honey bee venom [i1]	Thermo Fisher Scientific, USA
Honey bee venom [i1]	Siemens AG, Erlangen Germany
rApi m 1 [a45]	Siemens AG, Erlangen Germany
rVes v 1 [i211]	Thermo Fisher Scientific, USA
rVes v 5 [a670]	Siemens AG, Erlangen Germany
rVes v 5 [i209]	Thermo Fisher Scientific, USA
Wasp venom [i3]	Siemens AG, Erlangen Germany
Wasp venom [i3]	Thermo Fisher Scientific, USA

Appendix 5 – Original article

Šelb J, Kogovšek R, Šilar M, Košnik M, Korošec P. Improved recombinant Api m 1- and Ves v 5-based IgE testing to dissect bee and yellow jacket allergy and their correlation with the severity of the sting reaction. Clin Exp Allergy. 2016;46(4):621-30.

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