

***In vitro* testing to diagnose venom allergy and monitor immunotherapy: a placebo-controlled, crossover trial**

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Summary

Background In people with a history of sting allergy, only prior reaction severity and older age are known to predict subsequent reaction risk. Furthermore, no diagnostic test other than a deliberate sting challenge has been found to identify people in whom venom immunotherapy (VIT) has been unsuccessful.

Objective We aimed to assess the utility of a number of *in vitro* tests to diagnose venom allergy and to monitor immunotherapy.

Methods During a double-blind randomized placebo-controlled crossover trial of *Myrmecia pilosula* ant VIT the following venom-specific tests were performed at enrolment, and at completion of treatment prior to a diagnostic sting challenge; leucocyte stimulation index (SI), IL-4 production, IgE RAST, histamine release test (HRT), leukotriene release test (LRT) and basophil activation test (BAT). Intradermal venom skin testing (VST) was also performed at trial entry.

Results Only VST and HRT identified those at risk of sting anaphylaxis in the placebo group. Although IgE RAST, leucocyte SI and IL-4 production, LRT and BAT all correlated well with intradermal VSTs, they did not predict sting challenge outcome. After successful VIT, venom-induced leucocyte IL-4 production tended to fall, whereas IgE RAST increased and a natural decline in HRT reactivity was reversed. A confounding seasonal affect on laboratory results was suspected.

Conclusion The HRT warrants further assessment for diagnosis of venom allergy. Uninformative performance of the commercially available LRT and BAT tests may be due to pre-incubation with IL-3. None of the tests evaluated appear to be reliable markers of successful VIT.

Keywords anaphylaxis, basophil activation test, histamine release test, IL-4, intradermal skin testing, leucocyte proliferation, leukotriene release test, serum IgE RAST, venom allergy- diagnosis, venom immunotherapy

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Introduction

Three controlled studies have proven venom immunotherapy (VIT) to be effective in preventing insect sting anaphylaxis [1–3]. Current practice is to offer VIT to people with a history of severe systemic reactions and positive intradermal venom skin tests (VSTs). However, the rational application of VIT is hindered by the fact that only 25–50% of people with positive skin tests and a history of systemic sting reactions to bees and wasps react to subsequent stings [4, 5]. This reaction rate appears to be higher (~70%) for *Myrmecia pilosula* ant sting allergy [3, 6]. Therefore, using current criteria, many people undergoing VIT do not require this treatment. Furthermore, VIT failure rates ranging from 0% to 40% have been reported [7].

No diagnostic test or historical feature apart from prior reaction severity and older age appears to distinguish those at particular risk [4, 6]; however, one small study has suggested that a negative histamine release test (HRT) may identify people who have lost their clinical sensitivity [8]. Venom-specific IgE and IgG subclass titres do not predict reactivity or successful immunotherapy [4, 9, 10], and no *in vitro* mediator release test has been rigorously assessed for its ability to monitor immunotherapy and identify patients in whom VIT has failed. Any test used to exclude people from receiving VIT, or from carrying adrenaline following VIT, would need to be close to 100% sensitive for identifying those at risk of a severe reaction.

Various basophil activation and mediator release tests are now commercially available [11], and there has been strong support for use of the basophil activation test (BAT) in allergy diagnosis [12]. However, assessments of sensitivity and specificity for venom allergy diagnosis have been limited to comparisons with clinical history and skin tests. Given that intradermal tests correlate poorly with clinical sensitivity [4],

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such studies provide little rationale for changing current practice. Furthermore, the selection of diagnostic thresholds for mediator release and basophil activation have been based on the assumption that allergy is defined by the presence of specific IgE and clinical reaction history. Asymptomatic sensitization (development of a specific IgE response without clinical allergy) and loss of clinical reactivity despite the presence of specific IgE are well-recognized phenomena that lead to poor performance of skin tests, clinical assessment and venom-specific IgE when the aim is to predict subsequent reaction risk.

Our aims were to assess the clinical utility of venom-induced leucocyte proliferation, cytokine production, basophil activation and mediator release tests for safely excluding low-risk patients from VIT and for monitoring immunotherapy. We wished to do this in a well-defined and clinically relevant group, namely, those who are currently offered VIT on the basis of a positive reaction history and demonstrable venom-specific IgE.

Methods

Trial structure

Our randomized double-blind placebo-controlled crossover trial of *M. pilosula* VIT has been described in detail elsewhere [3]. The relevant ethics committees approved the trial and fully informed written consent was obtained from each participant. Otherwise healthy adults with a history of systemic allergic reactions to *M. pilosula* were assessed for trial inclusion. To be eligible, participants also had to have a positive intradermal VST at a venom concentration of 1.0 µg/mL or less.

Participants were allocated using a random number-based sequential allocation process to receive immunotherapy with either a placebo containing histamine to imitate local injection reactions or venom extract. Immunotherapy was given according to an outpatient 'clustered' or 'semi-rush' schedule over 3 months, aiming for a maintenance dose of 100 µg. One week after the second of two maintenance doses (given a week apart), a diagnostic sting challenge was performed to determine treatment efficacy. Participants initially allocated to placebo were offered crossover to VIT after which the sting challenge was repeated.

The initial trial arms (venom vs. placebo) were treated during spring and the early part of summer. The crossover treatment phase for participants initially given placebo was commenced mid summer and continued through to winter.

Venom

To ensure a standard venom preparation was used at all times, the venom used for skin testing and all *in vitro* analyses was obtained from a single large batch prepared locally by venom sac dissection. Sacs were dissected and placed in sterile water for injection BP. After rupturing sacs with a tissue homogenizer, the venom was pooled, filtered through a 0.22-µm low protein-binding filter, lyophilized and weighed, reconstituted in water for injection, dispensed into aliquots, then stored at -20°C until required. Venom aliquots

required for laboratory use were defrosted immediately prior to use and not refrozen.

Skin testing

VSTs were performed by intradermal injection of about 0.02 mL of venom solution to form a 3–5 mm bleb. A positive result was defined by wheal growth of at least 2 mm plus a flare greater than or equal to 10 mm at 20 min. An initial concentration of 0.01 µg/mL was used, increasing to 0.1 µg/mL and then 1.0 µg/mL to determine the venom concentration threshold giving a positive result.

Blood sampling

Blood samples were taken at trial entry and prior to each sting challenge. For logistical reasons a full set of pre- and post-immunotherapy laboratory assessments could be performed at only one trial site (the Royal Hobart Hospital), where the majority of participants were enrolled, and some tests were unavailable temporarily during the trial.

RAST

Venom-specific IgE was measured by RAST [13], using venom bound to CNBr-activated discs. Results were expressed as percentages of anti-IgE tracer uptake. Serum was stored until completion of the clinical trial so that all samples from any one patient were analysed for IgE in a single laboratory assay including positive and negative serum controls.

Basophil activation tests

BATs were performed using the Basotest[®] kit (ORPEGEN Pharma, Heidelberg, Germany) according to the manufacturer's instructions. Heparinized blood was primed with a stimulation buffer containing IL-3, incubated with negative control (buffer only) or positive control (*N*-formyl-Met-Leu-Phe-fMLP) or venom for 20 min at 37°C , stained with anti-CD63-FITC and anti-IgE-PE, and analysed using a Coulter EPICS Elite ESP flow cytometer (Coulter, FL, USA). The percentage of activated basophils was calculated by first gating the IgE positive granulocytes then determining the percentage of these cells expressing CD-63, defined by a marker set at the upper 2.5% of the negative control sample.

Histamine release tests

HRTs were performed on heparinized whole blood using histamine release kits and histamine ELISA (IBL, Hamburg, Germany) according to the manufacturer's instructions. Heparinized blood was incubated with negative control (buffer only), positive control (anti-IgE) or venom (without IL-3 priming) for 60 min at 37°C , or lysed to determine whole blood histamine content. Supernatants were stored at -20°C prior to analysis for histamine by ELISA. Results were expressed as the percentage of blood histamine released.

Leukotriene release tests (LRT)

LRTs were performed using the CAST-2000[®] kit (Bühlmann laboratories, Switzerland) according to the manufacturer's

instructions. After sedimentation of erythrocytes with dextran, leucocytes were separated by centrifugation, primed with IL-3, and incubated for 40 min at 37 °C with negative control (buffer only), positive control (anti-IgE) or venom. Supernatants were stored at –20 °C prior to analysis by ELISA using a monoclonal antibody recognizing LTC₄, LTD₄ and LTE₄. Results were expressed in pg/mL.

Venom concentrations used for BAT, HRT and LRT

We used venom concentrations of 0.001, 0.01 and 0.1 µg/mL for each test (BAT, HRT and LRT), as determined by a preliminary analysis for sensitivity and non-specific activation (which was evident at higher concentrations) in allergic volunteers and non-allergic controls.

Venom-induced lymphocyte proliferation and cytokine production

Peripheral blood mononuclear cells (PMBC) were isolated from heparinized blood using a LymphoprepTM gradient (Axis-Shield, Oslo, Norway), washed with phosphate-buffered saline and resuspended in RPMI-1640 medium containing 10% human AB+serum and gentamicin, and adjusted to 2×10^6 cells/mL.

For proliferation, 0.2×10^6 cells in 200 µL was incubated for 6 days at 37 °C (in a fully humidified atmosphere of 5% CO₂ in air) with no stimulation (control-buffer only), ConA at 12.5 µg/mL (positive control), or venom at 5, 1, 0.5 and 0.1 µg/mL. Venom concentrations were chosen on the basis of a preliminary analysis for sensitivity and cytotoxicity thresholds in allergic and non-allergic controls. 1 µCi of ³H-thymidine was added to each well for the final 16 h of culture. Proliferative responses were calculated as stimulation indexes (SIs), being the average c.p.m. of triplicate antigen stimulated cultures divided by the average c.p.m. of triplicate unstimulated cultures.

For cytokine production, 2×10^6 cells in 1 mL were incubated for 6 days at 37 °C (in a fully humidified atmosphere of 5% CO₂ in air) with no stimulation (control), phorbol myristate acetate (PMA) 1 µg/mL plus ionomycin 100 µg/mL (positive control), or *M. pilosula* venom at 1 µg/mL (a concentration chosen as it was associated with the maximum response in proliferation studies). Supernatant samples were taken at day 6, and stored at –80 °C for later cytokine determinations by indirect sandwich ELISA (PharMingen). Initially we also analysed cytokine production at day 2, but after an interim analysis indicated more reliable detection at day 6 we stopped performing day 2 sampling. For PMA/ionomycin cultures, IL-4 and IFN-γ were analysed. Only IL-4 was analysed in venom culture supernatants, as IFN-γ was not found in detectable amounts either before or after VIT.

Sting challenge

The sting challenge was performed in a resuscitation room with non-invasive physiological monitoring. Using a 10 mL syringe device [14], a live ant was induced to sting for 60 s. If no reaction occurred to the initial sting, another sting from a fresh ant was delivered 30 min later. A positive sting challenge result was determined by the occurrence of objective signs of

a systemic reaction. Hypotensive reactions were defined by a systolic blood pressure <90 mmHg.

Analysis

For missing data, cases were excluded on a test-by-test basis. We defined significance at a *P*-value of <0.05.

Baseline characteristics and between-test correlations

Baseline characteristics were compared between treatment groups using the Mann–Whitney *U*- and χ^2 -tests for continuous and categorical variables, respectively. The relationships between each baseline *in vitro* test and VST sensitivity threshold (1.0, 0.1 and 0.01 µg/mL in order of increasing sensitivity) were evaluated by Spearman's rank correlation.

Predicting sting challenge reactivity in the placebo group

Analyses of *in vitro* test ability to predict the sting challenge result were confined to baseline (trial entry) studies in the placebo group, to avoid the confounding effect that VIT might have on laboratory parameters and to match the real-life requirements of a test (i.e. to select untreated patients who would most benefit from VIT). The Pearson χ^2 - and Mann–Whitney *U*-tests were used to test for relationships between sting challenge result (occurrence of any grade reaction or occurrence of a hypotensive reaction) and skin test sensitivity and *in vitro* tests, respectively. A receiver–operator characteristics (ROC) curve analysis was performed for any test showing diagnostic potential, and 95% confidence intervals calculated for sensitivity and specificity.

In vitro parameter changes during VIT

Changes in each *in vitro* parameter were assessed with the Wilcoxon signed-ranks test. Differences between placebo and venom groups were assessed with the Mann–Whitney *U*-test. Because relatively few sting challenge reactions were expected after VIT, this analysis was confined to identifying *in vitro* parameters that changed with VIT and thus might have potential for further investigation as markers of successful/unsuccessful VIT.

Study power

To exclude patients from a therapy that may effectively prevent a life-threatening reaction to subsequent stings, a safe test for venom allergy would have to be ≥98% sensitive at the chosen diagnostic cut-off (i.e. identify ≥98% of those who would react to sting challenge). A clinically useful test would have specificity in the order of 50% to enable exclusion of a significant proportion of people from needless treatment. Calculations using the computer program Power and PrecisionTM [15] indicated our study would have 83–89% power at a significance level of 0.05 to identify a clinically useful diagnostic test according to these requirements, if 30–50% of 30 people in the placebo treatment group reacted to sting challenge. This reaction rate was assumed from bee and wasp sting challenge studies, because at the time of trial planning we did not know what the reaction rate to *M. pilosula* stings would be.

Table 1. Correlation with intradermal venom skin test sensitivity for each *in vitro* test according to venom concentration used

	Venom concentration ($\mu\text{g/mL}$) of <i>in vitro</i> test						N/A
	0.001	0.01	0.1	0.5	1.0	5.0	
Specific IgE							rs = 0.44* P < 0.001
SI			rs = 0.37 P = 0.193	rs = 0.37* P = 0.004	rs = 0.40* P = 0.002	rs = 0.34* P = 0.008	
IL-4					rs = 0.38* P = 0.002		
HRT	rs = 0.07 P = 0.608	rs = 0.25 P = 0.085	rs = 0.17 P = 0.248				
BAT	rs = 0.39* P = 0.003	rs = 0.55* P < 0.001	rs = 0.46* P < 0.001				
LRT	rs = 0.31* P = 0.019	rs = 0.40* P = 0.002	rs = 0.29* P = 0.026				

rs = Spearman's correlation coefficient. *Significance < 0.05.

Results

Clinical results

Our clinical results, including a detailed analysis of baseline clinical characteristics, have been described in detail before [3]. Briefly, 68 participants were recruited; 35 were allocated to VIT and 33 to placebo. After an interim analysis the placebo arm was terminated early. Of the 29 placebo-treated participants who underwent a sting challenge, 21 (72%) had objective clinical signs of a systemic reaction and in 8 (28%) the reactions were associated with hypotension. This observed reaction rate gave 79% power at a significance level of 0.05 to identify a clinically useful diagnostic test. After 35 sting challenges in the VIT group, there was only one reaction consisting of mild self-limiting urticaria. Thirty participants initially allocated placebo crossed over to VIT; of 26 people re-challenged, one had a mild self-limiting urticarial reaction.

Baseline laboratory characteristics

All baseline laboratory parameters were compared between placebo and VIT groups to detect any randomization bias not evident in the analysis of clinical characteristics. There were no significant between-group differences (data not shown) except for the HRT at a venom concentration of 0.01 $\mu\text{g/mL}$, where the median histamine releases were 7.5% and 2.3% in the placebo and venom groups, respectively ($P = 0.029$).

Correlations between laboratory parameters and skin testing

Spearman's correlation co-efficient values for the relationship between venom-specific laboratory parameters at each venom concentration performed at trial entry and VST threshold are outlined in Table 1. As can be seen, the HRT was the only venom-specific laboratory parameter that did not correlate significantly with VST. For BAT and LRT studies, the best correlation with VST sensitivity appeared to be at a venom concentration of 0.01 $\mu\text{g/mL}$.

Predicting sting challenge reactions in the placebo group

A cross-tabulation of VST sensitivity and sting challenge reactivity in terms of both reaction occurrence and severe (hypotensive) reactivity is presented in Table 2. Participants sensitive at a venom concentration of 0.01 $\mu\text{g/mL}$ were significantly more likely to experience a hypotensive reaction. A cross-tabulation of *in vitro* results at trial entry and sting challenge reactivity is presented in Table 3. Although there were generally higher median values for many tests in those experiencing systemic reactions, these differences were not statistically significant. Only the HRT differed significantly between non-reactors and reactors, at a venom concentration of 0.1 $\mu\text{g/mL}$. Using a venom concentration of 0.01 $\mu\text{g/mL}$, HRT values in those with hypotensive reactions were significantly higher than those with no or mild reactions.

ROC curve analyses for the HRT are presented with sensitivity, specificity, and positive and negative predictive values in Fig. 1. In the clinical context of this study, a high

Table 2. Baseline venom skin test sensitivity and reactivity to sting challenge

		Total	No reaction	Objective systemic reaction	
				Non-hypotensive	Hypotensive
Skin test sensitivity, $\mu\text{g/mL}$ *	0.01	11	1 (9%)	4 (36%)	6 (55%)
	0.1	11	4 (36%)	6 (55%)	1 (9%)
	1.0	7	3 (43%)	3 (43%)	1 (14%)
P-value (χ^2)			Any reaction: 0.210	Hypotensive reaction: 0.039	

*Venom concentration at which skin test noted to be positive; lower concentrations equal greater sensitivity.

Table 3. Laboratory test results and reactivity to sting challenge in the placebo group

	<i>n</i>	No reaction	Systemic reaction	<i>P</i>	Hypotensive reaction	<i>P</i>
		Median (range)	Median (range)		Median (range)	
Specific IgE	29	2.1% (1–27)	6.5% (1–35)	0.153	7.2% (3–35)	0.126
SI (0.1)	29	1.7 (0.5–25.5)	2.1 (0.4–28.4)	0.981	2.9 (1.5–28.4)	0.083
SI (0.5)	29	6.8 (1.9–37.6)	4.6 (0.7–53.2)	0.582	7.0 (3.2–33.4)	0.153
SI (1.0)	29	8.9 (2.9–45.0)	6.0 (1.1–47.7)	0.401	9.1 (4.2–35.1)	0.103
SI (5.0)	29	10.6 (2.3–36.8)	9.4 (1.0–82.5)	0.615	14.9 (3.2–48.7)	0.349
IL-4 pg/mL (1.0)	29	9.5 (0–109)	11.0 (0–425)	0.981	43 (0–425)	0.114
IL-4 pg/mL (PMA)	29	121 (0–2990)	288 (0–3880)	0.237	80 (5–950)	0.649
IFN- γ pg/mL (PMA)	29	1540 (0–8210)	1605 (0–19410)	0.793	1652 (0–19410)	0.867
HRT (0.001)	24	2.2% (0–7)	1.7% (0–15)	0.820	2.4% (0–6)	0.923
HRT (0.01)	24	5.5% (0–18)	11.9% (2–62)	0.280	17.6% (8–21)	0.027
HRT (0.1)	24	12.2% (0–33)	39.2% (8–67)	0.015	39.8% (18–67)	0.177
BAT (0.001)	26	5.4% (2–9)	5.1% (1–41)	0.778	6.4% (1–41)	0.494
BAT (0.01)	26	34% (3–53)	43% (7–82)	0.135	31% (7–82)	0.790
BAT (0.1)	26	36% (6–75)	64% (7–87)	0.152	62% (7–85)	0.836
LRT (0.001)	26	70 (0–287)	96 (0–715)	0.866	117 (0–715)	0.421
LRT (0.01)	26	187 (97–1585)	343 (31–1100)	0.364	493 (82–1100)	0.295
LRT (0.1)	26	326 (102–6554)	481 (0–3807)	0.120	593 (308–1041)	0.457

For each test performed (listed in left hand column), the number in brackets indicates the stimulus or venom concentration in $\mu\text{g/mL}$ used. Tests where there are significant differences are in bold. The *P*-values in this cross-tabulation are for comparisons of test values between those with no reaction and those with any grade of systemic reaction, and between those with no or mild reactions and those with hypotensive reactions.

sensitivity was required to detect potentially life-threatening allergy. Therefore, high sensitivity/moderate–low specificity points on the ROC curve were chosen as the optimal diagnostic thresholds. To screen for any degree of sting challenge reactivity, an HRT of $>10\%$ at a venom concentration of $0.1 \mu\text{g/mL}$ was optimal (sensitivity 94%, specificity 50%). Using the same diagnostic cut-off, the test was 100% sensitive for severe reactivity (i.e. any degree of respiratory compromise or hypotension). To screen for hypotensive reactions, an HRT of $>6.5\%$ at a venom concentration of $0.01 \mu\text{g/mL}$ was optimal (sensitivity 100%, specificity 39%). However, as noted in Fig. 1, the confidence intervals were wide.

Changes during immunotherapy

Box plots and significance values for laboratory parameter changes demonstrating significant changes during immunotherapy are presented in Fig. 2. For each of those parameters measured at multiple venom concentrations (HRT, BAT and LRT), only one representative concentration ($0.1 \mu\text{g/mL}$) is displayed; similar changes were seen at all concentrations but were most significant at $0.1 \mu\text{g/mL}$. The results may be summarized as follows:

- (i) Venom-specific IgE (RAST) remained unchanged during placebo treatment, but significantly increased

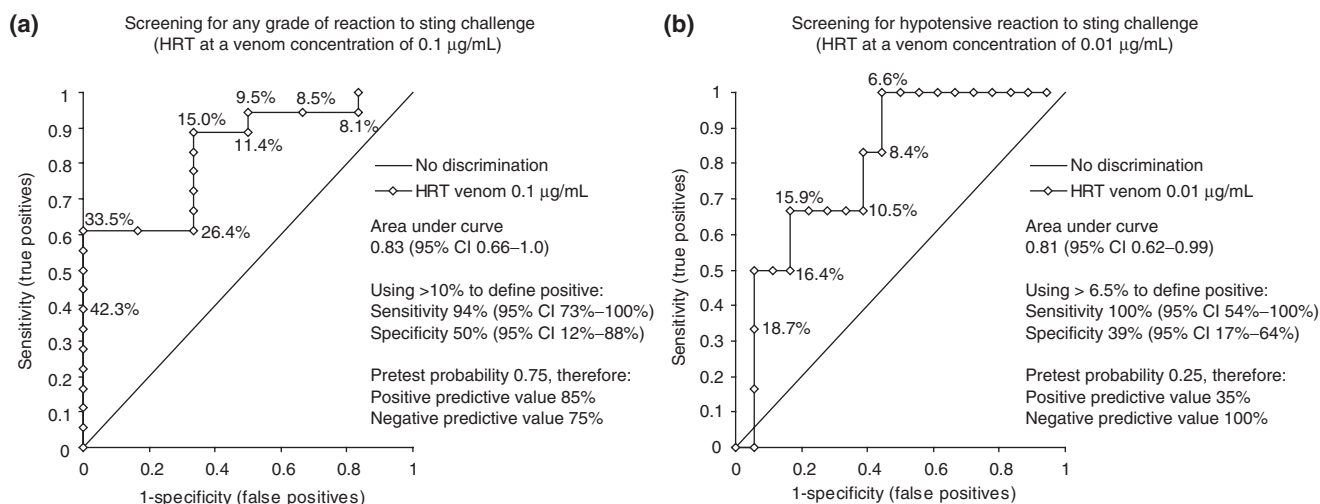


Fig. 1. Receiver–operator characteristics (ROC) curve analysis of HRT as a screening tool for sting challenge reactivity. ROC curves plot the performance of each possible diagnostic cut-off (the values that are plotted along the curve above) in terms of *sensitivity* (number of true positives divided by the number with the disease) and *specificity* (number of true negatives divided by the number without the disease). *1-Specificity* thus represents the number of false positives divided by the total number of subjects without the disease, that is the rate of false positives.

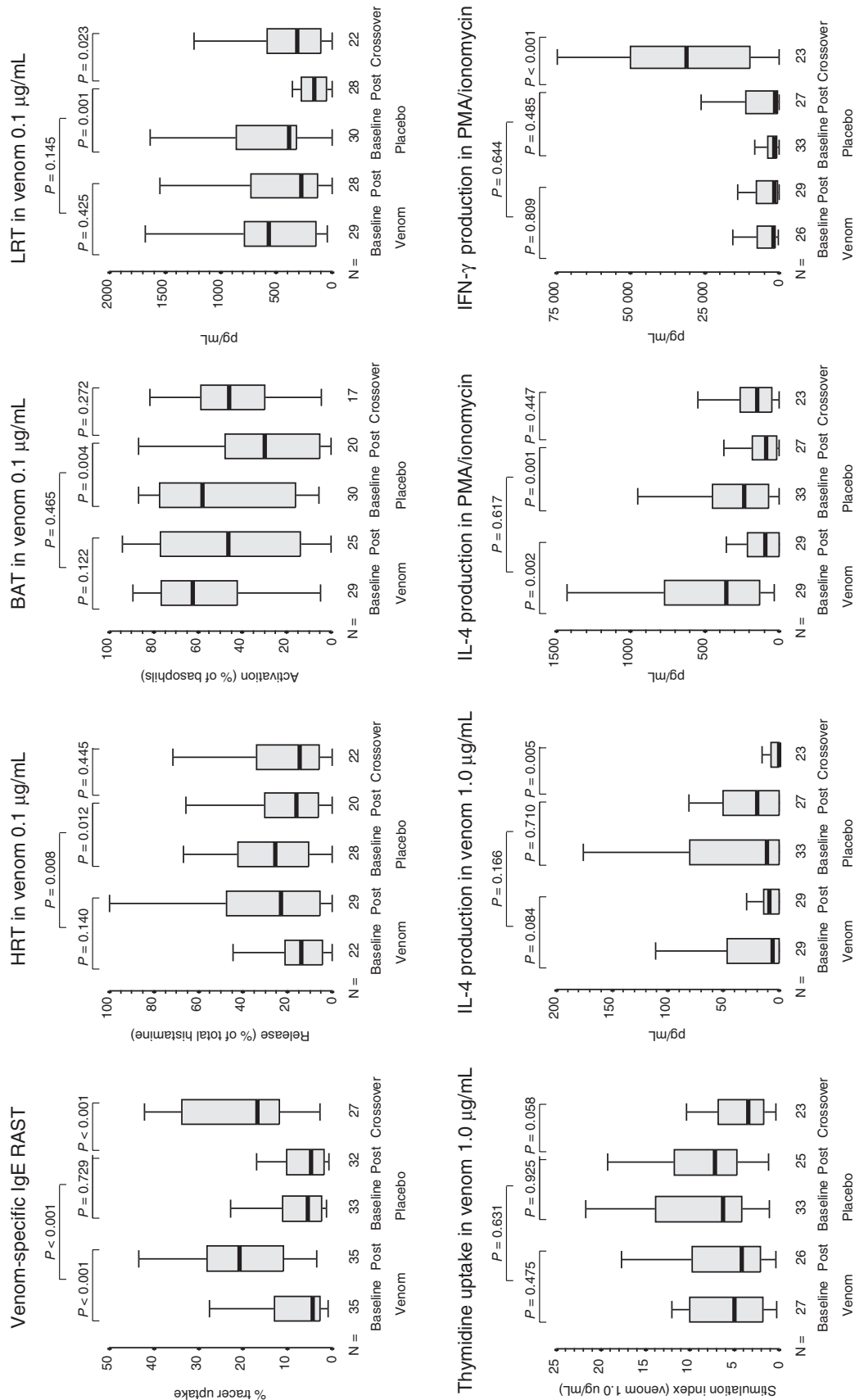


Fig. 2. Changes in laboratory parameters during immunotherapy. The three lower P -values for each graph are for changes between pre- and post-treatment values for each treatment arm (Wilcoxon signed-ranks test). The top P -value compares changes in the parameter between placebo and venom groups during the randomized phase of the trial (the Mann-Whitney U -test).

with immunotherapy during both the randomized and crossover phases.

- (ii) Reductions in the HRT, BAT and LRT were evident during placebo treatment. In comparison, during VIT (randomized and crossover), there was either an increase or a lesser decline. However, for the randomized phase statistically significant differences between venom and placebo groups were only evident with the HRT where median histamine release increased after VIT but fell with placebo treatment.
- (iii) Lymphocyte proliferative responses to venom did not differ significantly between venom and placebo groups. However, IL-4 production appeared to be reduced with VIT, with a reduction in the number of 'outliers' with very high IL-4 production in both groups undergoing VIT, statistically significant in the crossover group.
- (iv) IL-4 production in response to non-specific stimulation with PMA and ionomycin fell after treatment in both venom and placebo groups during the randomized phase, but did not change during the crossover phase. Conversely, IFN- γ production increased during crossover treatment, but no change was seen during the randomized phase.

Discussion

Diagnosis of venom allergy

We found that the results of venom-specific IgE RAST, SI, IL-4 production in response to venom, LRT and BAT all mirror VST sensitivity. However, only VST and HRT appeared to be predictive of sting challenge results. With a higher concentration of venom (0.1 $\mu\text{g/mL}$), release of > 10% of total blood histamine was a reasonably sensitive marker of any degree of sting challenge sensitivity, and 100% sensitive for severe reactivity. Using a lower venom concentration of 0.01 $\mu\text{g/mL}$, the release of > 6.5% of total blood histamine was less sensitive for any degree of reactivity, but was 100% sensitive for hypotensive anaphylaxis. However, because of small sample size, the confidence intervals for these estimates were wide. Although test imprecision in these dynamic *in vitro* studies is likely to be high, this factor was not responsible for the wide confidence intervals in our study where a defined cut-off determined the test result. However, imprecision could result in poor sensitivity and specificity due to inaccurate classification (positive vs. negative) of each individual result, a possibility that requires clarification in larger studies.

Our results are consistent with previous studies that have shown BAT and LRT tests to correlate well, but HRT to correspond less well, with VST in the setting of bee and yellow jacket venom allergy [16, 17]. However, our results are not consistent with one large sting challenge study performed in the setting of honeybee and vespid allergy that found VST did not predict sting challenge sensitivity [4]. Whether this inconsistency is due to chance, differences in VST methodology, or differences between honeybee, wasp and *M. pilosula* allergy is unknown. Significant differences in natural history [4, 5], diagnostic performance of skin tests and serum IgE analysis [18] and behaviour during VIT [19] have been observed in comparisons of honeybee and vespid wasp allergy. *M. pilosula* allergy appears to be unique in terms of

a high reaction rate to both accidental and deliberate stings when compared with bee and vespid allergy [3, 6], so differences in diagnostic test performance can also be anticipated. An explanation for our *in vitro* findings may lie with the use of IL-3 pre-incubation in the BAT and LRT (but not HRT) tests. One previous study showing some correlation between HRT and VST utilized an IL-3 pre incubation step during the HRT [17]. Alternatively, other factor(s) having an effect on the links between basophil activation and mediator release may be important.

Monitoring venom immunotherapy

During the trial, changes in the various laboratory parameters appeared to be somewhat erratic. When directly comparing the placebo and VIT groups, the only statistically significant changes we found during VIT were an increase in venom-specific IgE RAST and a reversal of the trend towards reduced HRT values seen in the placebo group. Although not reaching statistical significance, it appeared that VIT may also prevent a 'natural' decline in BAT and LRT reactivity seen in the placebo group. Although an effect of VIT on leucocyte IL-4 production was evident taking together the changes seen in both randomized and crossover phases, this did not reach statistical significance in the randomized phase. This suggests its utility, as a marker of successful VIT, is limited.

It is interesting that significant reductions in IL-4 production to non-specific stimulation (PMA/ionomycin) occurred in both VIT and placebo groups. This suggests the possibility of a confounding seasonal effect, related to an effect of light exposure on immune system function [20], or an unidentified change in laboratory conditions or reagents.

Mechanistic considerations

T cell responses are thought to play a central role in hyposensitization [21]. T cell proliferation has been found to fall transiently soon after rush VIT, rising again by 1 month and then declining again over the following 12 months. No significant change occurs with conventional VIT until 1 year of therapy has elapsed [22]. With both rush and conventional VIT, T helper (Th) cell cytokine production changes from an IL-4-dominated (Th2-type) to an IFN- γ -dominated (Th1-type) response to antigen [22–24]. This occurs within a few weeks of rush VIT and about 2 months after commencing conventional VIT [22]. Our results were consistent with these previous studies.

In our study, VIT reversed a trend toward reduced HRT seen in those not receiving therapy, suggesting that the factors involved in conferring protection in untreated VST-positive people (where protection was associated with lesser degrees of histamine release) may be different to the mechanisms by which VIT has an effect. It is likely that the mechanisms that determine sensitivity, and by which VIT confers protection, are so diverse that no single marker may be sufficient to determine risk or to monitor the effects of therapy.

Study strengths and limitations

Major strengths of this study were its randomized placebo-controlled design and use of a diagnostic sting challenge

(incorporating two stings) as the gold standard for diagnosis. We also performed the study in a clinically relevant group of patients, namely, those who are currently considered as candidates for immunotherapy, and used commercially available quality-controlled test kits. The concurrent placebo-controlled design controlled for the influence of confounding variables such as a natural history for reduced sensitivity over time and seasonal changes in Th1 to Th2 balance.

Main limitations of this study were that

- (i) Although diagnostic sting challenge is the best available test for venom allergy, it is not infallible because a small proportion of people (approximately 5%) with initially negative challenges may go on to react to subsequent field stings [25].
- (ii) The subgroup with hypotensive anaphylaxis was relatively small (necessary for ethical reasons).
- (iii) For logistical reasons tests could not always be performed.
- (iv) Laboratory assessments were confined to the initial 3-month period of immunotherapy, therefore changes in the longer term cannot be excluded.
- (v) We used a simple approach in assessing IL-4 release, namely venom stimulation of unselected cell cultures. Methods that select venom-reactive cells prior to non-specific stimulation may be more useful [23].
- (vi) We chose a concentration for lymphocyte cytokine production based on highest proliferative responses. In this context, it is notable that one of the major allergenic peptides in *M. pilosula* venom (pilosulin 1) is cytotoxic and may have influenced our results [26]. We cannot exclude the possibility that tests using different venom concentrations, sampled at different time-points of the lymphocyte culture, or using pre-selected venom-reactive cell lines followed by non-specific stimulation, could have performed better.

The high efficacy of VIT would have hindered testing a candidate marker for successful VIT. However, as no laboratory result consistently paralleled successful VIT, we were able to conclude that no such marker had emerged.

Clinical and research implications

Our results suggest that further research into the utility of the HRT is warranted. Although specificity appears to be poor, the inconvenience and cost of VIT means that even the exclusion of a small number of people who would otherwise be given VIT might make the test cost-effective. It may also be worthwhile investigating the utility of the HRT in determining reaction risk in people with negative VST – a group currently not offered immunotherapy but who may still be at a significant risk [27]. Caution should be applied however, as the discriminating concentrations, diagnostic thresholds and test utility may vary between insect venoms and populations (e.g. VST-positive vs. VST-negative people), and standardized venom preparations (in terms of allergen composition) must be used. Also, a larger definitive study of diagnostic sensitivity must include prospective follow-up of accidental stings and perhaps repeated sting challenges at a later date to identify those in whom allergic sensitivity re-develops after the initial sting challenge.

Lymphocyte proliferative responses and cytokine release patterns designed to assess the Th1/Th2 balance have been used to assess the efficacy of novel immunotherapies [28, 29]. Our results, as well as the uncertainty regarding immunotherapy mechanisms, indicate that no laboratory marker is yet able to replace deliberate challenge with allergen.

Conclusions

The HRT offers promise for diagnosis of people with a history of anaphylaxis to insect venom. The poor performance of other commercially available *in vitro* tests may be related to pre-incubation with IL-3. None of the *in vitro* tests appear to be reliable markers of successful immunotherapy in the 3-month period we examined.

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Conflicts of Interest

None.

Contributors

S. Brown and R. Heddle managed the clinical trial. S. Brown, M. Haas and G. Woods were responsible for the laboratory study design. M. Haas, J. Black and A. Parameswaran performed laboratory analyses under the supervision of S. Brown and G. Woods. S. Brown performed data analysis. All investigators reviewed the data and contributed to writing of the report.

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