

Journal of Immunological Methods

Array-based measurements of aero-allergen-specific IgE correlate with skin-prick test reactivity in asthma regardless of specific IgG4 or total IgE measurements.

--Manuscript Draft--

Manuscript Number:	JIM-D-20-00128R2
Article Type:	Research Paper
Keywords:	Allergic asthma; aero-allergen; IgG4; IgE; Microarray; skin prick test
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Abstract:	<p>Skin prick testing (SPT) and measurement of serum allergen-specific IgE (sIgE) are used to investigate asthma and other allergic conditions. Measurement of serum total IgE (tIgE) and allergen-specific IgG4 (sIgG4) may also be useful. The aim was to ascertain the correlation between these serological parameters and SPT. Sera from 60 suspected asthmatic patients and 18 healthy controls were assayed for sIgE and sIgG4 reactivity against a panel of 70 SPT allergen preparations, and for tIgE. The patients were also assessed by skin prick tests for reactivity to cat, dog, house dust mite and grass allergens. Over 50% of the patients had tIgE levels above the 75th percentile of the controls. 58% of patients and 39% of controls showed sIgE reactivity to ≥ 1 allergen. The mean number of allergens detected by sIgE was 3.1 in suspected asthma patients and 0.9 in controls. 58% of patients and 50% of controls showed sIgG4 reactivity to ≥ 1 allergen. The mean number of allergens detected by sIgG4 was 2.5 in patients and 1.7 in controls. For the patients, a strong correlation was observed between clinical SPT reactivity and serum sIgE levels to cat, dog, house dust mite (HDM) and grass allergens. SPT correlations using sIgE/sIgG4 or sIgE/tIgE ratios were not markedly higher. The measurement of serum sIgE by microarray using SPT allergen preparations showed good correlation with clinical SPT reactivity to cat, dog, HDM and grass allergens. This concordance was not improved by measuring tIgE or sIgG4.</p>
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Response to Reviewers:	

To the Editors,

We would be very grateful if this paper entitled 'Array-based measurements of aero-allergen-specific IgE correlate with skin-prick test reactivity in asthma regardless of specific IgG4 or total IgE measurements' could be considered for publication in the Journal of Immunological Methods.

In allergic asthma, the eliciting inhaled aero-allergens stimulate the immune system via the mucosal surface of the respiratory tract resulting a type 1 hypersensitivity response. Allergen-specific IgE is the key mediator of this response in the respiratory mucosa via its interaction with mucosal mast cells and with eosinophils. The systemic distribution of this allergen-specific IgE also means that it can also be detected cell-free in the blood using laboratory assays, and bound to epidermal mast cells using the clinical skin prick test (SPT). However, the correlation between these two types of tests is not absolute, and could be affected by the amount of total IgE (of all specificities) that an individual produces, and their production of other classes of antibodies that bind to the allergens, but do not cause an allergy (and may even help to prevent allergy) – particularly IgG4. In this study we therefore established a microarray assay to detect, in suspected asthmatic patients, numerous different classes of antibodies that bind to a large panel of allergens used for SPTs. We then compared SPT results for these patients with their blood levels of IgE specific for common aero-allergens (grass pollen, house dust mite, cat or dog danders), either alone, or in relation to their total IgE or aero-allergen-specific IgG4 levels. We found that the aero-allergen-specific IgE levels showed good correlation with the corresponding SPT results, and this correlation was not markedly improved by also considering the total IgE or aero-allergen-specific IgG4 levels. This is important to know in terms of being aware that aero-allergen-specific IgE alone is sufficient to give diagnostic information equivalent to performing an SPT in individuals with allergy affecting the respiratory mucosa, even if levels of total IgE and aero-allergen-specific IgG4 have regulatory effects at the mechanistic level.

We therefore believe that this study will be of particular interest to readers of the Journal of Immunological Methods

Thank you for your consideration.

Regards,

Lucy Fairclough

Journal of Immunological Methods – response to Editor/Reviewer comments.

Dear Professor Niewold,

We are very grateful for the helpful editor/reviewer comments. Responses to the are indicated below.

Editor and Reviewer comments:

Reviewer #1: The manuscript by Aljali Hamed et al describes an allergen array for assessment of specific IgE and specific IgG4 and correlation of these results with skin prick testing in patients with asthma versus healthy controls. The manuscript is primarily a methods paper and while correlations have been determined between SPT and specific IgE or IgG4, the clinical relevance of these comparisons is not clear. I have the following comments and suggestions:

1. Introduction: Define Treg and Breg at first use

Response: These definitions have been added.

2. 2.1: Which 'standard allergens' were used in the evaluation of the 18 controls.

Response: This information has been added – (cat hair, dog hair, grass pollen, tree pollen, house dust mite, *Aspergillus*).

3. 2.3: How was optimal serum dilution determined? Or mention what the optimal serum dilution was.

Response: The optimal dilutions have been added.

4. 3.5: What criteria were used to determine whether SPT was positive or negative? Include the minimum wheal and flare measurements for positivity.

Response: It is now stated that 'SPT positivity was defined as a wheal diameter \geq 3mm'.

5. Section 3.5 states that statistical analysis showed significant correlation between clinical SPT to cat and dog allergens; where is the data shown? If it is meant to be Table 3, please reference it here.

Response: This information has been added to the text (it's not in Table 3). Statement also added on some samples being SPT positive to *Aspergillus* and/or tree pollen (data not shown).

6. The description of results shown in Figure 5 is confusing. When describing the correlation between SPT and sIgE for individual subjects, include a few examples stating the subject ID number to make it easier for the reader to navigate this figure.

Response: Examples have been added to the text.

7. Table 2. Please either add a footnote to the table stating that no controls were in the 'high IgE' group, or state N/A (not applicable).

Response: N/A and footnote have been added.

8. Table 3. Table 3 is confusing - does the first column for each table indicate sIgE, sIgE/sIgG4 and sIgE/tIgE for that particular allergen, and do the rows indicate SPT for

the 7 allergens tested? If that is the case, the title "Cat hair SPT" (for example), is misleading. Please clarify.

Response: A footnote has been added to the table to clarify these points.

9. Figure 5D. Label the y-axis

Response: The labels on the right of the figure have been modified to clarify that these are SPT measurements.

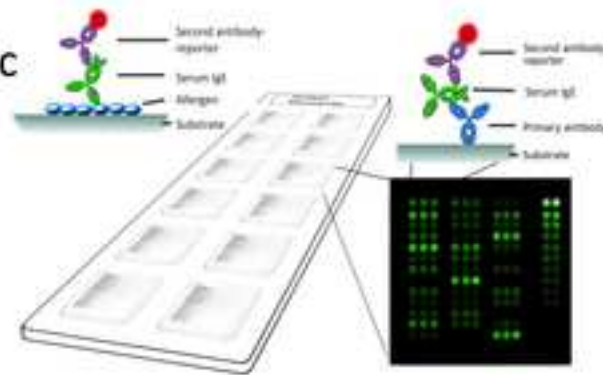
10. Overall, the manuscript describes a novel allergen array for the assessment of sIgE and sIgG4 for a variety of allergens. The data are interesting from the technical standpoint. A comparison of the array data with conventional sIgE testing platforms would be useful in order to determine the clinical utility of the allergen array.

Response: A sentence has been added to the Discussion to highlight the value of a comparison between our assay and those that are commercially available.

Thank you again for your consideration of this manuscript.

Regards,

Lucy Fairclough

Allergen-specific
IgE/IgG4 array

Total IgE array

A. Assay Development

B. Assay Validation

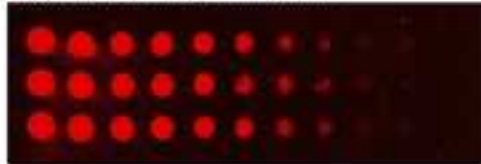
C. Assay Performance

i.

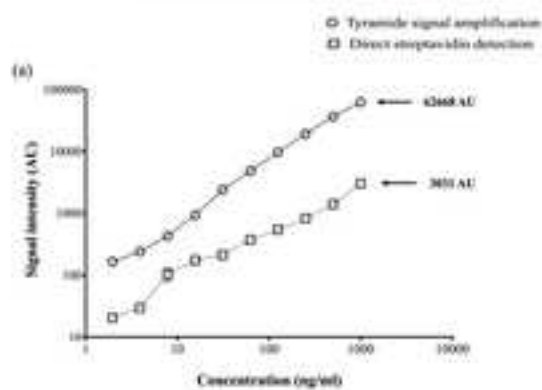
(a) Detection with streptavidin cy5



(b) Detection with tyramide signal amplification

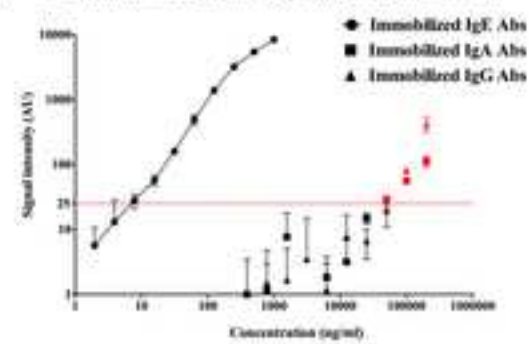


ii.

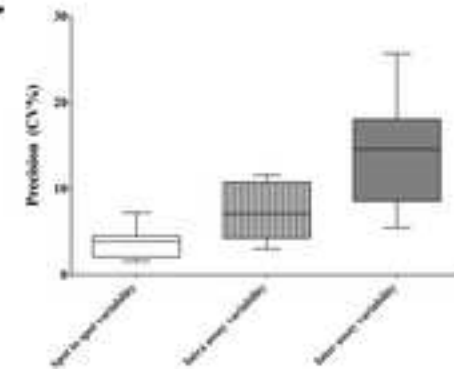


i.

Goat Anti-Human IgE Antibody (Invitrogen Corporation, USA)

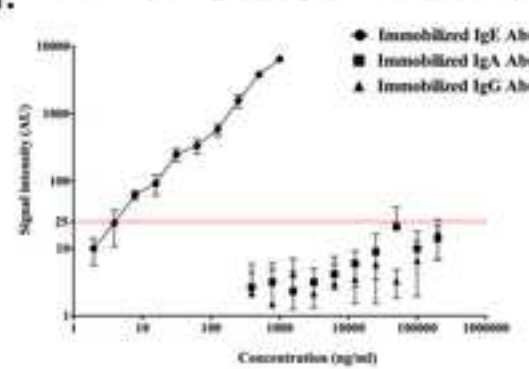


i.

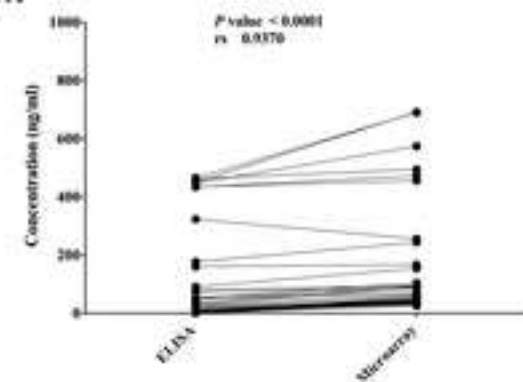


ii.

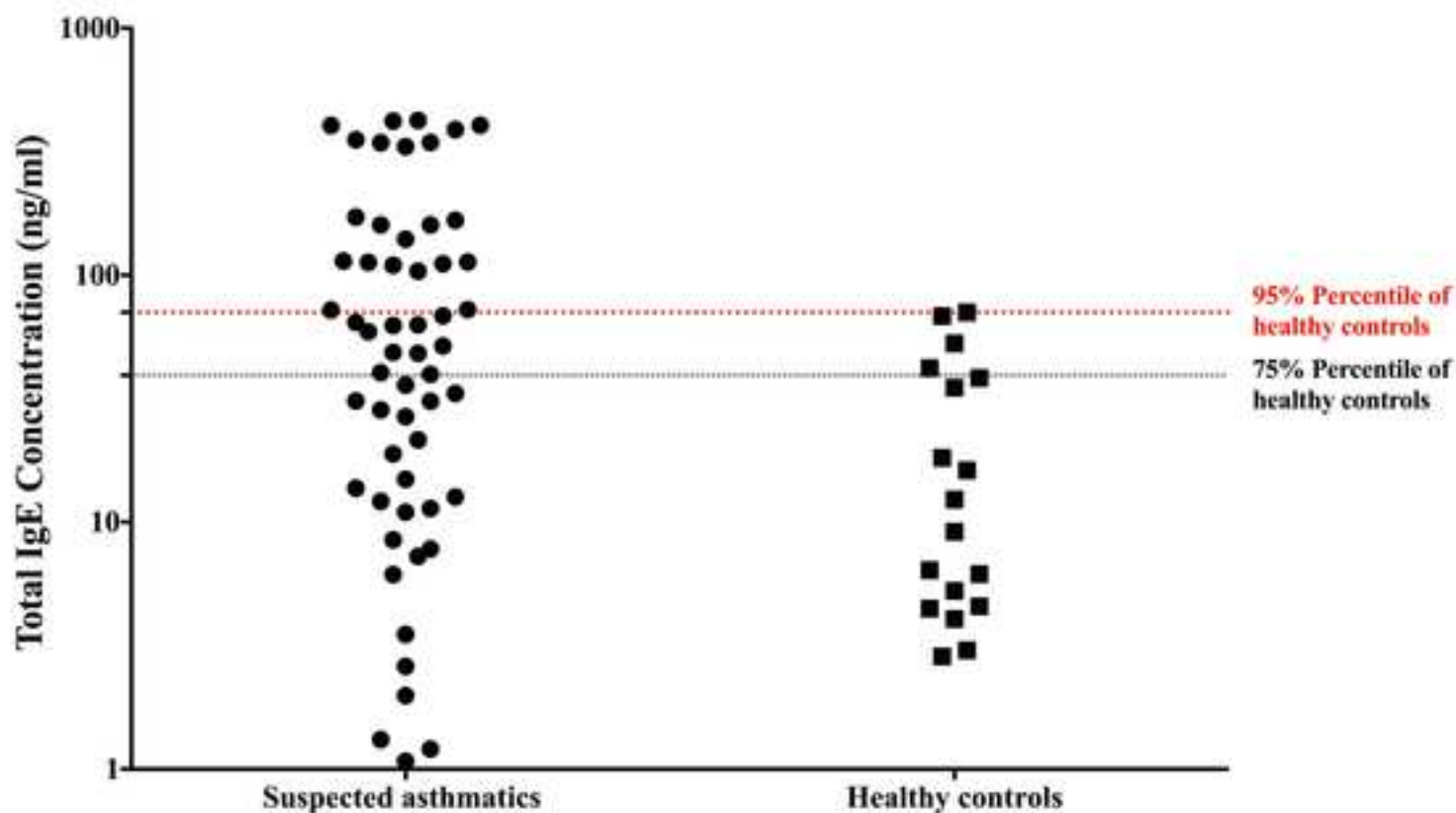
Goat Anti-Human IgE Antibody (Vector Laboratories, UK)



ii.



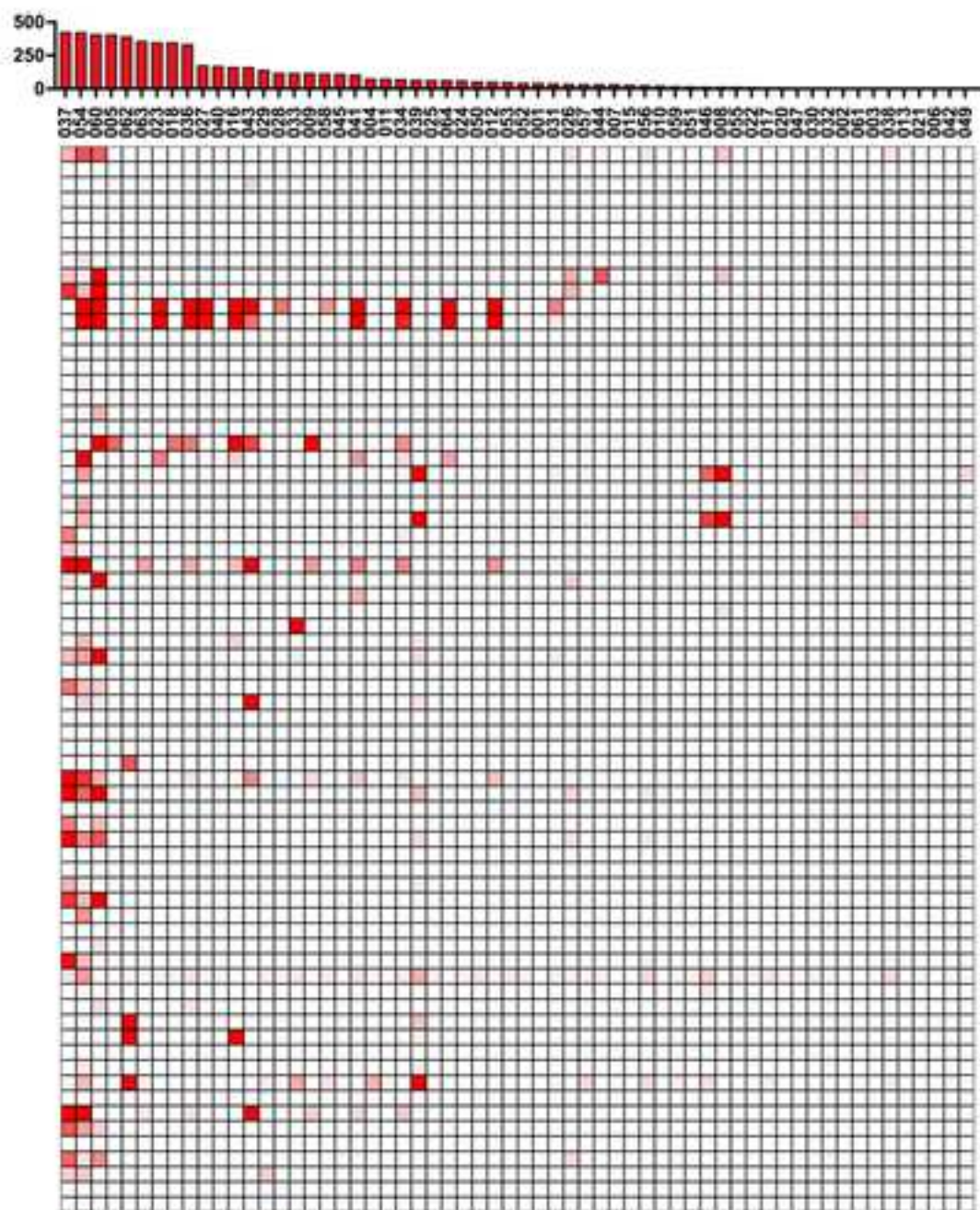
A.



B.

Cut-off	Number of samples above cut-off	
	Suspected Asthmatics	Healthy Controls
95 th Percentile	22 out of 60 (36.7%)	0 out of 18 (0%)
75 th Percentile	32 out of 60 (53.3%)	4 out of 18 (22%)

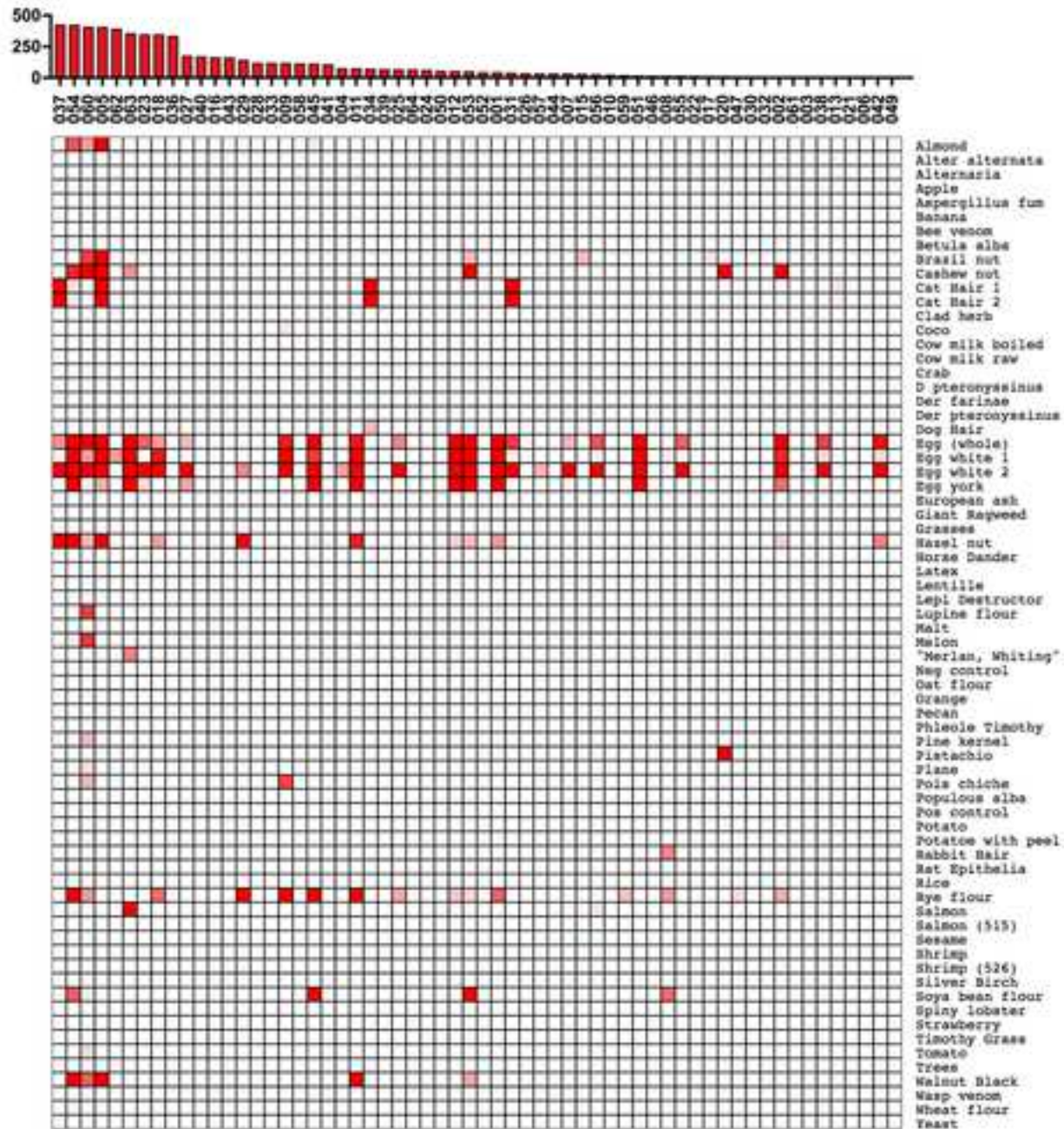
A. Suspected asthmatic patients



B. Healthy controls



A. Suspected asthmatic patients



B. Healthy controls

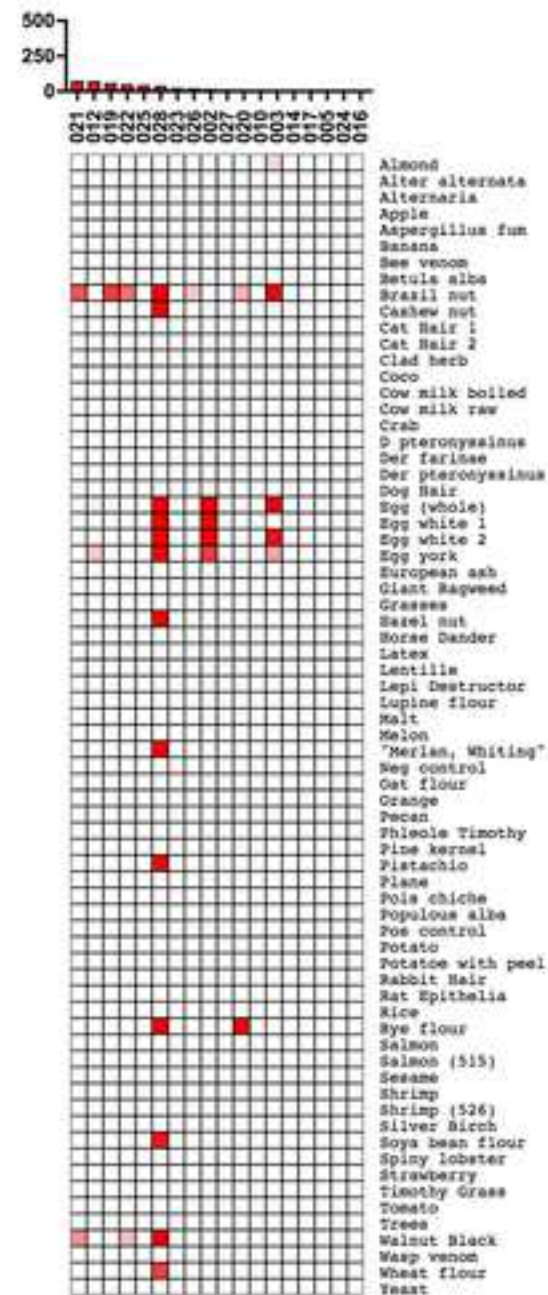
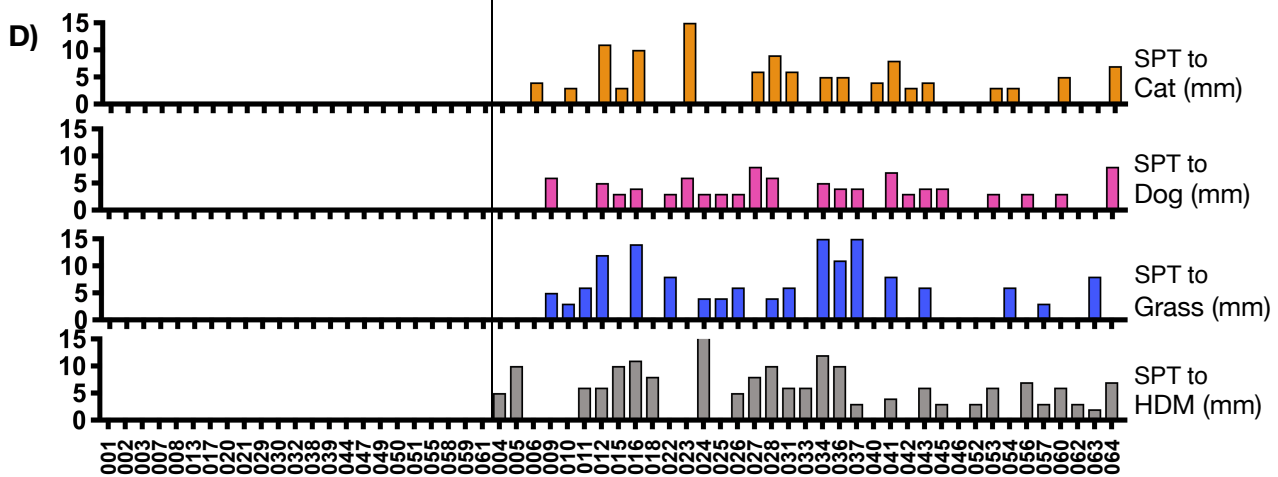
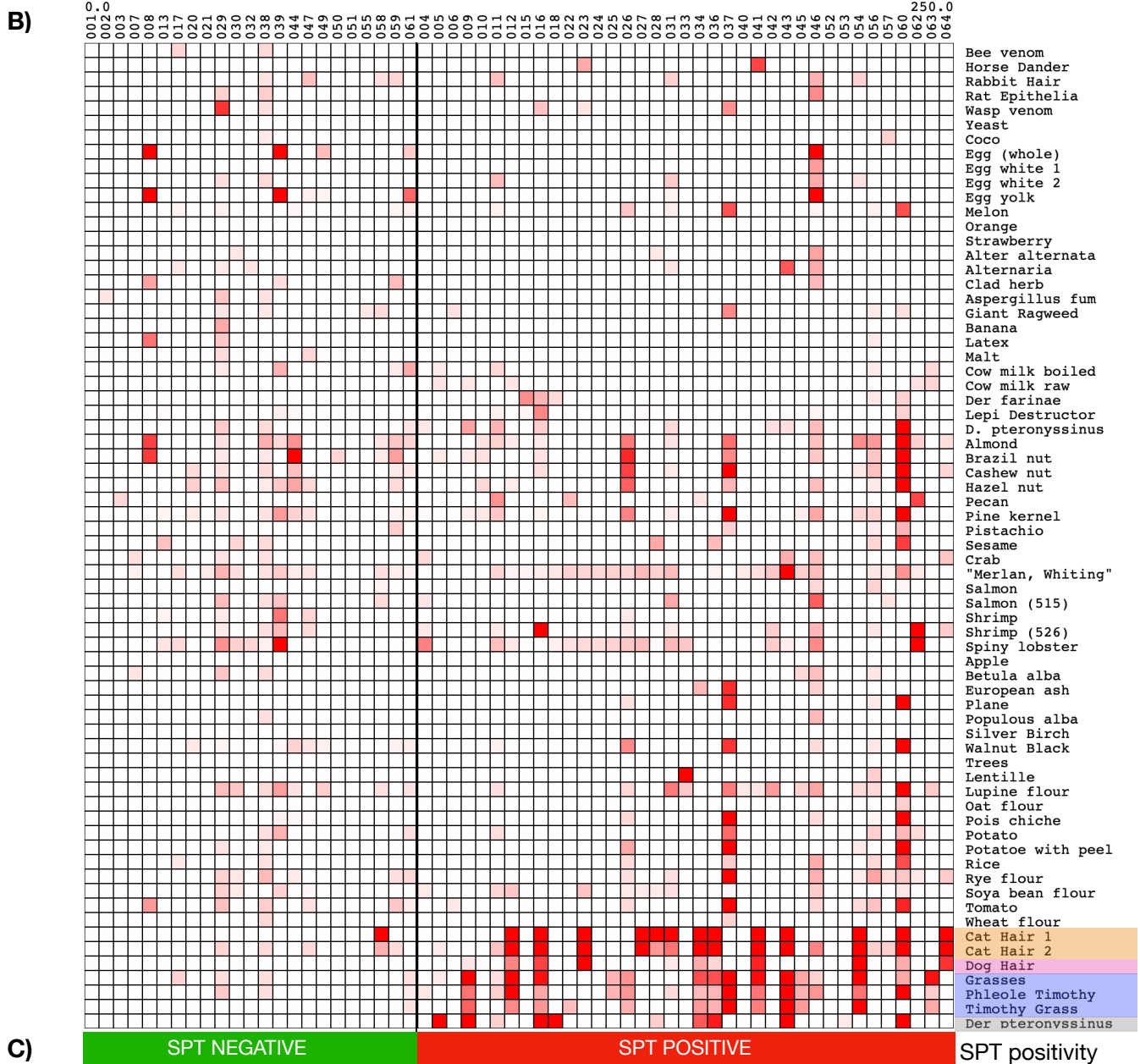
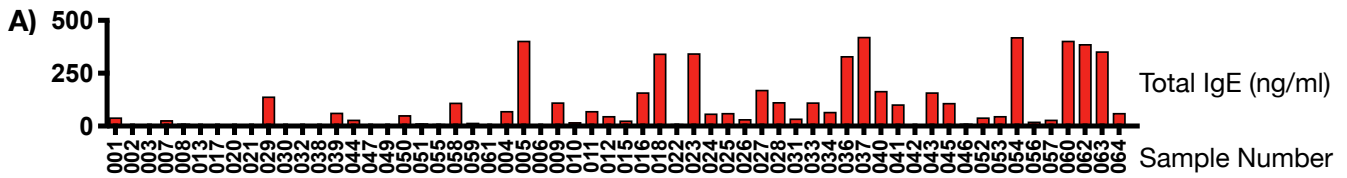


Figure 5 revised



Array-based measurements of aero-allergen-specific IgE correlate with skin-prick test reactivity in asthma regardless of specific IgG4 or total IgE measurements.

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Running title: SPT correlates well with specific IgE alone

ABSTRACT

Skin prick testing (SPT) and measurement of serum allergen-specific IgE (sIgE) are used to investigate asthma and other allergic conditions. Measurement of serum total IgE (tIgE) and allergen-specific IgG4 (sIgG4) may also be useful. The aim was to ascertain the correlation between these serological parameters and SPT. Sera from 60 suspected asthmatic patients and 18 healthy controls were assayed for sIgE and sIgG4 reactivity against a panel of 70 SPT allergen preparations, and for tIgE. The patients were also assessed by skin prick tests for reactivity to cat, dog, house dust mite and grass allergens. Over 50% of the patients had tIgE levels above the 75th percentile of the controls. 58% of patients and 39% of controls showed sIgE reactivity to ≥ 1 allergen. The mean number of allergens detected by sIgE was 3.1 in suspected asthma patients and 0.9 in controls. 58% of patients and 50% of controls showed sIgG4 reactivity to ≥ 1 allergen. The mean number of allergens detected by sIgG4 was 2.5 in patients and 1.7 in controls. For the patients, a strong correlation was observed between clinical SPT reactivity and serum sIgE levels to cat, dog, house dust mite (HDM) and grass allergens. SPT correlations using sIgE/sIgG4 or sIgE/tIgE ratios were not markedly higher. The measurement of serum sIgE by microarray using SPT allergen preparations showed good correlation with clinical SPT reactivity to cat, dog, HDM and grass allergens. This concordance was not improved by measuring tIgE or sIgG4.

Keywords: Allergic asthma, aero-allergen, IgG4, IgE, microarray, skin prick test

1. Introduction

In allergic asthma, sensitisation to potential allergens occurs when they cross epithelial barriers and are presented immunogenically by antigen presenting cells (APCs) that induce Th2-dominant responses (Galli et al., 2008). Both T and B regulatory cells play important roles in maintaining tolerance to allergens in non-allergic subjects and predominate both through natural allergen exposure and successful desensitising immunotherapy (Jutel et al., 2003; Oral et al., 2006; van de Veen et al., 2013). Both regulatory T cells (Treg) and regulatory B cells (Breg) produce immunosuppressive cytokines (particularly IL-10 and TGF β) that inhibit Th2 function and promote immunoglobulin class switching by B cells to produce IgG4 rather than IgE (Jeannin et al., 1998; Lebman and Edmiston, 1999; Chen et al., 2003; Akdis, 2012; van de Veen et al., 2013). In addition, IgG4 itself may serve a direct blocking or desensitising role by competing for allergen binding, combined with its inability to activate complement and its low affinity for Fc γ -receptors (Kemeny et al., 1986; Nakagawa, 1988; Calkhoven et al., 1991; Aalberse et al., 2009). Thus, allergen-specific IgG4 can serve as an indicator of tolerance due to its indirect association with regulatory lymphocyte activity and its direct allergen-neutralising activity. Indeed, high allergen-specific IgE/IgG4 ratios serve as a marker of allergic sensitization and, conversely, high IgG4/IgE ratios correlate with immune tolerance (Noh et al., 2007; Geroldinger-Simic et al., 2011; Okamoto et al., 2012). Accordingly, clinical trials have used high IgG4/IgE ratios as indicators of a positive clinical response to allergen-specific immunotherapy (Rossi et al., 2007; Jones et al., 2009; Lai et al., 2013).

Diagnostic procedures for allergic asthma begin with the patient's history, clinical symptoms and physical examination, followed by provocation tests and laboratory investigations. Thus, *in vivo* provocation tests and *in vitro* serological tests are used to confirm sensitization and identify the eliciting allergens (Deinhofer et al., 2004; Hamilton and Franklin Adkinson, 2004). Skin-prick testing can show good correlation with allergic disease: however, major drawbacks include identifying the clinically relevant allergens in a suitable soluble form, patient safety, test inapplicability in extensive skin diseases such as severe eczema, and the poor consistency in the clinical interpretation of results (Hamilton and Adkinson, 2003; Hamilton, 2010). In contrast, advantages of serological testing over skin prick testing

include lack of interfering bio-physiological or medical products that might affect provocation test results; laboratory tests are also more accurate and quantitative, safe for patients regardless of their age or clinical status, and samples can be handled, examined and stored for testing over the long-term. In addition, as there are hundreds of different identified allergens and an individual patient may be allergic to only one or a few of these, assessing all possible sensitivities by skin-prick testing is impractical.

Evaluation of IgE antibodies is the principal element in laboratory investigations of allergy because of the central role of IgE in triggering allergic reactions, and its level often correlates closely with concurrent allergic disease. Thus, the first step in the serological diagnosis of allergy is to quantify total IgE immunoglobulin levels in serum and then determine allergen-specific IgE antibody levels to identify the clinically relevant allergens. IgE measurement requires particularly accurate and sensitive assays as its serum concentration is typically low. Established assays for the detection and quantification of total and specific IgE include the radio-allergosorbent test (RAST) and ELISAs but, more recently, protein microarray technology has been applied to the detection and quantification of total and allergen-specific IgE (Bacarese-Hamilton et al., 2002; Fall et al., 2003; Jahn-Schmid et al., 2003; Deinhofer et al., 2004; King et al., 2007) with continued developments both commercially and academically (Melioli et al., 2011; Lupinek et al., 2014; Skrindo et al., 2015).

We previously reported the development of a autoantigen-specific microarray for the detection of autoantibodies (Shindi et al., 2017) and report here the development and application of a serological allergen microarray using commercial skin-prick test allergen preparations as the target antigens: these are all purified, natural allergens that detect allergen-specific IgE (sIgE) rather than recombinant antigens that may have structural and antigenic alterations. This approach also means that, if required, the allergen sensitivity of a patient can be confirmed by SPT using the same allergen preparation used in the laboratory assay. We also determined the levels of allergen-specific IgG4 (sIgG4) in the same serum samples using the allergen microarray, as well as the total IgE (tIgE) serum concentrations.

Serum samples from subjects who had undergone skin-prick testing for

reactivity to cat hair, dog hair, house dust mite and grasses were tested in the microarray assay to determine the degree of correlation between SPT and serum sIgE, and whether stronger correlation was seen between SPT and either the sIgE/sIgG4 ratio, or the sIgE/tIgE ratio.

2. Material and methods

2.1. Subjects

Serum from 60 individuals (age range 19-75 years) referred by general practitioners for specialist assessment of asthmatic status were collected at the Nottingham Biomedical Research Unit, City Hospital (Study ID: 53, Study Acronym: RCAM, RecReF: 12/EM/0241) under a study protocol approved in accordance with HTA regulations. Serum samples from 18 individuals (age range 51-81 years) who showed negative results by SPT (wheal diameter < 3mm) to standard allergens (cat hair, dog hair, grass pollen, tree pollen, house dust mite, *Aspergillus*) were collected at Leicestershire, Northamptonshire, Rutland (LNR), REC 10/H0406/65, under a protocol approved by the local research ethics committee: these samples were used as healthy (non-allergic) control subjects.

2.2. Total IgE Microarray

The Human IgE ELISA Quantitation Set (E80-108, Cambridge Biosciences) was used in an in-house microarray platform to quantify total IgE levels in serum samples. First, capture goat anti-human IgE (A80-108A) was diluted (20µg/ml) in printing buffer (PBS-Trehalose-Tween₂₀), transferred into a 384 well microtitre plate (Genetix, USA) and printed as regular arrays onto poly-L-lysine slides (Electron Microscopy Sciences, USA) using a Biorobotics Microgrid II 610 arraying robot. Subsequently, printed slides were blocked with 0.2% I-Block (Tropix, USA) for 1 hour. After incubation, blocking buffer was removed and the slides were washed 3 times with wash buffer (PBS, containing 0.05% Tween₂₀; PBS-tween). Eight serial dilutions (875 – 6.84 pg/ml) of IgE standard (RC80-108-6) were added to separate arrays. Serum samples (diluted 1:500 in antibody diluent (Dako, UK)) were added to separate arrays and incubated for 1 hour.

After incubation, the slides were washed as above and biotin-conjugated goat anti-human IgE antibody (0.25µg/ml; Vector Lab, UK) was incubated for 1 hour. Then, slides were washed again and 1:1000 diluted streptavidin-HRP (Bio- RAD, USA) was added for 15 min in the dark. Subsequently, the slides were washed as before and tyramide signal amplification reagent (Bio-Rad, UK) was added for 10 min in the dark (amplification reagent dilution ratio 4:1:4). Next, slides were washed 3 times with 20% DMSO (dimethyl sulfoxide, Sigma, UK) in PBS-Tween followed by 3 washes in PBS-0.05% Tween. Afterwards, streptavidin Cy5 (diluted 1:1000) (Ebioscience, UK) was added and incubated for 15 min in the dark. Slides were washed 3 times, then rinsed in ultrapure water for 3 min. Finally, slides were dried by centrifugation (1200rpm for 3 min) and scanned immediately with Axon GenePix® 4200AL scanner at 635nm.

2.3. Allergen-Specific Array

Human purified immunoglobulin standards (IgE, IgA, IgG, rIgG1, rIgG4) were serially diluted as described in PBS-trehalose-tween₂₀ and arrayed simultaneously onto poly-L-lysine slides in triplicate. Skin Prick Test (SPT) preparations (Table 1) were diluted two-fold in printing buffer and arrayed in triplicate. Printed slides were blocked with I-Block (Tropix, USA) for 1 hour and washed 3 times with PBS-0.05% Tween₂₀. Subsequently, 100µl of serum samples at the optimal dilution (1/100 for sIgE, 1/100 for sIgG4, 1/500 for tIgE) were added to the corresponding array. One array on each slide was used as a blank, where only the serum diluent (Dako, UK) was applied; samples were incubated for 1 hour. Afterwards, the slides were washed and 100µl of the corresponding biotinylated anti-human IgE or anti-human IgG4 detection antibodies was added per array and incubated for 1 hour. Then, slides were washed and 1:1000 diluted streptavidin-HRP (Bio- RAD, USA) was added for 15 min in the dark. Subsequently, the slides were washed as before and tyramide signal amplification reagent (Bio-Rad, UK) was added for 10 min in the dark (amplification reagent dilution ratio 4:1:4). Next, slides were washed 3 times with 20% DMSO (dimethyl sulfoxide, Sigma, UK) in PBS Tween followed by 3 washes in PBS-0.05% Tween₂₀. Afterwards, streptavidin Cy5 (diluted 1:1000) (Ebioscience, UK) was added and incubated for 15 min in the dark. Slides were washed 3 times, then rinsed in ultrapure water for 3 min. Finally, slides were dried by centrifugation (1200rpm for 3 min) and scanned immediately with Axon GenePix® 4200AL scanner at 635nm.

2.4. Statistical Analysis

Spot fluorescence was measured with Axon GenePix Pro 6 Microarray Image Analysis software (version 6, USA), and gpr files containing multi-parametric data were generated. Median fluorescence of each spot was measured (minus background). All spots with low signal and poor spot morphology (i.e., spot circularity < 65) were excluded. After filtration, data in gpr files were analysed in R (RPPAnalyzer, version 3.0.2, 2013).

Statistical analyses were performed using GraphPad Prism 7.0 software (GraphPad Software, USA). The correlation between ELISA and microarray techniques was performed with Pearson's r (two-tailed) test; subsequent multiple comparisons between the two techniques were done by the Wilcoxon t test. Comparisons between total IgE antibodies levels in the different serum groups were performed using median with interquartile range. A p -value ≤ 0.05 was considered statistically significant. Individual allergen-specific antibodies were presented semi-quantitatively by heat-maps and hierarchical clustering using Multiple Experiment Viewer Software (MEV, version 4.7.4). Correlations between SPT measurements and sIgE levels measured by microarray were determined by the non-parametric Spearman's Correlation test using SPSS software.

3. Results

3.1. Total and Allergen-Specific Assay development

To enable accurate and sensitive measurement of both total IgE (tIgE) and allergen-specific IgE (sIgE), two bespoke arrays were developed and validated. A variety of surface chemistries, as well as printing and blocking buffers, and amplification systems were tested. Figure 1a shows the greater signal sensitivity achieved with tyramide amplification compared to direct detection with streptavidin-Cy5. Numerous tests were conducted to validate the in-house arrays, including cross-reactivity of the detection antibodies (summarized in figure 1b). The assay performance was first tested using a small-scale trial of the validated assay with samples of known atopic

status. Inter- and intra-assay variation was within the acceptable limits (figure 1ci) and the correlation with ELISA was significant (figure 1cii).

3.2. Total IgE Measurements

Total IgE measurements were made in the serum samples of the suspected asthmatic patients and the healthy controls and two cutoffs were examined, the 95th and 75th percentiles of the healthy control group (Figure 2). For the suspected asthmatic patients, 36.7% had total IgE levels above the 95th percentile of the control group, whilst 53.3% had total IgE levels above the 75th percentile of the controls.

3.3. Allergen-Specific IgE Measurements

The heat-maps in figure 3 display the specific IgE (sIgE) reactivity profiles in the microarray against the 68 SPT allergens in the 60 patients and 18 controls. The subjects are ordered on the horizontal axis from high to low serum total IgE concentrations (left to right). Allergens are ordered alphabetically on the vertical axis (top to bottom). Based on the representation of data in the heat-maps, 58% of the patients show sIgE reactivity to at least one allergen (with many of these reactivities being strong), compared to 39% of the controls (in whom all the reactivities are medium to weak). The mean number of allergens detected by sIgE is 3.1 in the patients' serum samples and 0.9 in the control samples.

The subjects were also categorized into groups based on their serum levels of total IgE (high, medium, low): this gave three equally sized groups of patients (20 in each), and two groups of controls (6 medium and 12 low). Table 2 shows the percentages of patients and controls in the tIgE groups in whom sIgE to at least one allergen was detectable in the microarray, and the mean number of allergens detected by sIgE in the tIgE groups of patients and controls (based on the representation of data in the heatmaps). This shows that, as is subjectively apparent from the heat-maps in figure 3, the highest number of sIgE reactivities to allergens (and the strongest) are mainly present in the suspected asthmatic patients with the highest serum tIgE concentrations.

Amongst the allergens used in the microarray, 12 were each detected by sIgE in the sera of at least 10% of the patients (i.e. ≥ 6 patients): these were almond, both cat hair preparations, *Dermatophagoides pteronyssinus* dust mite, whole egg, egg yolk, grasses, lupine flour, both Timothy grass preparations, pine kernel, chickpea and

spiny lobster. Four of these same allergens were also detected (relatively weakly) by sIgE in at least 10% (≥ 2) of the control sera: these were both cat dander preparations, whole egg and spiny lobster.

3.4. Allergen-Specific IgG4 Measurements

The heat-maps in figure 4 display the specific IgG4 (sIgG4) reactivity profiles in the microarray against the 70 SPT allergens in the 60 patients and 18 controls. The ordering of subjects and allergens is the same as in figure 3. Based on the representation of data in the heat-maps, 58% of the patients show sIgG4 reactivity to at least one allergen (which is the same percentage as for sIgE), compared to 50% of the controls (which is a higher percentage than for sIgE). The mean number of allergens detected by sIgG4 is 2.5 in the patients' serum samples (which is lower than for sIgE) and 1.7 in the control samples (which is higher than for sIgE).

As discussed in the previous section for sIgE, Table 2 also shows the percentages of patients and controls in the tIgE groups in whom sIgG4 to at least one allergen was detectable in the microarray, and the mean number of allergens detected by sIgG4 in the tIgE groups of patients and controls (based on the representation of data in the heat-maps,). This shows that, in contrast to sIgE, and as is subjectively apparent from the heat-maps in figure 4, the sIgG4 reactivities to allergens are similarly represented in the high, medium and low tIgE groups. It is particularly apparent that high levels of sIgG4 to egg allergens (whole, white or yolk) are detectable in numerous patients and controls.

Amongst the allergens used in the microarray, 6 were each detected by sIgG4 in the sera of at least 10% of the patients (i.e. ≥ 6 patients): these were cashew nut, whole egg, both egg white preparations, egg yolk, hazel nut and rye flour. Six allergens were also each detected by sIgG4 in at least 10% (≥ 2) of the control sera: five of these were the same as those detected by sIgG4 in $\geq 10\%$ of patients ($\geq 10\%$ of controls showed sIgG4 reactivity to Brazil nut and black walnut, rather than cashew nut or hazel nut). Of these allergens, only whole egg and egg yolk were the same as those that were frequently detected by sIgE.

3.5. Comparison between SPT status and antibody measurements by microarray.

The general practitioner-referred suspected asthmatic patients were assessed by SPT for allergic reactivity to cat, dog, grass and house dust mite (HDM) allergens. Thirty-

seven of the 60 subjects showed clinically significant reactivity to at least one of these four SPT preparations. These data are presented in the bottom part of figure 5 as SPT wheal diameters (in mm), with the subjects divided into SPT-negative and SPT-positive. SPT positivity was defined as a wheal diameter ≥ 3 mm. Statistical analysis showed that there was significant correlation between clinical SPT positivity to cat and dog allergens ($r = 0.780$, $p < 0.0005$), indicating that numerous subjects were allergic to both. There were no other significant correlations between the SPT allergic reactivities to the four allergens. A small number of the SPT-positive samples also showed positivity for SPT to *Aspergillus* (five) or tree pollen (seven) (data not shown); just one of these (sample 046) did not show positivity to cat, dog, grass or HDM, but was positive to *Aspergillus* and so is included in the SPT positive group.

The upper part of figure 5 shows the corresponding serum tIgE concentrations for each subject. The central part of figure 5 shows the heat-map of the corresponding sIgE concentrations for each subject against the 70 allergens in the microarray assay; the allergens corresponding to those used in the clinical SPT (cat, dog, grasses, *Dermatophagoides pteronyssinus*) are shown at the bottom of the heat-map.

The heat-map in figure 5 shows that, in general, the SPT-positive subjects have more, and higher levels, of serum sIgE against a range of the allergens compared to the SPT-negative subjects; and this is particularly the case for allergens in the microarray corresponding to those used in the clinical SPT. For example, sample 060 (4th from the right in figure 5) was positive for clinical SPT to cat, dog and HDM; this sample was also positive for sIgE to cat, dog, HDM and grass and 22 other allergens included in the microarray. By contrast, sample 064 (on the extreme right of figure 5) was also positive by clinical SPT to cat, dog and HDM and was strongly positive for sIgE to cat and dog, and weakly positive to 5 other allergens. Numerous of the SPT-negative samples showed sIgE to none, or just a few, of the allergens (e.g. samples 001, 002, 003 and 007 on the extreme left of figure 5). However, other samples that were SPT-negative to cat, dog, grass and HDM were strongly positive for sIgE to other allergens: for example, sample 008 (5th from left in figure 5) showed sIgE reactivity to several allergens, including strong positivity to egg yolk, latex, almond and brazil nut.

A statistical analysis was performed to determine the degree of correlation between the clinical SPT positivity and the levels of serum sIgE to the corresponding allergens in the microarray assay. In addition, the corresponding calculations were also performed for correlation between clinical SPT positivity and the sIgE/sIgG4 ratios and the sIgE/tIgE ratios using the values from the microarray assays. These correlation coefficients and associated P-values are summarised in Table 3.

For the clinical SPT to cat allergen (Table 3a), a strong correlation is seen with sIgE alone and slightly higher still with the sIgE/tIgE ratio to both cat hair preparations used in the microassay. There were also correlations between the clinical SPT to cat and serum antibodies to dog hair allergen (Table 3a): this is consistent with the significant concurrence of clinical SPT reactivity to cat and dog allergens noted above. There were no significant correlations between SPT to cat allergen and serum antibodies to house dust mite or to grasses.

For the clinical SPT to dog allergen (Table 3b), the most significant correlation is seen with serum sIgE alone to dog hair allergen, but strong correlations are also seen with the sIgE/sIgG4 and sIgE/tIgE ratios to dog hair. SPT to dog allergen also correlates with serum antibodies to cat hair allergens (consistent with the concurrence of SPT to cat and dog allergens noted above), but not with serum antibodies to house dust mite or to grass allergens.

Significant correlation is seen between clinical SPT to grass allergen and serum sIgE alone to grasses, or the corresponding sIgE/sIgG4 and sIgE/tIgE ratios (Table 3c). Similar correlations are seen between clinical SPT to house dust mite allergen and serum antibodies to *Der pteronyssinus* (Table 3d); in this case the highest correlation coefficient is given by the sIgE/tIgE ratio. (The SPT preparation labelled 'D. pteronyssinus' showed relatively poor reactivity in the microarray, and so was not used for determining correlation with SPT.)

Overall, the results in Table 3 indicate that measurements of serum sIgE alone by microarray assay correlate very significantly with the clinical SPT reactivity to the corresponding allergens, and there is little or no advantage to be gained from using the serum sIgE/sIgG4 or sIgE/tIgE antibody ratios from the microarray data.

4. Discussion

In this study, we developed a bespoke microarray platform for detecting allergen-specific serum antibodies of defined isotypes, as well as for detecting serum total IgE. The development of this platform included testing different surface chemistries for the glass slides employed, as well as a variety of printing buffers, blocking buffers and amplification systems. This resulted in an assay system that was optimised for both sensitivity and specificity of antibody detection using small amounts of serum. It would clearly be of value to directly compare our bespoke assay with the commercially available assays for detecting allergen-specific sIgE that were outlined in Section 1 (Introduction).

The microarray platform was used to investigate the sera of 60 subjects with suspected asthma, and 18 non-allergic control subjects, for levels of total IgE immunoglobulins, as well as levels of allergen-specific IgE and IgG4 using panel of 70 SPT allergen preparations. Only about one third of the suspected asthmatic subjects had tIgE levels above the 95th percentile of the tIgE levels in the non-allergic controls (and about half above the 75th percentile, respectively). This may be partly because it is well known that the correlation between serum levels of total IgE and allergen-specific IgE are not absolute (Johansson et al., 2009; Hamilton et al., 2010; Grabenhenrich et al., 2016), as was also apparent in the present study (see figure 3). However, it is also likely that the breathing difficulties in a proportion of the subjects, as recorded by general practitioners, were not caused by allergic asthma; this is consistent with the findings that about one-third of these subjects did not have positive SPTs to any of the four common allergens cat, dog, grass or house dust mite (figure 5).

An advantage of the laboratory measurement of serum sIgE antibodies, particularly using a microarray platform, is the large number of allergens that can be tested at one time, compared with performing SPTs. This may highlight unusual, rare or multiple allergen reactivities which can then be further investigated clinically using SPT or allergen-challenge tests. This is exemplified in the present study, where some subjects were found to have many sIgE reactivities in the microarray assay (the largest number being 26 in subject 054 – see figure 3). Also, the identification of rare or unusual sIgE reactivities are exemplified in figure 3 by subject 044 who

showed high sIgE reactivity only to brazil nut out of the 70 allergens on the array; conversely, only one of the 60 subjects (062) showed strong sIgE reactivity to pecan.

Unlike sIgE (figure 3), sIgG4 (figure 4) showed no apparent relationship with tIgE levels, and was similarly prevalent in the suspected asthmatic subjects and the non-allergic controls. It is also interesting that serum IgG4 specific to egg allergens was found to be particularly prevalent, possibly as a result of egg being a prominent dietary component from an early age, and being a fairly strong allergen (Caubet and Wang, 2011).

It is apparent from figure 5 that SPT reactivity, to the four common allergens tested, generally corresponded with higher serum levels of total IgE and the detection of allergen-specific IgE. Conversely the SPT-negative subjects generally had low serum levels of total IgE and only a few showed strong reactivity of sIgE with allergens in the microarray panel. Many studies have examined the relationship between clinical SPT reactivity and laboratory measurements of allergen-specific serum IgE. These studies have variously reported high, moderate or low concordance (Cho et al., 2014; Mohammad et al., 2016; Chauveau et al., 2017; Griffiths et al., 2017; Scadding et al., 2017; Sookrung et al., 2019). This is not surprising given the very different physiological bases to the tests: SPT depends on the degree of expression of allergen-specific IgE antibodies on the surface of mast cells present in the skin epidermis, whereas laboratory assays detect sIgE in the circulation (Santos and Brough, 2017). The degree of correlation between SPT and serum sIgE measurements is also affected by numerous other factors, including the nature and sensitivity of the assays used, the patient population examined, and the types of allergens and the nature of their preparations. It should also be borne in mind that the half-life of specific IgE in the circulation is only about one-tenth that of mast cell-associated specific IgE in the skin (2 days versus 20 days) (Lawrence et al., 2017).

Some studies have also considered the influence of serum total IgE concentrations on the detection and significance of food allergen-specific IgE and have therefore investigated sIgE/tIgE concentration ratios (Mehl et al., 2005; Gupta et al., 2014; Grabenhenrich et al., 2016). In this context, the proportion of IgE specific for an allergen relative to the total IgE concentration may affect its representation on the surface of mast cells (and therefore its detection by SPT or by oral challenge) as well as the sensitivity of its detection in serum by laboratory assays. However, only one of the three studies referenced above found there to be improved diagnostic

power of sIgE/tIgE ratios compared to sIgE alone (Gupta et al., 2014), whereas the other two did not (Mehl et al., 2005; Grabenhenrich et al., 2016). On the other hand, lower sIgE/tIgE ratios have been reported to correlate with the response to treatment with the anti-IgE monoclonal antibody omalizumab (Johansson et al., 2009; Hamilton et al., 2010).

Levels of allergen-specific IgG4 has also been considered in numerous studies. As outlined in the Introduction, sIgG4 may directly compete with, and antagonise, the effects of sIgE, as well as higher sIgG4 levels being an indirect consequence of cell regulatory moves away from sIgE production. Indeed, many allergen desensitization studies have shown correlation of decreased allergen-specific sIgE/sIgG4 ratios (or increased sIgG4/sIgE ratios) with successful desensitization (Vazquez-Ortiz et al., 2014; Salmivesi et al., 2016; Stylianou et al., 2016; Sugimoto et al., 2016; Wright et al., 2016; Zeng et al., 2016; Akashi et al., 2017; Jones et al., 2017; Kukkonen et al., 2017; Palmer et al., 2017; Perez-Rangel et al., 2017; Perezabad et al., 2017; Wei-Liang Tan et al., 2017).

Therefore, in the present study, we used the advantages of the multiplexing possibilities of the microarray platform to measure SPT allergen-specific sIgE, sIgG4 and tIgE in all the suspected asthmatic subjects (both SPT positive and negative). We found good correlation between clinical SPT reactivity to cat, dog, house dust mite or grass allergens and the serum levels of sIgE to these allergens (table 3). For cat allergen, a slightly more significant correlation was observed with the sIgE/tIgE ratio than with sIgE alone; for dog allergen, a slightly higher correlation was seen with the sIgE/sIgG4 ratio than with sIgE alone. No improvement was seen with either ratio compared with sIgE alone for correlation to SPT for house dust mite or grass allergens. Overall, we found good correlation between serum allergen-specific IgE and clinical SPT measurements, with no clear advantage to be gained from additional measuring allergen-specific IgG4 or total IgE.

The absence of added value from measuring sIgG4 may seem surprising in view of the correlation of sIgE/sIgG4 ratios with successful allergen desensitization, as discussed above. However, a key difference is that, during desensitization, a change in allergic status is being monitored whereas, in our study, the allergic status of the subjects is likely to be relatively stable. In this 'stable' situation, levels of IgG4 may be less informative than when IgG4 levels are being actively raised in relation to the tolerizing effects of desensitization therapy. This proposal is consistent with a

closer examination of the sIgE and sIgG4 levels in Figures 3 and 4, respectively. For example, for the four major allergens also investigated by SPT (cat hair, dog hair, Der pteronyssinus and grasses), for which numerous individuals had high levels of sIgE, there was little or no expression of sIgG4 (only for cat hair did some individuals show high sIgG4). Furthermore, the findings in our study are consistent with the report that sIgG4/sIgE ratios do not improve prediction of peanut allergy (nor its severity) compared with sIgE alone (Datema et al., 2019).

Conclusion

The microarray we have developed using SPT allergen preparations for detection of allergen-specific serum IgE shows good correlation with *in vivo* SPT reactivity to cat, dog, house dust mite and grass allergens. The concordance of the microarray results with SPT was not clearly improved by also measuring total IgE or allergen-specific IgG4 in serum in addition to allergen-specific IgE. The microarray system described, facilitates the determination of serum IgE reactivity against a wide range of potential allergens, whose clinical significance can then be further investigated.

Funding

The Jones 1986 Charitable Trust contributed to the funding of this project. AH was funded by Omar Al-Mukhtar University, Libya.

Author contributions

Individual contributions to the work are as follows: conception and study design (LCF, AH, PJT, IT, TH, RJP); data acquisition and analysis (AH, LCF, PJT, IT, TH); drafting of the manuscript (AH, LCF, IT, PJT); editing of the manuscript (TH, RJP); critical revision of the manuscript (LCF, AH, PJT, IT, TH, RJP). All authors have approved the final manuscript.

Declaration of Competing Interest

The authors declare that they have no conflicts of interest.

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TABLE 1. Skin Prick Test solutions used in the microarray assay.

Skin Prick Test Solutions	Manufacturer
Dog Hair, Cat Hair, Plane Tree, Cat Hair, European ash, White poplar, 3-Trees (Alnus/Betula/Corylus), Wheat flour, Wasp venom, Potato with peel, Cow milk (raw), Cow milk (boiled), Almond, Silver Birch, Latex (Hevea brasiliensis), Oat flour, HDM (Dermatophagoides farina), HDM (Dermatophagoides pteronyssinus), Honey bee venom, Aspergillus fumigatus, Hazel nut, 6-Grasses (Avena, Dactylis, Poa, Festuca, Lolium, Phleum), Strawberry, Banana, Egg white, Horse Dander, Shrimp, Timothy Grass, Tomato, Cladosporium herbarum, Glycine max (Soya bean flour)	Soluprick, ALK-Abello Ltd, UK
Yeast, Brazil nut, Cashew nut, Salmon, Melon, Cantaloupe, Walnut, Black (food)	Hollister-Stier LLC
Giant Ragweed, Rat Epithelia, Shrimp, Salmon	MERCK, UK
Alter. alternata	LETIPharma, Portugal
Apple, Malt, Pistachio, Pine kernel, Sesame, Pecan, Rye (flour), Potato, Orange, Rice, Egg (white), Egg (yolk), Egg (whole), Coco, Rabbit Hair, Lepi. Destructor, D. pteronyssinus, Betula alba, Birch, Timothy grass, Alternaria, Chickpea, Lentille, Lupine flour, Spiny lobster, Crab, Merlan (Whiting)	Alyostal, Praha

TABLE 2. Recognition of allergens in the microarray by specific IgE (sIgE) and specific IgG4 (sIgG4) in the sera of the suspected asthmatic patients and the healthy controls in relation to the corresponding serum total IgE (tIgE) concentrations.

Serum tIgE conc.	sIgE		sIgG4		sIgE		sIgG4	
	% pts. with sIgE to ≥ 1 allergen	% ctrls. with sIgE to ≥ 1 allergen	% pts. with sIgG4 to ≥ 1 allergen	% ctrls. with sIgG4 to ≥ 1 allergen	Mean no. of allergens detected in pts.	Mean no. of allergens detected in ctrls.	Mean no. of allergens detected in pts.	Mean no. of allergens detected in ctrls.
High	90	N/A*	60	N/A	6.0	N/A	3.8	N/A
Mid	50	67	60	83	2.3	1.0	2.3	3.2
Low	35	25	55	33	1.0	0.8	1.5	1.0

*N/A – not applicable (because no control samples were in the ‘High tIgE’ group).

TABLE 3. Correlation coefficients for correlation between SPT reactivity and serum levels of sIgE alone, sIgE/sIgG4 or sIgE/tIgE determined by microarray assay.[†]

a) CAT HAIR SPT

	Cat hair Prep A	Cat hair Prep B	Dog hair	Der pter- onyssinus	Grasses	Timothy grass A	Timothy grass B
sIgE	0.621**	0.603**	0.666**	0.249	0.094	0.101	0.170
sIgE/sIgG4	0.455*	0.317	0.676**	0.262	0.266	0.317	0.304
sIgE/tIgE	0.727***	0.712***	0.515*	0.171	0.122	0.032	0.102

b) DOG HAIR SPT

	Cat hair Prep A	Cat hair Prep B	Dog hair	Der pter- onyssinus	Grasses	Timothy grass A	Timothy grass B
sIgE	0.703***	0.581**	0.784**	0.242	0.182	0.028	0.228
sIgE/sIgG4	0.484*	0.283	0.663***	0.174	0.129	0.126	0.259
sIgE/tIgE	0.758***	0.661***	0.650**	0.175	0.118	-0.005	-0.067

c) GRASS POLLEN SPT

	Cat hair Prep A	Cat hair Prep B	Dog hair	Der pter- onyssinus	Grasses	Timothy grass A	Timothy grass B
sIgE	0.386	0.296	0.339	0.168	0.707***	0.616**	0.704***
sIgE/sIgG4	-0.019	-0.185	0.010	0.202	0.664**	0.666**	0.741***
sIgE/tIgE	0.366	0.264	0.222	0.065	0.699***	0.545*	0.670**

d) HOUSE DUST MITE SPT

	Cat hair Prep A	Cat hair Prep B	Dog hair	Der pter- onyssinus	Grasses	Timothy grass A	Timothy grass B
sIgE	0.318	0.405*	0.158	0.694***	-0.101	-0.297	-0.095
sIgE/sIgG4	0.019	0.142	0.104	0.717***	-0.066	-0.039	-0.041
sIgE/tIgE	0.363	0.407*	0.128	0.778***	-0.069	-0.277	-0.161

* $P \leq 0.05$ for positive correlation

** $P \leq 0.01$ for positive correlation

*** $P \leq 0.001$ for positive correlation

[†]In each of the four tables (a-d), the columns indicate the target allergens in the microarray and the rows indicate the antibody isotypes detected in the microarray, either individually (sIgE) or as ratios (sIgE/sIgG4 or sIgE/tIgE). The table titles indicate the corresponding clinical SPTs for the correlation analysis. Significant correlations are shown in bold type. For example, in Table (a), sIgE antibodies specific for Cat hair Prep A show significant correlation with the clinical SPT results to Cat Hair with $r = 0.621$ ($p \leq 0.01$).

FIGURE LEGENDS

FIGURE 1. Development, validation and performance of microarrays for measuring total IgE (tIgE) and allergen-specific IgE and IgG4 (sIgE and sIgG4) in serum. A) Assay Development. A variety of surface chemistries, as well as printing and blocking buffers, and amplification systems were tested. i. Images represent the IgG standard curves, with (a) and without (b) amplification. ii. Graphical representation of the increased signal intensity observed with tyramide amplification. B) Assay Validation. Numerous tests were conducted to validate the in-house arrays, including cross-reactivity of the detection antibodies and setting of limits of detection. i. & ii. Comparison of cross-reactivity of two anti-IgE detection antibodies. C) Assay Performance. i. Inter and intra assay variation was within the acceptable limits. ii. The correlation with the gold-standard ELISA was significant.

FIGURE 2. Total IgE measurements in the two participant groups. Two cutoffs were examined, the 95th and 75th percentile of the ‘healthy control group’. N=60 suspected asthmatic patients, and N=18 healthy control subjects.

FIGURE 3. Heat-map displaying the specific IgE reactivity profiles against 68 SPT allergens in the 60 suspected asthmatic patients (A), and the 18 healthy control subjects (B). The subjects are ordered from highest to lowest total IgE levels (left to right), as indicated by the histograms at the top of the figure (tIgE concentrations in ng/ml). The allergens are listed on the vertical axis in alphabetical order, and the serum samples on the horizontal axis. Colour intensity is proportional to the sIgE reactivity, i.e. strong red – high; pale red – medium; white – negative. (‘Salmon’ was disregarded due to high non-specific reactivity with this preparation.)

FIGURE 4. Heat-map displaying the specific IgG4 reactivity profiles against 68 SPT allergens in the 60 suspected asthmatic patients (A), and the 18 healthy control subjects (B). The subjects are ordered from highest to lowest total IgE levels (left to right), as indicated by the histograms at the top of the figure (tIgE concentrations in ng/ml): this is identical to the order in figure 3. The allergens are listed on the vertical axis in alphabetical order, and the serum samples on the horizontal axis. Colour intensity is proportional to the sIgE reactivity, i.e. strong red – high; pale red –

medium; white – negative. ('Salmon' was disregarded due to high non-specific reactivity with this preparation.)

FIGURE 5. Results of serum IgE and SPT measurements in the 60 suspected asthmatic patients. A: Total IgE levels. B: Heat-map displaying the specific IgE reactivity profiles against 68 SPT allergens. Colour intensity is proportional to the sIgE reactivity, i.e. strong red – high; pale red – medium; white – negative. C: Clinical decision on SPT positivity (Red=positive or Green=negative). SPT positivity was defined as a wheal diameter ≥ 3 mm. D: Individual positivity (SPT response wheal diameter in mm) to each of 4 SPT allergens as indicated (cat, dog, grass, house dust mite). The allergens used in the microarray corresponding to the ones used for SPT are listed towards the bottom of the heatmap with colour coding corresponding to that used in the SPT histograms (orange – cat; pink – dog; blue – grass; grey – house dust mite). A small number of the SPT-positive sera were positive to tree pollen or *Aspergillus* (not show).