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Title page

Title:

A comparative study on basophil activation test, histamine release assay and passive sensitization histamine release assay in the diagnosis of peanut allergy

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Keywords

Allergy tests

Basophil

Food allergy

IgE

Peanut

Abbreviations

BAT	Basophil Activation Test
fMLP	Formyl-methionyl-leucyl-phenylalanine
HR	Histamine Release Assay
NPV	Negative Predictive Value
OFC	Oral Food Challenge
Passive HR	Passive sensitization Histamine Release Assay
PMA	Phorbol 12-myristate 13-acetate
PPV	Positive Predictive Value
ROC	Receiver Operator Characteristics
sIgE	Allergen-specific IgE
SPT	Skin Prick Test

Abstract

Background: Allergy can be diagnosed using basophil tests. Several methods measuring basophil activation are available. This study aimed at comparing basophil activation test (BAT), histamine release assay (HR) and passive sensitization histamine release assay (passive HR) in the diagnosis of peanut allergy.

Methods: BAT, HR, and passive HR were performed on eleven peanut allergic and fourteen non-allergic subjects. Blood was incubated with peanut extract or anti-IgE and tests performed as follows: BAT - CD63-upregulation assessed by flow cytometry; HR - released histamine quantified by a glass fiber-based fluorometric method; Passive HR - IgE-stripped donor basophils were incubated with participants' serum and histamine release quantified as HR.

Results: CDsens, a measure of basophil allergen sensitivity, was significantly higher for BAT (80.1 ± 17.4) compared to HR (23.4 ± 10.31) and passive HR (11.1 ± 2.0). BAT, HR, and passive HR had a clinical sensitivity of 100%, 100%, and 82%, and specificity of 100%, 100%, and 100%, respectively when excluding inconclusive results. BAT identified 11 of 11 allergic patients, HR 10 and passive HR 9. Likewise, BAT recognized 12 of 14 non-allergic subjects, HR 10 and passive HR 13. However, the tests' diagnostic performances were not statistically different. Interestingly, non-releasers in HR but not in BAT had lower basophil count compared to releasers (249 vs. 630 counts/min).

Conclusion: BAT displayed a significant higher CDsens compared to HR and passive HR. The basophil tests' diagnostic performances were not significantly different. Still, BAT could diagnose subjects with low basophil number in contrast to HR.

Introduction

Food allergy is commonly diagnosed based on a clear clinical history of an immediate allergic reaction supplied with a positive skin prick test (SPT), detection of serum allergen-specific IgE (sIgE) and/or a positive oral food challenge (OFC) (1). OFCs are time-consuming and have an inherent risk of eliciting severe reactions. Even though both SPT and sIgE measurements are easy to perform, reliable and standardized, they are not always optimal. SPT requires that the suspected allergen source is non-toxic and is unsuited for screening reactivity to many different allergens simultaneously. sIgE measurements require that the suspected allergen is available as an allergosorbent such as ImmunoCAP. In addition, sIgE measurements only provide information on allergen sensitization but not on biological reactivity. Blood tests using basophil granulocyte reactivity as a measure of allergy overcome the limitations of SPT and sIgE measurements. Furthermore, basophil tests do not put the patients at risk of inducing a severe allergic reaction in contrast to OFC. Consequently, basophil tests could add to the diagnostic armamentarium in selected cases.

Basophil degranulation induced by allergen cross-linkage of IgE bound to FcεRI receptors has been used for more than fifty years to study the mechanism of IgE-mediated allergic reactions. Upon IgE-mediated activation human basophils rapidly release granule stored mediators (e.g. histamine) and upregulate cell surface receptors like CD63 and CD203c (2,3). Several *in vitro* diagnostics tests based on basophil activation have been developed to

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accurately diagnose patients suffering from type-1 allergies. One method to assess basophil degranulation in response to culprit allergen is a glass fiber-based fluorometric method, histamine release assay (HR) (4-6). The principle of HR is that released histamine from activated basophils binds to a solid glass-fiber phase with unbound blood constituents being removed by washing the solid phase repeatedly. Trapped histamine is eluted from the solid phase by increasing pH and quantified fluorometrically after forming complexes with o-phthaldialdehyde. Thus, HR measures the amount of released histamine from activated basophils. A modified procedure of this method is the passively sensitized basophil histamine release (passive HR) (7-9). It was developed to overcome the problem of non-releasing basophils and to include samples which are not possible to analyze within 24 hours. In passive HR, serum from a patient is used for sensitizing basophils from a healthy donor from which the autologous IgE has been removed. Basophils are then activated by the allergen of interest and histamine quantified as described for HR. With the identification of the granular associated tetraspan glycoprotein marker CD63, a flow cytometry-based method was later developed for measuring basophil activation and is commonly known as basophil activation test (BAT) (10-12). The principle of BAT is the patients' blood is incubated with suspected allergen extract to elicit a reaction. Basophils are then identified by flow cytometry from other blood cells based on expression of specific cell surface markers (e.g. CD123, CD193, CD203c, CD294 and/or FcεRI) and the activation status is determined by upregulated expression of CD63 and/or CD203c.

HR and passive HR are being used for allergy testing and BAT is considered for clinical diagnostics (4,13-15). However, it has to our knowledge not been investigated whether these three cellular allergy test yields similar diagnostic results. In this study, we wanted to compare the diagnostic performance of BAT, HR, and passive HR on peanut allergic patients and non-allergic individuals

Methods

Study population

Peanut allergic patients were recruited from the Allergy Clinic, Gentofte University Hospital, Denmark based on positive oral food challenge tests (4 subjects) or a convincing medical history of a severe immediate allergic reaction to peanut (7 subjects) and a peanut-specific IgE concentration above 3.5 KU/l within the last 5 years. The healthy control population

consisted of non-allergic, non-atopic individuals. The study was approved by the committees on health research ethics in the capital region of Denmark (H-15001321). Written informed consent was obtained from all participants.

sIgE measurement

sIgE towards common inhalation allergens, peanut, and Ara h2 as well as peanut-specific IgG and peanut-specific IgG4 were quantified using the Phadia ImmunoCAP immunoenzymatic assay (ThermoFisher Scientific, Uppsala, Sweden).

Peanut Extract for stimulation assays

Peanut extract was prepared as previously described (16). In brief, raw peanuts (Procudan, Kolding, Denmark) were ground in ice-cold acetone (Sigma-Aldrich, St Louis, MO, USA) for 2 min and stored for 2 hours at -20 °C. The solution was defatted, filtered, and then resuspended in 2M NaCl and homogenized at 24000 RPM for 10 min, using a Yellow Line DI 18 Basic homogenizer (VWR international, Soeborg, Denmark). The peanut homogenate was extracted for 3 h at pH 8.5 at ambient temperature with constant stirring, centrifuged initially at 4000g for 10 min and then at 18400g for 10 min, and sterilized by filtration through a 0.22 µm pore filter (Millipore, Billerica, MA, USA). Lipopolysaccharides were removed with Detoxi-Gel removing columns (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. The protein concentration of the extract was determined spectrophotometrically. Endotoxin levels were assessed by limulus amebocyte lysate (LAL) assay (Lonza, Basel, Switzerland) and the peanut extract exhibited levels <0.001 endotoxin units/ml in stimulation assays.

Basophil activation test (BAT)

Heparinized whole blood was incubated with RPMI 1640 (Sigma-Aldrich) supplied with 0.5 % (v/v) human serum albumin (CSL Behring GmbH, Marburg, Germany) and polyclonal goat anti-human IgE (KPL, Gaithersburg, MD, USA), formyl-methionyl-leucyl-phenylalanine (fMLP) (Sigma-Aldrich) or peanut extract for 30 min at 37 °C and concurrently stained with anti-CD63-phycoerythrin (H5C6), anti-CD123-brilliant violet 650 (7G3) and anti-CD193-brilliant violet 421 (5E8) all from BD Biosciences, San Jose, CA, USA. Erythrocytes were lysed with BD FACSTM lysing solution (BD Biosciences) and cells were washed, fixed using

BD CellFix™ (BD Biosciences) and acquired on a BD Fortessa flow cytometer (BD Biosciences). The blood was processed within 4 h of collection. Basophils were gated as CD123+CD193+ cells of single cells. The expression of CD63 was evaluated by setting the gate to 2% CD63 positive cells on unstimulated control samples. Data were analyzed with FlowJo software version 10 (TreeStar, Ashland, OR, USA).

Histamine release assay (HR)

HR was performed as previously described (17). In brief, heparinized whole blood was centrifuged 600g, 10 min, and plasma replaced with 1,4-Piperazinediethanesulfonic acid (PIPES) buffer (RefLab, Copenhagen, Denmark). Fifty μ l blood and 50 μ l stimulant (polyclonal goat anti-human IgE (KPL), peanut extract or phorbol 12-myristate 13-acetate (PMA) + ionomycin (both from Sigma-Aldrich) were added to glass fiber-coated microtiter plates (RefLab) and incubated at 37 °C for 60 min. Plates were washed repeatedly with demineralized water, incubated with 0.4% (w/v) SDS (RefLab) for 10 min and washed with demineralized water. Plates were incubated with 0.1 mg/ml o-phthalaldehyde (RefLab) at alkaline pH to allow formation o-phthalaldehyde:histamine fluorescent complexes. After 10 min the reaction was stopped by decreasing pH with 0.59% (w/v) perchloric acid (Hospital Pharmacy) and the fluorescence intensity of each well were quantified on a Histareader (Reflab). The measured fluorescence intensity was converted to amounts of released histamine using a standard curve. Percent histamine release (HR (%)) was calculated as the released histamine of stimuli divided by maximum histamine release induced by PMA + ionomycin stimulation.

Passive histamine release assay (Passive HR)

Buffy coats were obtained from the local blood bank (Rigshospitalet, Copenhagen, Denmark). One ml buffy coat blood was washed with PIPES buffer, centrifuged 600g, 10 min and resuspended in 3 ml PIPES buffer. Buffy coats were screened for histamine release to polyclonal goat anti-human IgE (KPL), PMA + ionomycin (both from Sigma-Aldrich), peanut extract and common inhalation-, mite- and food allergens (Reflab) using the fluorescence method described above. A donor with > 100 ng/ml histamine release to anti-IgE and PMA + ionomycin stimulation and no release to peanut extract and common allergens was selected and incubated overnight with 10 pg/ml recombinant human IL-3 (R&D Systems, Minneapolis, MN, USA). Buffy coat blood was washed in ten volumes 9

mg/ml sodium chloride (Fresenius Kabi AG, Bad Homburg, Germany), centrifuged 1000g, 10 min, 11°C and resuspended in ten volumes ice-cold stripping buffer (Reflab) to remove IgE on donor basophils. The cell suspension was immediately centrifuged 1000g, 10 min, 11°C, washed in ten volumes PIPES buffer and finally resuspended in one volume PIPES buffer. IgE-stripped cells were incubated with serum for 1 h at 37 °C. Cell suspension (25 µl), and stimulant (25 µl) were added to glass fiber-coated microtiter plates and released histamine was quantified as described earlier.

Classification of responses and calculation of CDsens

Participants who responded to anti-IgE stimulation with less than 10% basophil reactivity (< 10% CD63 upregulation or < 10% HR) were designated non-releasers. Non-releaser basophils did respond to non-IgE-mediated activation (PMA + ionomycin or fMLP stimulation) in the three basophil tests. Participants not responding to two consecutive peanut extract doses with ≥ 10 % basophil reactivity were regarded non-reacting. A test was regarded conclusive if the basophils reacted to anti-IgE or peanut extract stimulation, and inconclusive if the participant was both non-releaser and non-reacting. CDsens was calculated, on conclusive test results, as the inverted peanut extract concentration eliciting 50 % of maximum response times 100 as defined previously by S.G.O. Johansson et al. (18). The CDsens value was set to 0.01 for non-reacting participants (highest peanut extract concentration inverted times 100).

Statistical analysis

Data were analyzed and plotted using GraphPad Prism 6 (GraphPad Software Inc., La Jolla, CA, USA). Statistical analysis was done using Fishers' exact test for categorical variables and Mann-Whitney U test for continuous variables. Cochran's Q test was used to compare matched dichotomous outcomes between the three diagnostic tests. One-way repeated measures ANOVA with Tukey's post hoc test was used to compare quantitative outcomes of the three basophil tests. Significance was determined using a 2-sided α level of 0.05. Statistical analysis was performed with GraphPad Prism 6 and SAS Enterprise Guide 7 (SAS Institute Inc., Cary, NC, USA).

Results

Study population

This study aimed at comparing three cellular allergy test; BAT, HR, and passive HR. Eleven peanut allergic and fourteen non-allergic age and gender matched subjects were included in the study. The demographic features of the subjects are listed in table 1. None of the non-allergic participants had detectable levels of sIgE to peanut or Ara h 2, whereas all peanut allergic participants had more than 5.4 kUA/l sIgE to peanut (table 1). One of the peanut allergic subjects had <0.35 kUA/l Ara h2 sIgE in serum (table 1).

Table 1. Characteristics of the study population

Demographic features	Non-allergic (n = 14)	Peanut allergic (n = 11)	P-value
Age (y)	25 (20 - 42)	21 (18 - 50)	0.08 [#]
Female sex. no. (%)	10 (71)	8 (73)	1.00
Other food Allergies. no (%)	0 (0)	6 (55)	0.0026
Urticaria. no. (%)	0 (0)	2 (18)	0.18
Atopic Eczema. no. (%)	0 (0)	9 (82)	<0.0001
Asthma. no. (%)	0 (0)	7 (64)	0.0007
Pollen allergy. no. (%)	0 (0)	11 (100)	<0.0001
Total IgE (kU _A /l)	37.7 (5.3 - 3527)	339 (34.8 - 4111)	0.0002[#]
sIgE to peanut (kU _A /l)	<0.35 [‡]	42.7 (5.4 - 99.7)	<0.0001[#]
sIgE to Ara h 2 (kU _A /l)	<0.35 [‡]	19.4 (<0.35 - 107) [†]	<0.0001[#]

Values are shown as numbers (percentages) or medians (range). Statistics were done using Fisher's exact test or Mann-Whitney *U* test (#). Bold indicate statistically significant differences. [‡] designate below the detection limit for all non-allergic donors. [†] One peanut allergic participant had a specific IgE to Ara h 2 <0.35 kU_A/l.

Number of non-releasers differs between BAT and HR

The number of non-releaser donors, that is donors responding with basophil reactivity less than 10% to anti-IgE activation, differed among the three tests. The BAT detected two (8 %) non-releasers whereas HR resulted in five (20 %) (figure S1 and table S1). Passive HR where autologous IgE is stripped off from anti-IgE responsive donor basophils and substituted with participant IgE did only lead to restored anti-IgE response in 22 out of 25 cases. The lack of release was seen for both non-allergic and peanut allergic subjects and was not due to a lower total IgE concentration in the serum of the non-releaser participants (table S1 and figure S2A).

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All peanut allergic participants reacted in BAT

BAT and HR were carried out on blood cells from non-allergic and peanut allergic subjects whereas serum from the same subjects was used for passive HR. The dose-response curves for peanut extract stimulation showed that all peanut allergic participants reacted to peanut in BAT, one failed to react in HR and two did not react in passive HR (figure 1, table S1). The peanut allergic subject not reacting to peanut extract in HR was also a non-releaser to anti-IgE stimulation, whereas the two non-reacting subjects in passive HR responded to anti-IgE activation (figure S1 and table S1). These two allergic subjects had lower peanut-specific IgE compared to responding peanut allergic subjects included in the study (median 9.1 vs. 56.7 kU_A/l), which could explain the lack of response even though the difference in sIgE did not reach statistical significance, $p = 0.07$ (figure S2B). The levels of peanut-specific IgG and IgG4, which have been shown to inhibit the ability of basophils to degranulate upon allergen stimulation, were not significantly elevated in serum from non-reacting peanut allergic participants (figure S3) (19,20). Likewise, the ratios of peanut-specific IgG/IgE and peanut-specific IgG4/IgE were not significantly augmented in serum from peanut allergic subjects not reacting to peanut (data not shown). Accordingly, inhibitory mechanism of IgG-IgE cross-linkage is less likely to be involved in the lack of response from these two peanut allergic subjects in passive HR. None of the non-allergic participants consistently reacted to peanut extract stimulation in the three cellular tests (figure 1, table S1).

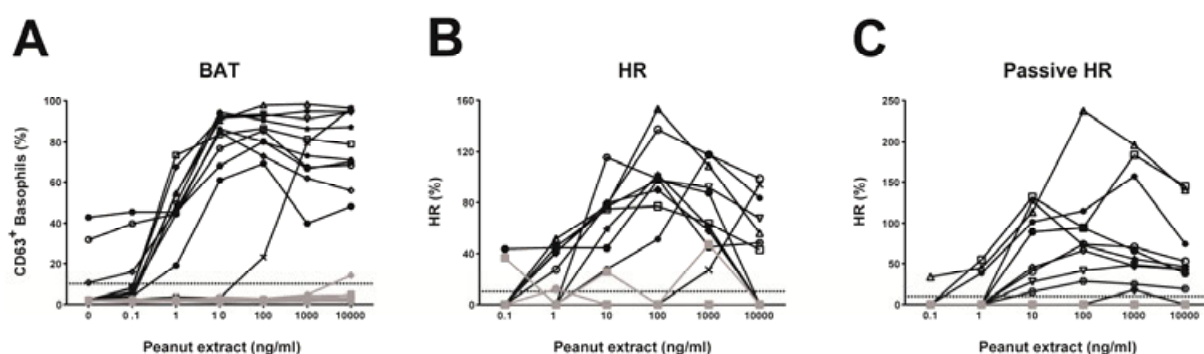


Figure 1. Basophils response to peanut extract stimulation. Basophils from non-allergic ($n = 14$) and peanut allergic ($n = 11$) participants were stimulated with peanut extract. (A) BAT: Degranulation was quantified by CD63 upregulation on CD123⁺CD193⁺ cells using flow cytometry. (B) HR: Degranulation was quantified by measuring released amount of histamine from participants' basophils. (C) Passive HR: Degranulation was quantified by measuring released amount of histamine from passively sensitized donor basophils. Grey = no allergy, Black = peanut allergy. Dotted black line indicate 10% basophil reactivity.

BAT displays a significantly higher CDsens compared to HR and passive HR

CDsens was calculated for each of the dose-response curves for all three tests (table S2). The CDsens of peanut allergic participants was significantly higher for BAT compared to HR and passive HR showing that the concentration of peanut extract needed to elicit half-maximum basophil activation is significantly lower in BAT (figure 2).

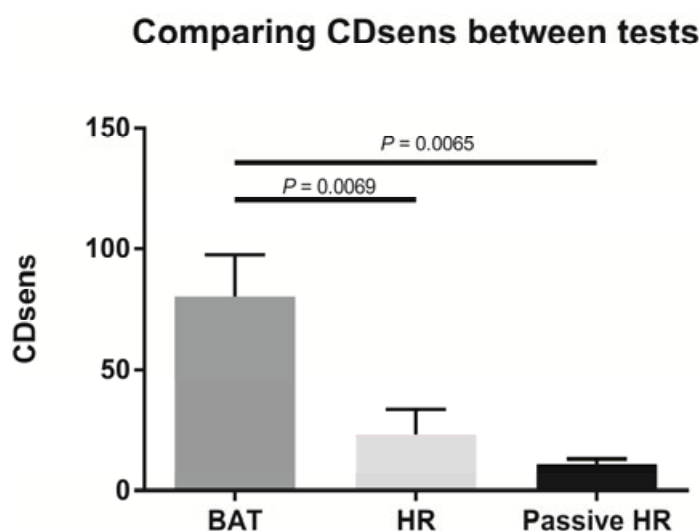


Figure 2. BAT display a significantly higher CDsens compared to HR tests. CDsens was calculated from each peanut allergic participants' dose-response curve to peanut extract (n = 11). Statistical calculation was done using One-way repeated measures ANOVA with Tukey's post hoc test. CDsens is plotted as mean ± SEM.

BAT, HR, and passive HR demonstrate comparable performance when assessing parameters for diagnostic test evaluation when excluding inconclusive test results

Based on CDsens for the dose-response curves (table S2), receiver operator characteristics (ROC) were calculated for all three cellular tests. Subjects with inconclusive tests, defined as individuals presenting as non-releaser to anti-IgE stimulation and not reacting to peanut extract were excluded in this analysis (table S1 and table S2). Numbers of excluded subjects are 2, 5 and 1 for BAT, HR and passive HR, respectively. The ROC curves presented in figure S4 was used to determine the optimal cutoff CDsens values for each test. Parameters for evaluation the three allergy tests are summarized in table 2. The area under curves for ROC, sensitivity, specificity, positive predictive value, negative predictive value, and diagnostic accuracy were identical for BAT and HR. However passive HR demonstrated slightly reduced performance compared to BAT and HR. Thus, when excluding inconclusive test from the analysis, BAT and HR had similar diagnostic strength.

Table 2. Performance of different tests in the diagnosis of peanut allergy, excluding inconclusive tests

Diagnostic tests	CDsens cutoff	AUC ROC	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Diagnostic accuracy (%)
BAT (n=23)	0.11	1.0 (1.0-1.0)	100 (71.5-100)	100 (73.5-100)	100 (71.5-100)	100 (73.5-100)	100 (85.2-100)
HR (n=20)	0.02	1.0 (1.0-1.0)	100 (69.2-100)	100 (69.2-100)	100 (69.2-100)	100 (69.2-100)	100 (83.2-100)
Passive HR (n=24)	3.65	0.91 (0.77-1.0)	81.8 (48.2-97.7)	100 (75.3-100)	100 (66.4-100)	86.7 (59.5-98.3)	91.7 (73.0-99.0)

Values in parentheses represent 95% CI. Numbers of excluded subjects are 2, 5 and 1 for BAT, HR and passive HR, respectively. Abbreviations: AUC ROC, area under the receiver operator characteristic curve; PPV, positive predictive value; NPV, negative predictive value; BAT, basophil activation test; HR, histamine release assay, passive HR, passive sensitization histamine release assay.

Comparing diagnostic performance of BAT, HR and passive HR

The diagnostic performance of the tests showed that BAT was able to identify a positive reaction to peanut in all 11 peanut allergic individuals, whereas HR identified 10 and passive HR only 9 of the 11 (figure 3A and table S2). Conversely, when studying non-allergic subjects BAT correctly identified 12, HR 10 and passive HR 13 of 14 non-allergic individuals (figure 3B and table S2). The overlap between the tests shows that all subjects diagnosed by HR were also diagnosed with BAT and that two subjects could only be classified as non-allergic by passive HR. Interestingly, when combining HR and passive HR all 11 peanut allergic subjects and 14 non-allergic subjects were identified (figure 3). The same result is obtained when combining BAT and passive HR. Nevertheless, there were no significant differences between the performances of the three tests in detecting peanut allergic or non-allergic subjects.

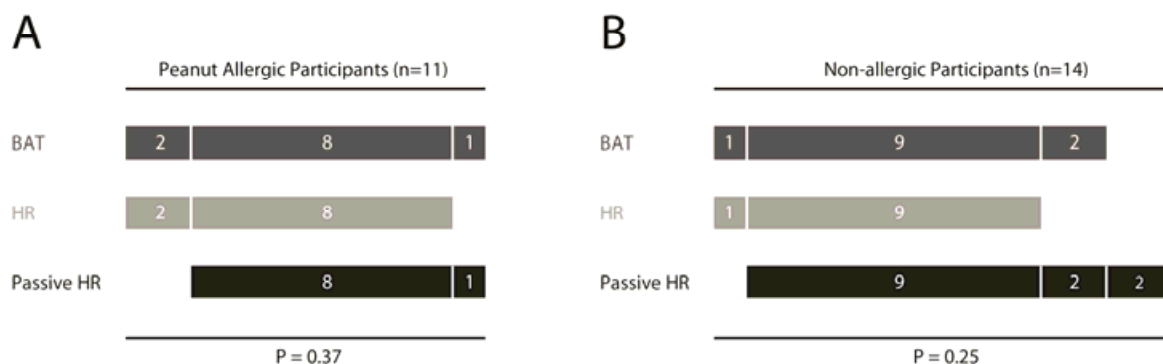


Figure 3. Diagrams depicting the number of participants who could be diagnosed using the three cellular tests. (A) The number of peanut allergic subjects correctly diagnosed with peanut allergy using BAT, HR, and passive HR. **(B)** The number of non-allergic subjects correctly diagnosed as non-allergic by BAT, HR, and passive HR. Subjects similarly diagnosed by all three tests appears centrally, whereas discordant test results are shown left and right to the vertical white lines. Statistical analysis to test if there are differences between the numbers of participant diagnosed as respectively peanut allergic and non-allergic with the three tests was performed using Cochran's Q test.

BAT is capable of diagnosing patients with low basophil number

Reliable detection of basophil activation with HR requires the release of more than 15 ng of histamine. Figure 4 shows that the number of basophils in peripheral blood varies more than 10 fold between donors and that non-releasers identified by HR, but not BAT, have a significantly lower number of basophils compared to releasers identified by both tests (figure 4). This indicates that subjects with a low basophil number, and thereby a low quantity of released histamine, might be falsely categorized as non-releasers by the HR assay resulting in inconclusive test results. Since BAT is a single cell analysis this problem is not seen here.

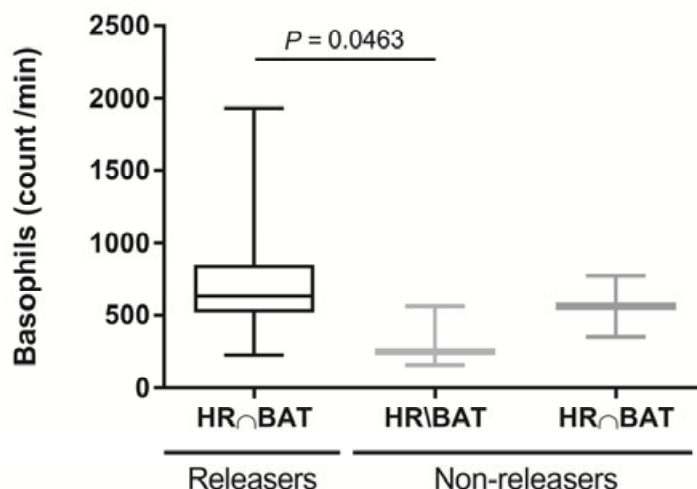


Figure 4. Subjects with a non-releaser profile in HR but not BAT have a significantly lower number of basophils compared to releasers found in both HR and BAT. Basophil number was arbitrary enumerated in each individual based on the number of basophils running through the flow cytometer in 60 sec at equal flowrate. The statistic was performed using Mann-Whitney *U* test. Box plots show median, interquartile range, as well as maximum and minimum (whiskers). HR∩BAT indicate that subjects were detected as releasers (*n* = 20) or non-releasers (*n* = 2) with both HR and BAT tests. HR\BAT signify subjects detected as non-releasers with HR but not BAT test (*n* = 3).

Discussion

In this present study, we compared three basophil diagnostic tests; BAT, HR, and passive HR. We showed that BAT displayed higher CDsens compared to HR and passive HR, and was able to diagnose allergic subjects with a low basophil number in contrast to HR. However, the differences in diagnostic performance of the three tests did not reach statistical significance in the scale of this study. One limitation of the present study was that the peanut allergic subjects all had a high sIgE to peanut (>3.5 kUA/l). Thus, allergic individuals with positive but intermediate levels of peanut-specific IgE were not included in the study (peanut-specific IgE between 0.35 - 3.5 kUA/l). Subjects with lower peanut specific IgE levels would be more likely to be sensitized but tolerant to peanut and would probably have challenged the diagnostic tests in their ability to discriminate between non-allergic and true peanut allergic subjects (21-23).

IL-3 is able to boost the sensitivity of human basophils resulting in basophils releasing more histamine and reaction at a lower concentration of stimuli (24,25). Consequently, IL-3 is frequently used in basophil testing to increase the sensitivity of the detection of cellular

activation (17,26-28). In this study, IL-3 was only added to prime basophils in the passive HR test and not used for BAT or HR. One could speculate that increasing the amount of released histamine by IL-3 priming in HR would result in fewer inconclusive test results as patients with a low basophil number are more likely to release sufficient histamine to be detected by the HR test.

The observation that some individuals do not respond to anti-IgE activation in the different basophil tests may be due to several causes. The two non-releasers detected in BAT did also present as non-releasers in HR. Hence, the lack of response is likely to be impaired intrinsic signaling mechanism such as reduced expression of the SYK downstream of FcεRI receptor signaling, which has been shown to be a hallmark of non-releaser basophils (32). The three non-releasers to anti-IgE stimulation found in HR but not BAT is very likely not non-releasers but properly classified as such due to lower assay sensitivity of HR. Subjects having low numbers of basophils might hence be identified as non-releasers and omitting IL-3 only increases this possibility due to lack of histamine release potentiation.

BAT was able to discriminate all 11 peanut allergic patients from non-allergic subjects whereas HR and passive HR needed to be run consecutively to give the same diagnostic accuracy. The current recommendation for BAT is to process the blood less than 4 hours from sampling as the reactivity and sensitivity drop over time (27). This short timespan makes it difficult to use BAT in a clinical setting as the outpatient clinic and laboratory need to be close and highly coordinated. Furthermore, BAT requires an advanced knowledge of flow cytometry to run the analysis and interpret the results and the procedure is not yet standardized (13,14). In contrast, the HR procedure is standardized and it can be performed within 24 hours after the blood has been sampled making it possible to mail samples. Passive HR is performed with patient serum and therefore does not require viable cells from the patient which gives this test the largest timespan from sampling blood to testing. Passive HR overcomes the intrinsic disadvantage of basophil tests that subjects with non-releaser basophils cannot be diagnosed with direct basophil testing. However, passive HR does not take the cellular characteristics of individual patients into account. In this study, the passive HR test left only one inconclusive test result but also lead to two peanut allergic subjects being identified as non-allergic. Peanut-

allergic subjects not reacting to peanut extract stimulation were predominantly observed for allergic donors displaying moderate levels of peanut-specific IgE (figure 2SB). Consequently, one could speculate that peanut allergic patients with a peanut specific IgE in the range of 0.35 to 3.5 kU_A/l would be difficult to diagnose using passive HR. Thus, passive HR should be used as a second test to BAT and HR, and only to confirm peanut allergy, not to exclude it.

The clinical utility of BAT in diagnosing peanut allergy has been thoroughly investigated in recent years (22,29-31). BAT has been stated to display a high sensitivity and specificity ranging from 83-92% and 77-100%, respectively (22,29-31). However, it is very important to consider the inherent disadvantage of inconclusive test results (subjects not reacting to anti-IgE and suspected allergen source) in basophil tests especially when comparing diagnostics test performance with SPT and sIgE testing. BAT might display higher sensitivity and specificity than SPT and sIgE testing when excluding subjects given inconclusive tests, but would end up with a reduced number of individuals been diagnosed (in this study 92%). Thus, even though BAT gave a very good sensitivity and specificity it would still be necessary to run a second test in 8% of the cases to be able to obtain a correct diagnosis.

In conclusion, our study showed that BAT displayed a significantly higher CDsens compared to HR and passive HR. When excluding inconclusive test results, the sensitivity, specificity, PPV, NPV, and diagnostic accuracy were comparable between the three basophil tests. The number of peanut allergic patients diagnosed with peanut allergy and the number of non-allergic subjects diagnosed as healthy was not significantly different between the three basophil tests. However, BAT was able to diagnose subjects with low basophil number in contrast to HR.

Acknowledgement

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Conflicts of Interest Statement

The authors declare no conflicts of interests.

Authors Contributions

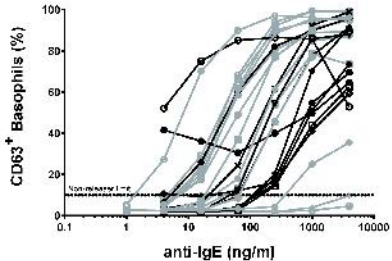
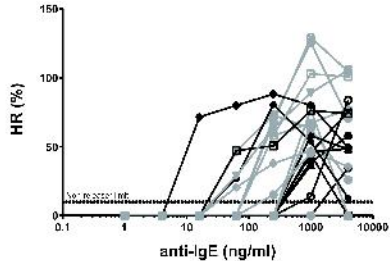
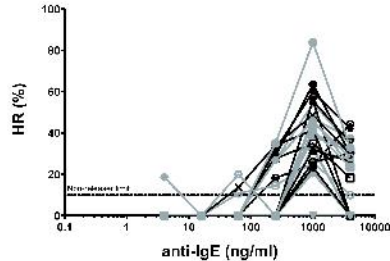
LFL, ENCM, RVR, LKP, and BMJ designed the study. LFL and BMJ developed the methodology. LFL, NJB, and KSH performed the clinical contribution to the study. LFL, and NJB collected the data and performed the analysis. LFL drafted the manuscript. NJB, KSH, ENCM, RVR, LKP, and BMJ critically reviewed the manuscript and all authors approved the final version.

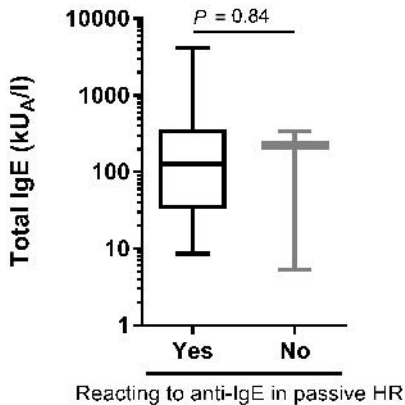
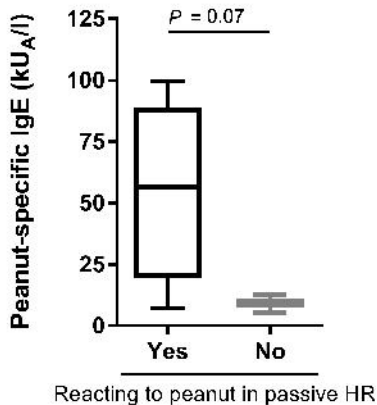
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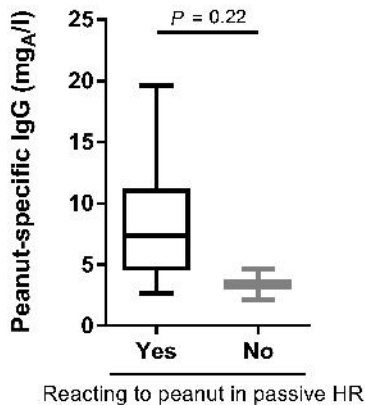
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A**BAT****B****HR****C****Passive HR**

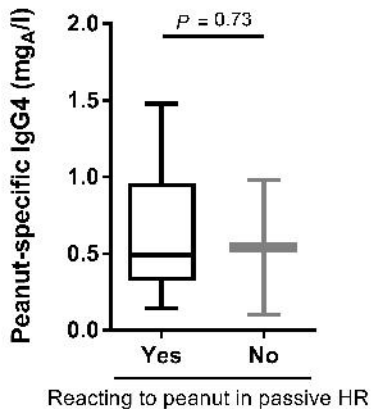
A**Passive HR response
and level of total IgE****B****Passive HR response and
level of peanut-specific IgE**

A

Passive HR response and
level of peanut-specific IgG

**B**

Passive HR response and
level of peanut-specific IgG4



CDsens

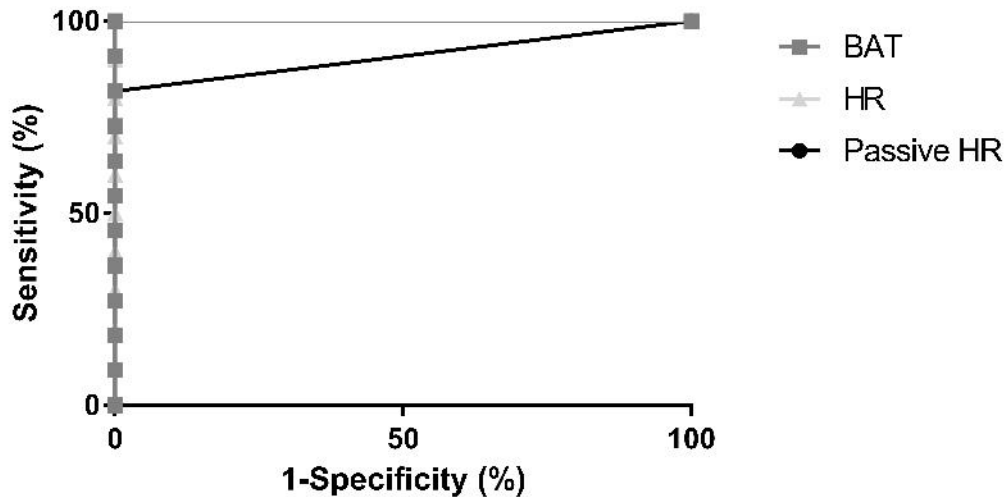


Table S1. Response to anti-IgE and peanut extract, and distribution of conclusive and inconclusive tests.

	Subject	Anti-IgE Response			Peanut Extract Response			Conclusive and Inconclusive Tests		
		BAT	HR	Passive HR	BAT	HR	Passive HR	BAT	HR	Passive HR
Peanut Allergic Subjects	1	+	+	+	+	+	+	C	C	C
	2	+	+	+	+	+	+	C	C	C
	3	+	+	+	+	+	+	C	C	C
	4	+	+	+	+	+	+	C	C	C
	5	+	-	-	+	-	+	C	I	C
	6	+	+	+	+	+	+	C	C	C
	7	+	+	-	+	+	+	C	C	C
	8	+	+	+	+	+	-	C	C	C
	9	+	+	+	+	+	-	C	C	C
	10	+	+	+	+	+	+	C	C	C
	11	+	+	+	+	+	+	C	C	C
Non-allergic Subjects	12	+	-	+	-	-	-	C	I	C
	13	+	+	+	-	-	-	C	C	C
	14	+	+	+	-	-	-	C	C	C
	15	+	+	-	-	-	-	C	C	I
	16	+	-	+	-	-	-	C	I	C
	17	+	+	+	-	-	-	C	C	C
	18	-	-	+	-	-	-	I	I	C
	19	+	+	+	-	-	-	C	C	C
	20	+	+	+	-	-	-	C	C	C
	21	+	+	+	-	-	-	C	C	C
	22	-	-	+	-	-	-	I	I	C
	23	+	+	+	-	-	-	C	C	C
	24	+	+	+	-	-	-	C	C	C
	25	+	+	+	-	-	-	C	C	C

Anti-IgE response; **minus sign (-)** signify non-releaser (< 10% basophil reactivity), **plus sign (+)** designate releaser ($\geq$ 10% basophil reactivity). Peanut extract response; **minus sign (-)** specify non-reacting individuals (Subjects not showing $\geq$ 10% basophil reactivity to two successive doses), **plus sign (+)** denotes reacting participants (subjects displaying $\geq$ 10% basophil reactivity to two successive doses). C; conclusive tests (subject responding to anti-IgE or peanut extract stimulation). I; inconclusive tests (subject not responding to anti-IgE and peanut extract stimulation)

Table S2. CDsens and test outcome for each subject using BAT, HR and passive HR

		CDsens			Test Outcome		
Subject		BAT	HR	Passive HR	BAT (0.11)	HR (0.02)	Passive HR (3.65)
Peanut Allergic Subjects	1	20.27	12.02	10.79	P	P	P
	2	170.81	115.37	18.72	P	P	P
	3	110.78	10.17	7.30	P	P	P
	4	104.27	33.93	11.22	P	P	P
	5	77.34	I	13.14	P	I	P
	6	102.90	0.49	14.81	P	P	P
	7	114.56	11.48	18.18	P	P	P
	8	143.63	50.02	0.01	P	P	N
	9	0.20	0.03	0.01	P	P	N
	10	22.93	5.73	17.39	P	P	P
	11	13.49	18.18	10.75	P	P	P
Non-allergic Subjects	12	0.01	I	0.01	N	I	N
	13	0.01	0.01	0.01	N	N	N
	14	0.01	0.01	0.01	N	N	N
	15	0.01	0.01	I	N	N	I
	16	0.01	I	0.01	N	I	N
	17	0.01	0.01	0.01	N	N	N
	18	I	I	0.01	I	I	N
	19	0.01	0.01	0.01	N	N	N
	20	0.01	0.01	0.01	N	N	N
	21	0.01	0.01	0.01	N	N	N
	22	I	I	0.01	I	I	N
	23	0.01	0.01	0.01	N	N	N
	24	0.01	0.01	0.01	N	N	N
	25	0.01	0.01	0.01	N	N	N

Numbers in parenthesis designate CDsens cutoff values. N; negative test (CDsens value < CDsens cutoff), P; positive test (CDsens value > CDsens cutoff), I; Inconclusive test (subject not responding to anti-IgE and peanut extract stimulation)

Supplementary Tables and Figures

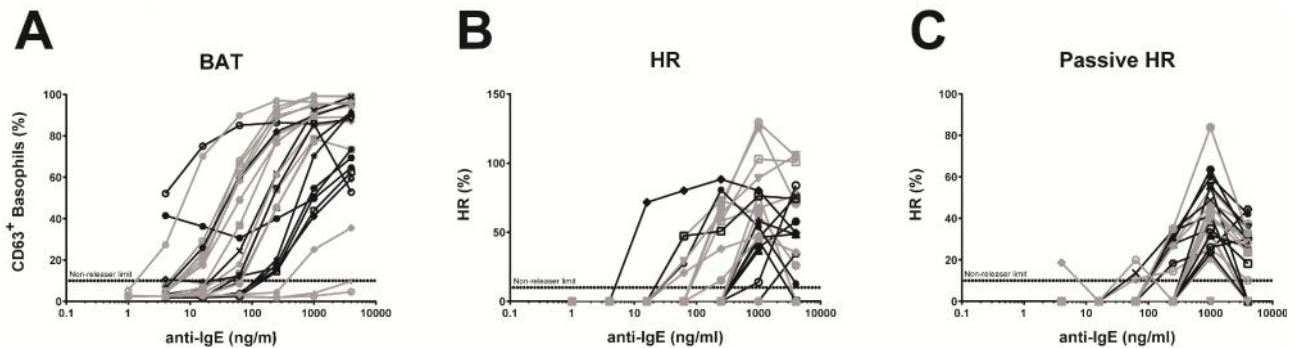


Figure S1. Basophils response to anti-IgE stimulation. Basophils from non-allergic (n = 14) and peanut allergic (n = 11) participants were stimulated with anti-IgE. **(A)** BAT: Degranulation was quantified by CD63 upregulation on CD123⁺CD193⁺ cells using flow cytometry. **(B)** HR: Degranulation was quantified by measuring released amount of histamine from participants' basophils. **(C)** Passive HR: Degranulation was quantified by measuring released amount of histamine from passively sensitized donor basophils. Grey = no allergy, Black = peanut allergy, Dotted black line indicate 10% basophil reactivity.

Table S1. Response to anti-IgE and peanut extract, and distribution of conclusive and inconclusive tests.

	Subject	Anti-IgE Response			Peanut Extract Response			Conclusive and Inconclusive Tests		
		BAT	HR	Passive HR	BAT	HR	Passive HR	BAT	HR	Passive HR
Peanut Allergic Subjects	1	+	+	+	+	+	+	C	C	C
	2	+	+	+	+	+	+	C	C	C
	3	+	+	+	+	+	+	C	C	C
	4	+	+	+	+	+	+	C	C	C
	5	+	-	-	+	-	+	C	I	C
	6	+	+	+	+	+	+	C	C	C
	7	+	+	-	+	+	+	C	C	C
	8	+	+	+	+	+	-	C	C	C
	9	+	+	+	+	+	-	C	C	C
	10	+	+	+	+	+	+	C	C	C
	11	+	+	+	+	+	+	C	C	C
Non-allergic Subjects	12	+	-	+	-	-	-	C	I	C
	13	+	+	+	-	-	-	C	C	C
	14	+	+	+	-	-	-	C	C	C
	15	+	+	-	-	-	-	C	C	I
	16	+	-	+	-	-	-	C	I	C
	17	+	+	+	-	-	-	C	C	C
	18	-	-	+	-	-	-	I	I	C
	19	+	+	+	-	-	-	C	C	C
	20	+	+	+	-	-	-	C	C	C
	21	+	+	+	-	-	-	C	C	C
	22	-	-	+	-	-	-	I	I	C
	23	+	+	+	-	-	-	C	C	C
	24	+	+	+	-	-	-	C	C	C
	25	+	+	+	-	-	-	C	C	C

Anti-IgE response; **minus sign (-)** signify non-releaser (< 10% basophil reactivity), **plus sign (+)** designate releaser ($\geq$ 10% basophil reactivity). Peanut extract response; **minus sign (-)** specify non-reacting individuals (Subjects not showing $\geq$ 10% basophil reactivity to two successive doses), **plus sign (+)** denotes reacting participants (subjects displaying $\geq$ 10% basophil reactivity to two successive doses). C; conclusive tests (subject responding to anti-IgE or peanut extract stimulation). I; inconclusive tests (subject not responding to anti-IgE and peanut extract stimulation).

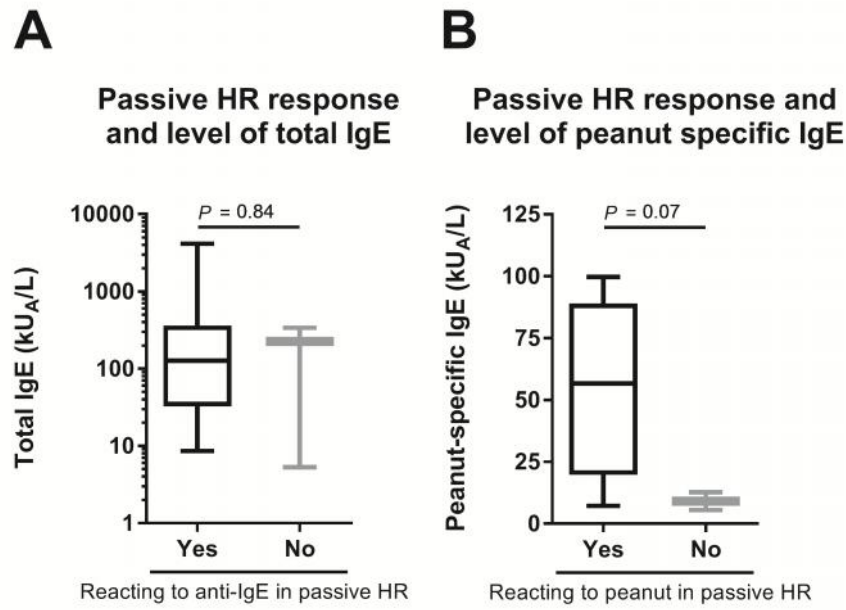


Figure S2. Influence of total- and peanut-specific IgE concentration in serum on the reactivity to anti-IgE and peanut extract in passive HR. (A) Total IgE concentration in serum of releasers ($n = 22$) and non-releasers ($n = 3$) in passive HR. **(B)** Peanut-specific IgE concentration in serum of peanut-allergic patient responding ($n = 9$) and not responding ($n = 2$) to peanut extract activation in passive HR. The statistic analysis was conducted using Mann-Whitney U test. Data is shown as boxplots with median, interquartile range and min/max.

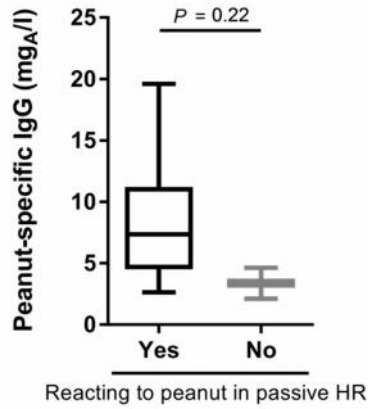
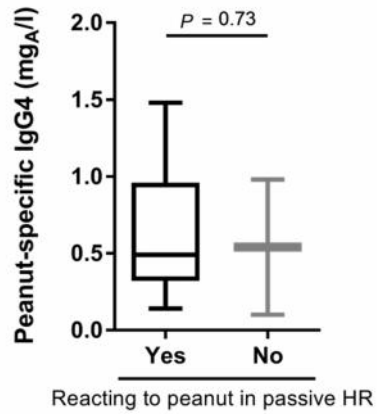
A**Passive HR response and
level of peanut specific IgG****B****Passive HR response and
level of peanut specific IgG4**

Figure S3. Influence of peanut-specific IgG and peanut-specific IgG4 concentration in serum on the reactivity to peanut extract in passive HR. (A) Peanut-specific IgG and (B) peanut-specific IgG4 concentrations in serum of peanut-allergic patient responding ($n = 9$) and not responding ($n = 2$) to peanut extract activation in passive HR. The statistical analysis was conducted using Mann-Whitney U test. Data is shown as boxplots with median, interquartile range and min/max.

Table S2. CDsens and test outcome for each subject using BAT, HR and passive HR

		CDsens			Test Outcome		
	Subject	BAT	HR	Passive HR	BAT (0.11)	HR (0.02)	Passive HR (3.65)
Peanut Allergic Subjects	1	20.27	12.02	10.79	P	P	P
	2	170.81	115.37	18.72	P	P	P
	3	110.78	10.17	7.30	P	P	P
	4	104.27	33.93	11.22	P	P	P
	5	77.34	I	13.14	P	I	P
	6	102.90	0.49	14.81	P	P	P
	7	114.56	11.48	18.18	P	P	P
	8	143.63	50.02	0.01	P	P	N
	9	0.20	0.03	0.01	P	P	N
	10	22.93	5.73	17.39	P	P	P
	11	13.49	18.18	10.75	P	P	P
Non-allergic Subjects	12	0.01	I	0.01	N	I	N
	13	0.01	0.01	0.01	N	N	N
	14	0.01	0.01	0.01	N	N	N
	15	0.01	0.01	I	N	N	I
	16	0.01	I	0.01	N	I	N
	17	0.01	0.01	0.01	N	N	N
	18	I	I	0.01	I	I	N
	19	0.01	0.01	0.01	N	N	N
	20	0.01	0.01	0.01	N	N	N
	21	0.01	0.01	0.01	N	N	N
	22	I	I	0.01	I	I	N
	23	0.01	0.01	0.01	N	N	N
	24	0.01	0.01	0.01	N	N	N
	25	0.01	0.01	0.01	N	N	N

Numbers in parenthesis designate CDsens cutoff values. N; negative test (CDsens value < CDsens cutoff), P; positive test (CDsens value > CDsens cutoff), I; Inconclusive test (subject not responding to anti-IgE and peanut extract stimulation).

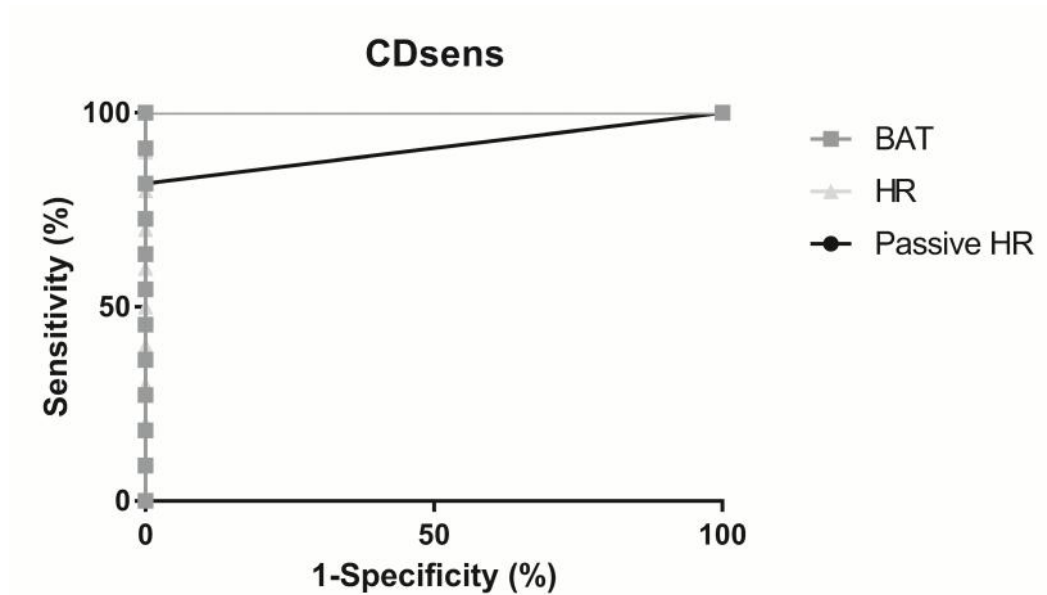


Figure S4. Receiver operator characteristic curves for CDsens to evaluate the diagnostic value of BAT (n = 23), HR (n = 20) and passive HR (n = 24). CDsens was calculated on each individual dose-response curve and applied for generation ROC curves. Inconclusive tests were excluded from the analysis. Numbers of excluded subjects are 2, 5 and 1 for BAT, HR and passive HR, respectively.