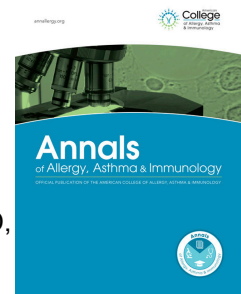


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TITLE: The Use of Microarray and Other Multiplex Technologies in the Diagnosis of Allergy

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Objective: To give an overview and describe strengths/weaknesses of IgE microarray and other multiplex assays that have been developed and are being used for allergy diagnostics.

Data Sources: Queries for IgE microarray and multiplex assays were conducted with PubMed and Google Scholar searching for primary articles and review papers.

Study Selections: We focused on articles written in English on commercially-available IgE multiplex assays which were reported in the allergy and immunology literature. **Results:** Several commercial IgE assays that use microarray or other multiplex technology have been developed and some have been implemented into clinical practice in Europe and Asia, with the ISAC being the most widely studied. Results of these assays generally correlate with results using “singleplex” IgE assays (eg , ImmunoCAP), though there can be variability between products and between allergens. A strength of the microarray technology is that IgE to a large number of allergens can be detected simultaneously in a single test and only a small amount of patient serum is required. Cost, inadequate sensitivity under some scenarios, and difficulties with data interpretation, in some cases of 100 or more allergens, can be limitations.

Conclusion: IgE microarray assays are already a valuable tool in research applications. These assays, and also other forms of IgE multiplex assays, are likely to play an important role in the clinical practice of allergy in the future. Additional studies focused on clinical outcomes and development of more targeted allergen panels could facilitate increased clinical use.

1 Introduction

Prior to the discovery of IgE as the “reaginic” substance in 1967, skin testing was the primary method for determining allergen sensitization. Since that time, serum-based *in vitro* tests have become an important tool for identifying specific IgE, a hallmark of allergic disease^{1, 2}. IgE testing is now conducted routinely in clinical laboratories with high sensitivity and specificity. Over the last two decades the emergence of molecular allergology has led to further advances in the field of IgE diagnostics^{3, 4}. Historically, *in vitro* IgE testing was limited to crude allergen extracts, which represent a complex mixture of many proteins and other biomolecules. However, many tests are now available which use individual, highly-purified allergen components on the solid phase of the assay. The use of allergen components has been referred to as component-resolved diagnostics (CRD), or more recently as precision allergy molecular diagnostics (PMAD), and has helped paved the way for high-throughput multiplex assays in which IgE to several allergens components (and in some cases extracts) can be measured simultaneously in a single assay^{3, 5}. Most IgE multiplex assays use microarray technology (*ie*, microchip-based), though multiplex bead-based platforms and other technologies have also been developed. These assays have not been introduced for routine clinical use in the USA, however a number of different multiplex assays are available in Europe and Asia. Our goal here is to give an overview of IgE microarrays and other multiplex assays, with a specific emphasis on the strengths and weaknesses of using these assays for clinical and research applications. A comprehensive analysis of all studies using multiplex assays is beyond the scope of this review. Rather, our aim has been to survey the literature and highlight major studies that have evaluated this emerging technology and to summarize key findings.

Microarray and other multiplex IgE assays: the nuts and bolts

Traditional assays for measuring allergen-specific IgE, including the ImmunoCAP from Thermo Fisher, the IMMULITE from Siemens, and the HYTEC-288 from Hycor Biomedical, have relied on a “singleplex” approach in which a single allergen extract or component is conjugated to the solid phase of the assay (see Fig 1)^{6,7}. Patient serum (or plasma) is incubated with the allergen-containing “resin”, and IgE which remains bound after a wash step can be detected by means of an anti-IgE secondary antibody linked to a luminescent or fluorescent tag. This approach has become the gold standard for *in vitro* IgE detection because it affords high sensitivity and specificity and also has a quantitative read-out which can be expressed in standardized units (1 kU_A/L = 2.4 ng/mL)^{4,8,9}. Currently, the lower level of detection of ImmunoCAP and other singleplex assays is 0.1 kU_A/L, with the well-established IgE class system ranging from class 0 to class 6 (Fig 1)⁹.

Despite the strengths of the singleplex assays, there are also limitations such as cost and the volume of serum required to investigate IgE to multiple allergens. Multiplex IgE assays have recently emerged as an alternative method for measuring IgE that can help address these shortcomings. Technological advances in chip-based microarrays and molecular allergology have made it possible to simultaneously detect IgE specific for a broad array of allergens in a single test. The microarray assays uses natural and/or recombinant allergen components on the microchip, though some recent assays also include extracts (Fig 1). It has been proposed that these assays will be useful for managing patients who are poly-sensitized and/or have complex clinical histories. Generally, the assay principle in multiplex IgE tests is similar to that of singleplex IgE tests (Fig 2)¹⁰. For microarray assays several allergens, in some cases over 100, are spotted onto specific sites of a single microchip. In turn, patient serum (or plasma) is added

to the face of the microchip, and IgE which remains bound to the microchip after a wash step can be detected by means of an anti-IgE secondary antibody linked to a luminescent or fluorescent tag. Subsequently the signal from each spotted allergen is measured with the use of a microarray scanner and an internal calibration curve. The IgE read-out can be semi-quantitative or quantitative, depending on the specific assay. Bead-based and immunoblot-based multiplex assays have also been developed. These differ in some details compared to microarray, but in general substitute a different solid phase substrate in lieu of a planar microchip. Considering all multiplex assays, a major difference compared with the singleplex approach is the amount of allergen that is conjugated to the solid-phase. Whereas singleplex IgE assays use microgram levels of allergen, multiplex assays often use considerably lower levels on the solid phase¹¹⁻¹³. One of the consequences of this difference is that multiplex assays can be less sensitive for detecting low-level IgE. The explanation for this relates to the fact that specific IgG and/or IgG4 antibodies can block access to allergen when the amount of allergen on the solid phase is limited. On the other hand, multiplex assays offer a number of advantages over singleplex assays in regard to the number of specific IgE that can be tested and the amount of serum sample required. Thus, multiplex assays could be particularly well suited for situations where sample volume is limited, such as the management of pediatric patients. The question of cost is not straightforward, however it has been estimated that for investigation of sensitization to 12 or more allergens that multiplex approaches could become cost-effective compared to singleplex assays^{14, 15}. Relative strengths and weakness of singleplex and multiplex IgE assays are described in Table 1 and discussed more extensively in a recent review of molecular allergology put together by an EAACI task force⁷.

The most widely used and best characterized multiplex IgE assay is almost certainly the

Immuno solid-phase allergen chip (ISAC) from Thermo Fisher. Originally introduced in the early 2000's and currently available for use in Europe and some other overseas markets (but not the United States), the assay has undergone some minor modifications since it was first introduced^{10, 16-18}. The current assay utilizes 112 allergen components representing over 50 allergen sources. The Med-ALL assay is an offshoot of the ISAC which includes an even greater array of allergen components, particularly targeting food, and has been used for research purposes^{13, 19}. Other commercial microarrays which have been described include the Allergy Explorer (ALEX) and the Microtest. The Multiplex IgE-QBA is a bead-based array and the EUROLINE is a multiplex test that is immunoblot-based. A brief description of these assays is given in Table 2. To date, none of these tests have been approved for clinical use in the United States. It is important to note that there are several other IgE multiplex assays which have been described and may be available in countries outside of the United States. A description of all such tests is outside the scope of the current article, but examples include the AdvanSure Alloscreen (LG Life Sciences, Seoul, Korea) and the "GOLD chip" microarray.²⁰

Performance of IgE microarray assays

There are two inter-related considerations when thinking about the analytical performance of multiplex assays. First, how do the results compare to the "gold" standard of *in vitro* IgE measurement - singleplex assays⁸? Second, how do multiplex assays perform in predicting and managing allergic disease? The latter question is likely most relevant to practicing clinicians. On the other hand, most studies have focused on assessing microarray results in relation to singleplex assays. An important consideration is that performance characteristics will vary for different allergens, and thus the utility of multiplex assays may also vary depending on the clinical scenario. This could be particularly important when evaluating food allergy where subtle

differences in component sensitization profiles can significantly impact management. To facilitate the discussion we have included separate sections for each of the major multiplex assays.

Recent studies that investigated ISAC

Several reports on the ISAC IgE microarray have been published over the last two decades^{17, 21-31}. Our focus here is on relatively recent papers, in part as the assay has changed over time and in part as Westwood and colleagues carried out a systematic review of relevant literature published prior to 2015¹⁵. Huss-Marp et al compared results of ISAC (using a cut-off 0.3 ISU/mL) with results of ImmunoCAP extract assays (using a cut-off 0.35 kU_A/L) in sera from 101 adults with hayfever³². For this study and others, ISAC was considered positive for a given allergenic source if any constituent component that was included on the microarray was positive. For the 12 allergen sources investigated agreement between the assays varied between 60 and 100%. For example, percent positive agreement (PPA) was high for Timothy grass (PPA = 100%), birch (PPA = 94%) and cat (PPA = 94%) but less so for dust mite (*D. pteronyssinus*) (PPA = 64%)³². The authors concluded that the lower correlation with mite could have reflected inadequate component representation in the ISAC microchip. They also indicated that the agreement between assays for dust mite improved when considered at a higher and perhaps more clinically relevant ImmunoCAP cut-off of 1.0 kU_A/L. Both of these conclusions were supported by Koch et al., who investigated 215 dust mite allergic patients and compared findings using ISAC and the ImmunoCAP extract assay to *D. pteronyssinus*³³. Importantly, that investigation also used the ALEX microarray and ImmunoCAP component testing. They found that overall ISAC sensitivity was 88.8%, but that sensitivity increased to 97.9% when considering samples with class 3 IgE (*ie*, >3.5 kU_A/L) or greater. They also determined that ISAC, which has the components Der p 1,

Der p 2, Der p 10 on the microarray (but not Der p 23), missed 4.7% of the cases who were mono-sensitized to Der p 23³³. Önell et al compared ISAC (and also Microtest) to ImmunoCAP using serum samples from 71 asthmatic children¹⁴. Concordance of ISAC with the ImmunoCAP was generally high, with Spearman's correlation values (Rs) exceeding 0.85 for IgE to each of cat, birch, Timothy grass and peanut. They did not report results for dust mite, but did find a more modest association for cow's milk (Rs = 0.80) and egg (Rs = 0.57), though the number of positive samples were fewer and levels of IgE to cow's milk and egg using ImmunoCAP were relatively low compared to the other allergens. Our group reported a similar finding in an investigation of children at mid-childhood who were part of a birth cohort³⁴. Agreement between ImmunoCAP and ISAC was quite strong for cat, dust mite and peanut, but strikingly lower for milk and egg. A secondary analysis of that data helps to illustrate a few points which are likely applicable to other multiplex assay: i) ISAC results correspond much better with ImmunoCAP at IgE levels of class 3 or above (as compared to class 1 or 2) and ii) when restricting the analysis to low-level IgE (ie, class 1 or 2) agreement can vary markedly for different allergens (**Table 3**). There are two major explanations that are likely involved in these findings. As previously mentioned, it is possible that not all relevant allergenic components are included on the microarray. Additionally, some allergens, particularly food, are recognized by high levels of concomitant IgG and IgG4, which can block IgE binding to the small amount of allergen present on the microchip. That latter explanation is important to be aware of if using microarray assays to study or manage a disease like eosinophilic esophagitis, where IgE sensitization is common but specific IgG4 levels can be very high^{35, 36}. An investigation of nut allergy in a Spanish cohort also suggests that regional variations in allergen sensitization can impact multiplex assay performance characteristics. In this Mediterranean population, where

sensitization to lipid transfer proteins is prevalent and Ara h 9 is an important peanut allergen, ISAC results were negative in 33% of the 39 peanut allergy cases that were investigated.³⁷

Studies that have investigated ALEX Explorer

The ALEX IgE “macroarray” was recently introduced and has been the subject of recent publications. Buzzilini et al. investigated sera from 120 subjects presenting for evaluation of respiratory or food allergy³⁸. ImmunoCAP extract assays were carried out on those sera in which it was clinically indicated and ALEX was run on all 120 samples. When comparing ALEX results with samples that tested positive by ImmunoCAP extract, there was strong agreement for grass pollen (22/26 = 85%), birch (20/23 = 87%), *D. pteronyssinus* (14/16 = 88%), peach (29/29 = 100%) and walnut (18/19 = 95%), but less agreement for mugwort (2/10 = 20%), ragweed (2/14 = 14%), peanut (6/19 = 32%) and hazelnut (13/18 = 72%). The authors suggested cross-reactive carbohydrate determinants (CCD) as a possible explanation in cases with lower agreement between the assays. Of note, CCDs present in some allergen extracts and/or naturally purified allergen components can be a cause of “false positive” results³⁹. The ALEX microarray addresses this by having a CCD inhibitor (*ie*, a MUXF3 glycoprotein) and also an internal CCD reference allergen (ISAC does not use an inhibitor but does have an internal CCD reference allergen)⁴⁰. Recently Hoang et al. investigated three different multiplex platforms focusing on nut sensitization⁴¹. As part of that analysis they found strong regression coefficients between ALEX and ImmunoCAP extract assays for peanut ($R^2 = 0.89$) and walnut ($R^2 = 0.92$), but less so for almond ($R^2 = 0.70$) and hazelnut ($R^2 = 0.66$). We are also aware of two studies that compared ALEX with ISAC^{40, 42}. Generally, both studies found good agreement between the two microarrays, albeit with varying discrepancies for some allergens.

Studies that have investigated Microtest

As previously mentioned, Önell et al compared Microtest (and also ISAC) to ImmunoCAP using serum samples from 71 asthmatic children¹⁴. Concordance of Microtest with the ImmunoCAP was generally high, and, similar to the performance of ISAC, Spearman's correlation values (Rs) exceeded 0.85 for IgE to each of cat, birch, Timothy grass and peanut. In keeping with results using ISAC, for cow's milk (Rs = 0.52) and egg (Rs = 0.64) the associations were less strong. A study by Williams et al that included 103 allergic adults investigated with Microtest and ImmunoCAP found that Spearman's correlation values for dust mite = 0.80, Timothy grass = 0.93 and peanut = 0.73²⁶.

Studies that investigated other multiplex assays

Di Fraia et al. reported on the performance of the EUROLINE customized pollen multiplex immunoblot that included both extracts and components²⁸. Spearman correlations with ImmunoCAP exceeded 0.9 for Timothy grass and birch and also for most of the major component allergens. The EUROLINE was also used as a comparator in the previously mentioned study by Hoang et al⁴¹. The key finding was that the EUROLINE reported more positive sensitizations to certain allergens, including hen's egg, cow's milk and almond, than either ALEX or the ISAC-related MeDALL chip. This may well be explained by the fact that the amount of allergen on the solid phase is greater for the immunoblot-based EUROLINE than for the microarray assays²⁸. The multiplex IgE-QBA from Indoor Biotechnologies is a bead-based array that can measure IgE to up to 11 allergens and also total IgE in a single test⁶. Working with a similar system, King et al. used a six-plex bead array to investigate sera from 95 allergic subjects and found reasonable correlations with results from ELISA for Der p 1 (Rs=0.80), Der p 2 (Rs = 0.88), Bet v 1 (Rs = 0.73), Phl p 5 (Rs = 0.76) and Fel d 1 (Rs = 0.85), but less so for Can f 1 (Rs = 0.44)⁴³. The authors noted that only 9 sera were sensitized to Can f 1, which could

explain the weak correlation. Pomponi et al used a similar 16-plex bead-based array and compared results with ISAC⁴⁴. Collectively, results of the bead-based array correlated well with ISAC ($R_s = 0.87$). It is important to note that the bead-based assays reported here used much more allergen on the solid phase, ranging from 0.1 μg to over 100 μg in some cases, than microarrays⁴³.

Clinical scenarios that could favor IgE microarray

In thinking about the utility of IgE microarrays there are certain clinical scenarios where the ability to test a large panel of allergens in a single test could have real advantages. These include work-up of idiopathic anaphylaxis⁴⁵, pollen-food allergy syndrome⁴⁶, local allergy⁴⁷⁻⁵⁰ and food allergy^{51, 52}. Heaps et al investigated 110 cases labeled as idiopathic anaphylaxis based on standard evaluation and found that ISAC testing identified likely culprits in an additional 20% of these cases²⁵. The pollen-food allergy syndrome, often also referred to as the oral allergy syndrome, can present diagnostic challenges because there are many allergens in pollen and food that can cross-react with each other. The large array of PR-10 and profilin components that are included in some microarrays can help address this with a single test⁵³. Another situation where multiplex testing could be advantageous is for the diagnosis of local allergic disease, where test samples (*eg*, tears, nasal secretions) are precious and the volume that can be obtained would be insufficient for testing for IgE to multiple allergens by singleplex. A role for IgE microarray has also been proposed for monitoring allergy immunotherapy⁵⁴. Although IgE levels do not typically drop significantly during allergy immunotherapy (AIT) (as determined by ImmunoCAP studies), the amount of IgE detected will decrease when detected by microarray. Seemingly a paradox, this finding is explained by the much smaller amount of allergen on the solid phase of

the microarray as compared to singleplex. As a result, IgE binding to the microchip can more readily be inhibited by “blocking” antibodies of the IgG1 and/or IgG4 subclass which are induced by AIT, a phenomena which does not occur with the high-capacity ImmunoCAP^{55, 56}. An alternative approach to monitoring AIT would be to directly measure levels of IgG and/or IgG4, as is possible with some microarrays (*eg*, ISAC). This has been taken advantage of by groups studying AIT⁵⁷ and also in studies on the natural history of allergy development⁵⁸

Future Developments

Multiplex IgE assays have made major strides over the last 2 decades and it is likely that there will be more developments which will help address unmet needs of practicing allergists. The diagnosis and management of food allergy is complicated by the fact that IgE sensitization is relatively common on a population basis, and there are rarely ideal IgE cut-offs that have both high sensitivity and high specificity for predicting clinical reactions⁵⁹. Further understanding of which components are associated with allergic reactions will be facilitated by use of multiplex assays in research studies. In addition, microarray assays have already been utilized in research settings to assess differences in IgE binding to linear versus conformational allergen epitopes^{4, 52}, with current evidence suggesting that IgE to linear epitopes carries greater risk for clinical reactivity⁵¹. This line of work could open up novel diagnostic opportunities that could significantly improve patient care.

To date, many of the major IgE multiplex assays that have been developed and studied involve large allergen panels (*eg*, ISAC, MeDALL, ALEX). These represent powerful research tools that will undoubtedly shed novel insights into the causes of allergic disease. On the other hand, it is not clear that measurement and interpretation of 112 or an even greater number of

specificities of IgE antibodies will be practical for day-to-day allergy care. By contrast, microchip or bead-based multiplex assays that could measure IgE to a focused allergen panel could provide significant benefit. For example, a mini-array for measuring IgE to peanut and tree nut components could have great utility in the management of a patient with confirmed or suspected nut allergy. Such a tool could facilitate testing for high-risk (eg, Ara h 1 or Ara h 2) versus lower-risk peanut sensitization (eg, Ara h 8), and at the same time provide information about concomitant tree nut risk. Similarly, a mini-array of profilins and PR-10 molecules could aid in the management of a patient with suspected pollen-food allergy syndrome. There are already manufacturers who are moving into this niche, as exemplified by the EUROLINE immunoblot, and Multiplex IgE-QBA.

Conclusion

Although not currently available in the USA for clinical use, IgE microarray and other multiplex assays are emerging and offer some important advantages over traditional singleplex assays. In the short term the major utility of these assays likely resides in research studies, particularly for microarrays in cohort studies where large amounts of information can be gleaned from a limited biological sample. It will not be surprising to see further debates over the relative merits of currently available *in vitro* multiplex diagnostics, including the number of sensitizations that should be measured concurrently. However, over time we can expect to see these assays adopted as part of clinical care⁶⁰.

References

1. Platts-Mills TA, Heymann PW, Commins SP, Woodfolk JA. The discovery of IgE 50 years later. *Ann Allergy Asthma Immunol.* 2016;116:179-182.
2. Johansson SGO. The discovery of IgE. *J. Allergy Clin. Immunol.* 2016;137:1671-1673.
3. Steering Committee A, Review Panel M. A WAO - ARIA - GA(2)LEN consensus document on molecular-based allergy diagnosis (PAMD@): Update 2020. *World Allergy Organ J.* 2020;13:100091.
4. Hamilton RG, Hemmer W, Nopp A, Kleine-Tebbe J. Advances in IgE Testing for Diagnosis of Allergic Disease. *J Allergy Clin Immunol Pract.* 2020;8:2495-2504.
5. Treudler R, Simon JC. Overview of component resolved diagnostics. *Curr Allergy Asthma Rep.* 2013;13:110-117.
6. Chapman MD, Wuenschmann S, King E, Pomes A. Technological Innovations for High-Throughput Approaches to In Vitro Allergy Diagnosis. *Curr. Allergy Asthma Rep.* 2015;15:36.
7. Matricardi PM, Kleine-Tebbe J, Hoffmann HJ, et al. EAACI Molecular Allergy User's Guide. *Pediatr. Allergy Immunol.* 2016;27:1-250.
8. Ansotegui IJ, Melioli G, Canonica GW, et al. IgE allergy diagnostics and other relevant tests in allergy, a World Allergy Organization position paper. *World Allergy Organ J.* 2020;13:100080.
9. Popescu FD, Vieru M. Precision medicine allergy immunoassay methods for assessing immunoglobulin E sensitization to aeroallergen molecules. *World J Methodol.* 2018;8:17-36.
10. Deinhofer K, Sevcik H, Balic N, et al. Microarrayed allergens for IgE profiling. *Methods.* 2004;32:249-254.
11. Ahlgrim C, Gutermuth J, Onell A, et al. Comparison of Molecular Multiplex and Singleplex Analysis of IgE to Grass Pollen Allergens in Untreated German Grass Pollen-Allergic Patients. *J Investig Allergol Clin Immunol.* 2015;25:190-195.
12. van Hage M, Hamsten C, Valenta R. ImmunoCAP assays: Pros and cons in allergology. *J. Allergy Clin. Immunol.* 2017;140:974-977.
13. Lupinek C, Wollmann E, Baar A, et al. Advances in allergen-microarray technology for diagnosis and monitoring of allergy: The MeDALL allergen-chip. *Methods.* 2014;66:106-119.
14. Onell A, Whiteman A, Nordlund B, et al. Allergy testing in children with persistent asthma: comparison of four diagnostic methods. *Allergy.* 2016.
15. Westwood M, Ramaekers B, Lang S, et al. ImmunoCAP (R) ISAC and Microtest for multiplex allergen testing in people with difficult to manage allergic disease: a systematic review and cost analysis. *Health Technol Asses.* 2016;20:1-+.
16. Jahn-Schmid B, Harwanegg C, Hiller R, et al. Allergen microarray: comparison of microarray using recombinant allergens with conventional diagnostic methods to detect allergen-specific serum immunoglobulin E. *Clin. Exp. Allergy.* 2003;33:1443-1449.
17. Gadisseur R, Chapelle JP, Cavalier E. A new tool in the field of in-vitro diagnosis of allergy: preliminary results in the comparison of ImmunoCAP(c) 250 with the ImmunoCAP(c) ISAC. *Clin. Chem. Lab. Med.* 2011;49:277-280.

18. Hamilton RG. Microarray Technology Applied to Human Allergic Disease. *Microarrays (Basel)*. 2017;6.
19. Skrindo I, Lupinek C, Valenta R, et al. The use of the MeDALL-chip to assess IgE sensitization: a new diagnostic tool for allergic disease? *Pediatr Allergy Immunol*. 2015;26:239-246.
20. Jeon H, Jung JH, Kim Y, Kwon Y, Kim ST. Allergen Microarrays for In Vitro Diagnostics of Allergies: Comparison with ImmunoCAP and AdvanSure. *Ann Lab Med*. 2018;38:338-347.
21. Wohrl S, Vigl K, Zehetmayer S, et al. The performance of a component-based allergen-microarray in clinical practice. *Allergy*. 2006;61:633-639.
22. Ott H, Baron JM, Heise R, et al. Clinical usefulness of microarray-based IgE detection in children with suspected food allergy. *Allergy*. 2008;63:1521-1528.
23. D'Urbano LE, Pellegrino K, Artesani MC, et al. Performance of a component-based allergen-microarray in the diagnosis of cow's milk and hen's egg allergy. *Clinical and Experimental Allergy*. 2010;40:1561-1570.
24. Bronnert M, Mancini J, Birnbaum J, et al. Component-resolved diagnosis with commercially available D. pteronyssinus Der p 1, Der p 2 and Der p 10: relevant markers for house dust mite allergy. *Clinical and Experimental Allergy*. 2012;42:1406-1415.
25. Heaps A, Carter S, Selwood C, et al. The utility of the ISAC allergen array in the investigation of idiopathic anaphylaxis. *Clin. Exp. Immunol*. 2014;177:483-490.
26. Williams P, Onell A, Baldracchini F, Hui V, Jolles S, El-Shanawany T. Evaluation of a novel automated allergy microarray platform compared with three other allergy test methods. *Clin. Exp. Immunol*. 2016;184:1-10.
27. Griffiths RLM, El-Shanawany T, Jolles SRA, et al. Comparison of the Performance of Skin Prick, ImmunoCAP, and ISAC Tests in the Diagnosis of Patients with Allergy. *International Archives of Allergy and Immunology*. 2017;172:215-223.
28. Di Fraia M, Arasi S, Castelli S, et al. A new molecular multiplex IgE assay for the diagnosis of pollen allergy in Mediterranean countries: A validation study. *Clinical and Experimental Allergy*. 2019;49:341-349.
29. Sastre J, Landivar ME, Ruiz-García M, Andregnette-Rosigno MV, Mahillo I. How molecular diagnosis can change allergen-specific immunotherapy prescription in a complex pollen area. *Allergy*. 2012;67:709-711.
30. Patelis A, Borres MP, Kober A, Berthold M. Multiplex component-based allergen microarray in recent clinical studies. *Clin. Exp. Allergy*. 2016;46:1022-1032.
31. van Hage M, Schmid-Grendelmeier P, Skevaki C, et al. Performance evaluation of ImmunoCAP(R) ISAC 112: a multi-site study. *Clin. Chem. Lab. Med*. 2017;55:571-577.
32. Huss-Marp J, Gutermuth J, Schaffner I, et al. Comparison of molecular and extract-based allergy diagnostics with multiplex and singleplex analysis. *Allergo J Int*. 2015;24:46-53.
33. Koch L, Laipold K, Arzt-Gradwohl L, et al. IgE multiplex testing in house dust mite allergy is utile, and sensitivity is comparable to extract-based singleplex testing. *Allergy*. 2020;75:2091-2094.
34. Wilson JM, Workman L, Schuyler AJ, et al. Allergen sensitization in a birth cohort at midchildhood: Focus on food component IgE and IgG4 responses. *J. Allergy Clin. Immunol*. 2018;141:419-423 e415.

35. Schuyler AJ, Wilson JM, Tripathi A, et al. Specific IgG4 antibodies to cow's milk proteins in pediatric patients with eosinophilic esophagitis. *J. Allergy Clin. Immunol.* 2018;142:139-148 e112.
36. Wright BL, Kulis M, Guo R, et al. Food-specific IgG4 is associated with eosinophilic esophagitis. *J. Allergy Clin. Immunol.* 2016;138:1190-1192. e1193.
37. Goikoetxea MJ, D'Amelio CM, Martinez-Aranguren R, et al. Is Microarray Analysis Really Useful and Sufficient to Diagnose Nut Allergy in the Mediterranean Area? *J Investig Allergol Clin Immunol.* 2016;26:31-39.
38. Buzzulini F, Da Re M, Scala E, et al. Evaluation of a new multiplex assay for allergy diagnosis. *Clin. Chim. Acta.* 2019;493:73-78.
39. Altmann F. Coping with cross-reactive carbohydrate determinants in allergy diagnosis. *Allergo J Int.* 2016;25:98-105.
40. Heffler E, Puggioni F, Peveri S, Montagni M, Canonica GW, Melioli G. Extended IgE profile based on an allergen macroarray: a novel tool for precision medicine in allergy diagnosis. *World Allergy Organization Journal.* 2018;11.
41. Hoang JA, Celik A, Lupinek C, et al. Modelling the conversion between specific IgE test platforms for nut allergens in children and adolescents. *Allergy.* 2020.
42. Bojcukova J, Vlas T, Forstenlechner P, Panzner P. Comparison of two multiplex arrays in the diagnostics of allergy. *Clinical and Translational Allergy.* 2019;9.
43. King EM, Vailes LD, Tsay A, Satinover SM, Chapman MD. Simultaneous detection of total and allergen-specific IgE by using purified allergens in a fluorescent multiplex array. *J Allergy Clin Immunol.* 2007;120:1126-1131.
44. Pomponi D, Bernardi ML, Liso M, et al. Allergen micro-bead array for IgE detection: a feasibility study using allergenic molecules tested on a flexible multiplex flow cytometric immunoassay. *PLoS ONE.* 2012;7:e35697.
45. Cardona V, Guilarte M, Labrador-Horrillo M. Molecular diagnosis usefulness for idiopathic anaphylaxis. *Curr. Opin. Allergy Clin. Immunol.* 2020;20:248-252.
46. Ebo DG, Bridts CH, Verweij MM, et al. Sensitization profiles in birch pollen-allergic patients with and without oral allergy syndrome to apple: lessons from multiplexed component-resolved allergy diagnosis. *Clin. Exp. Allergy.* 2010;40:339-347.
47. Hochwallner H, Alm J, Lupinek C, et al. Transmission of allergen-specific IgG and IgE from maternal blood into breast milk visualized with microarray technology. *J Allergy Clin Immunol.* 2014;134:1213-1215.
48. Leonardi A, Borghesan F, Faggian D, Plebani M. Microarray-based IgE detection in tears of patients with vernal keratoconjunctivitis. *Pediatr. Allergy Immunol.* 2015;26:641-645.
49. Castelli S, Arasi S, Tripodi S, et al. IgE antibody repertoire in nasal secretions of children and adults with seasonal allergic rhinitis: A molecular analysis. *Pediatr. Allergy Immunol.* 2020;31:273-280.
50. Gokkaya M, Schwierzeck V, Tholken K, et al. Nasal specific IgE correlates to serum specific IgE: First steps towards nasal molecular allergy diagnostic. *Allergy.* 2020;75:1802-1805.
51. Caubet JC, Sampson HA. Beyond skin testing: state of the art and new horizons in food allergy diagnostic testing. *Immunol Allergy Clin North Am.* 2012;32:97-109.
52. Lin J, Bardina L, Shreffler WG, et al. Development of a novel peptide microarray for large-scale epitope mapping of food allergens. *J. Allergy Clin. Immunol.* 2009;124:315-322, 322 e311-313.

53. Alessandri C, Ferrara R, Bernardi ML, et al. Molecular approach to a patient's tailored diagnosis of the oral allergy syndrome. *Clinical and translational allergy*. 2020;10:22.
54. Shamji MH, Kappen JH, Akdis M, et al. Biomarkers for monitoring clinical efficacy of allergen immunotherapy for allergic rhinoconjunctivitis and allergic asthma: an EAACI Position Paper. *Allergy*. 2017;72:1156-1173.
55. Wollmann E, Lupinek C, Kundi M, Selb R, Niederberger V, Valenta R. Reduction in allergen-specific IgE binding as measured by microarray: A possible surrogate marker for effects of specific immunotherapy. *J. Allergy Clin. Immunol.* 2015;136:806-809 e807.
56. Schmid JM, Wurtzen PA, Dahl R, Hoffmann HJ. Pretreatment IgE sensitization patterns determine the molecular profile of the IgG4 response during up dosing of subcutaneous immunotherapy with timothy grass pollen extract. *J. Allergy Clin. Immunol.* 2016;137:562-570.
57. Uotila R, Kukkonen AK, Greco D, Pelkonen AS, Makela MJ. Peanut oral immunotherapy increases IgG4 to Ara h 1, 2, and 6 but does not affect IgG4 to other allergens. *Pediatr. Allergy Immunol.* 2019;30:248-252.
58. Schwarz A, Panetta V, Cappella A, et al. IgG and IgG4 to 91 allergenic molecules in early childhood by route of exposure and current and future IgE sensitization: Results from the Multicentre Allergy Study birth cohort. *J. Allergy Clin. Immunol.* 2016;138:1426-1433 e1412.
59. Chokshi NY, Sicherer SH. Interpreting IgE sensitization tests in food allergy. *Expert Rev. Clin. Immunol.* 2016;12:389-403.
60. Jensen-Jarolim E, Jensen AN, Canonica GW. Debates in allergy medicine: Molecular allergy diagnosis with ISAC will replace screenings by skin prick test in the future. *World Allergy Organization Journal*. 2017;10.

Table 1. Strengths and weaknesses of IgE microarray/multiplex assays in relation to singleplex (*eg*, ImmunoCAP) assays

Strength	Weakness
Information about several allergens with single test	Interpreting large amounts of data
Only requires small amount of biological sample	Cost
Can more readily account for IgE binding to cross-reactive carbohydrate determinants (CCD), by use of recombinant components, internal controls (<i>eg</i> , MUXF3) and/or CCD inhibitors*	Not currently available in US for clinical use
Small amount of allergen on solid-phase → May be prone to inhibition by blocking antibodies (<i>eg</i> , IgG or IgG4) and thus greater specificity for predicting allergic symptoms	Small amount of allergen on solid-phase → May be prone to inhibition by blocking antibodies (<i>eg</i> , IgG, IgG4) and thus lower sensitivity for detecting low-level IgE

*See adjunct on carbohydrate epitopes at www.allergen.org

Table 2. Examples of allergen IgE multiplex assays

Format	Assay	Notes*	References
Microarray	ISAC 112 (Thermo Fisher/Phadia)	Chip microarray with longest track record and most publications; includes 112 allergen components from 51 sources; available for clinical use in Europe; assay requires 30 μ L of sample and read-out is semi-quantitative in ISU; each component assayed in triplicate; The ISAC 112i includes α -Gal	17, 27, 31, 32
	Allergy Explorer - ALEX (MacroArrayDX, Wien, Austria)	Chip microarray which includes 157 allergen extracts and 125 allergen components; assay requires 100 μ L of sample and read-out is quantitative in kU_A/L ; each allergen assayed in singlicate; includes a CCD inhibitor (MUXF3 glycoprotein) and can also measure total IgE; some models are fully automated	38, 40
	Microtest (Microtest Dx, London, UK)	Chip microarray which includes 19 allergen extracts and 16 allergen components, representing 26 different allergen sources; assay requires 100 μ L of sample and read-out is quantitative in kU_A/L ; each allergen assayed in triplicate	14, 26
Immunoblot	EUROline (EUROIMMUN, Lübeck, Germany)	Chip immunoblot which includes 54 allergens; assay requires 100 μ L of sample read-out is semi-quantitative, with 6 classes corresponding to ImmunoCAP	28
Bead-based	Multiplex IgE-QBA (Indoor Biotechnologies, Charlottesville, VA, USA)	Bead-based array which uses Luminex xMAP system to measure total IgE and up to 11 allergen components; assay requires 50 μ L of sample read-out is quantitative in IU/mL	43, 44

*All of these assays have a calibrator curve and the description about quantitative versus semi-quantitative is not evidence-based

Table 3. Comparison of results with ImmunoCAP and ISAC among 300 children in a birth cohort pre-screened for having at least one IgE sensitization (adapted and modified from Wilson et al³⁴).

Allergen extract (ImmunoCAP) and components (ISAC)		ImmunoCAP Class 1-2	ImmunoCAP Class 3+	ImmunoCAP Any positive
<u>Rye grass (g5)</u> <i>Cyn d 1, Phl p 1, Phl p 2, Phl p 4, Phl p 5, Phl p 6, Phl p 11</i>	ImmunoCAP, n	59	22	81
	ISAC positive, n	41	21	62
	ISAC positive, %	69.5	95.5	76.5
<u>Dust mite (d2)</u> <i>Der f 1, Der f 2, Der p 10</i>	ImmunoCAP, n	63	122	185
	ISAC positive, n	36	114	150
	ISAC positive, %	57.1	93.4	81.1
<u>Cat dander (e1)</u> <i>Fel d 1, Fel d 2, Fel d 4</i>	ImmunoCAP, n	42	84	126
	ISAC positive, n	36	84	120
	ISAC positive, %	85.7	100	95.2
<u>Cow's milk (f2)</u> <i>Bos d 4, Bos d 5, Bos d 6, Bos d 8, Bos d lactoferrin</i>	ImmunoCAP, n	93	3	96
	ISAC positive, n	3	2	5
	ISAC positive, %	3.2	66.7	5.2
<u>Hen's egg white (f1)</u> <i>Gal d 1, Gal d 2, Gal d 4</i>	ImmunoCAP, n	44	6	50
	ISAC positive, n	5	4	9
	ISAC positive, %	11.4	66.7	18
<u>Peanut (f13)</u>	ImmunoCAP, n	52	33	85

<i>Ara h 1, Ara h 2, Ara h 3, Ara h 6, Ara h 8, Ara h 9</i>	ISAC positive, n	30	29	59
	ISAC positive, %	57.7	87.9	69.4

Table 4. Examples of applications where allergen microarray could enhance clinical care

Application	Comment	References
Idiopathic anaphylaxis	Causal allergens that were not otherwise apparent were identified in 20% of cases in a report by Heaps et al.	25, 45
Pollen-food allergy syndrome	An array of PR-10 and profilin components on the microarray can facilitate detailed investigation into IgE sensitization profiles.	27, 46, 53
Local Allergy	The small sample volume can facilitate analysis of IgE in secretions, such as tears and nasal fluid.	47-50
Monitoring allergy immunotherapy	The efficacy of allergy immunotherapy is often monitored by measuring specific IgG and/or IgG4 “blocking” antibodies. In multiplex assays that have small amounts of antigen on the solid-phase, this can be measured indirectly, as IgE levels will drop as blocking antibody levels increase. This phenomena is not true when using the high-capacity singleplex assays such as the ImmunoCAP.	55, 56
Food allergies	Assessment of component-specific sensitization profiles; identifying linear versus conformational allergen epitopes.	4, 52

FIGURE LEGENDS

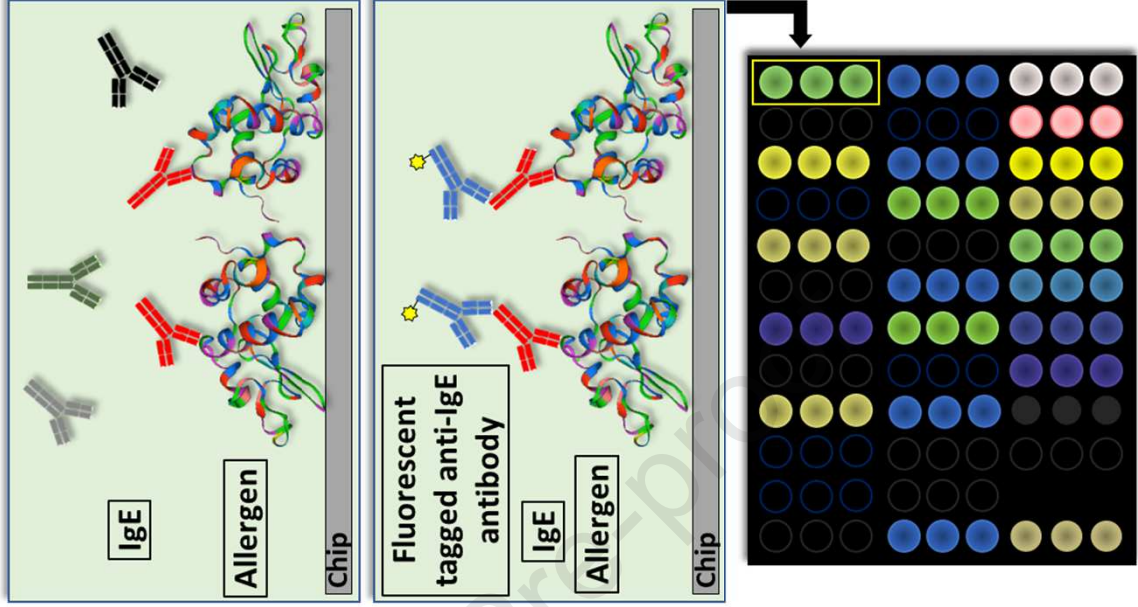
Fig 1. Comparison of representative singleplex and multiplex IgE assays*, where distinct allergens on the microchip are represented by unique colors.

* Here we have chosen the ImmunoCAP as the representative singleplex assay because it has a major market share of IgE assays conducted in North America and it also served as the reference assay for the majority of studies that evaluated multiplex assays. We chose ISAC as the representative multiplex assay because it is the microarray which has been most studied in the literature.

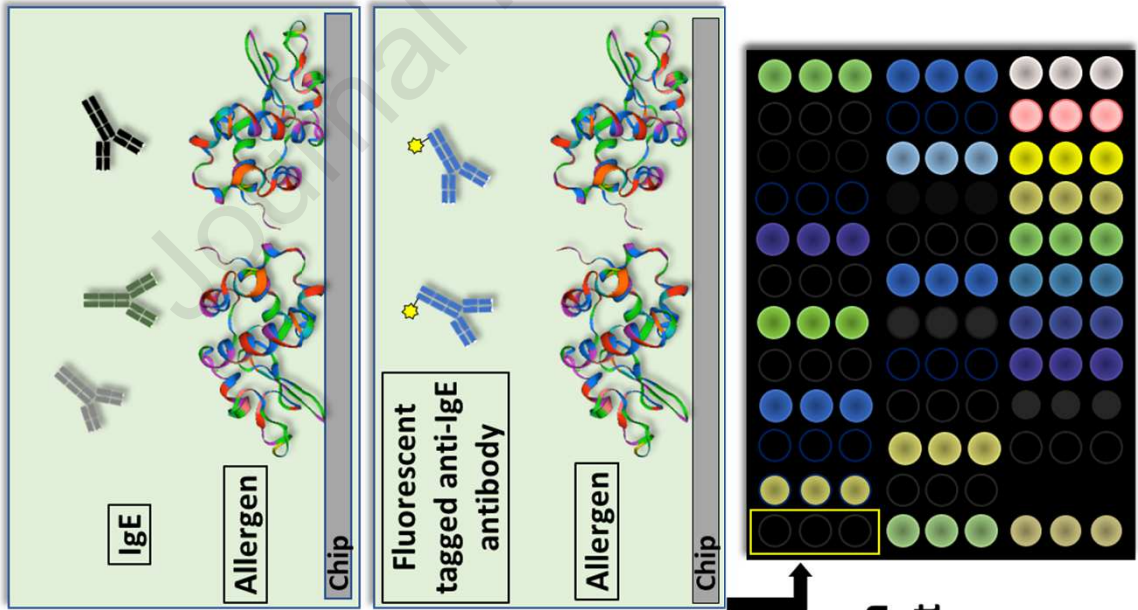
Fig 2. Layout and principles of IgE microarray assay. Top panels represent spots on a microchip that have been incubated with serum that has specific IgE or that lacks specific IgE to a given allergen component. The bottom panel shows two microchips that have been analyzed with a fluorescence microarray scanner (colors represent amount of specific IgE detected, ranging from black as negative to red/white as strong positive, as depicted by calibration curve on bottom of each microchip).

	Singleplex extract	Singleplex component	Multiplex components
			
Commercial assay	ImmunoCAP		ISAC
Materials	Cellulose matrix/resin		Polymer-coated glass slide
Amount of Allergen	~1-2 µg/ImmunoCAP		~100 pg/spot
Number of Allergens	mixture	1/ImmunoCAP	112/chip
Amount of serum	40 µl/ImmunoCAP		30 µl/chip
Sensitivity	0.1 kU _A /L		0.3 ISU
Interference from IgG	No		Yes
Read-out	Quantitative		Semi-quantitative
Assays unit and scale	Class 0: <0.1 kU _A /L Class 0/1: 0.1-0.34 kU _A /L Class 1: 0.35-0.70 kU _A /L Class 2: >0.70-3.5 kU _A /L Class 3: >3.5-17.5 kU _A /L Class 4: >17.5-50 kU _A /L Class 5: >50-100 kU _A /L Class 6: >100 kU _A /L		Low: 0.3-1.0 ISU/mL Moderate: >1.0-15 ISU/mL High: >15 ISU/mL

Microarray spot using serum sample
with IgE specific to allergen component



Microarray spot using serum sample
lacking IgE specific to allergen component



1°
phase

2°
phase

Read-out in
fluorescent
scanner

Learning Objectives: At the conclusion of this activity, participants should be able to:

- Appreciate that microarray and other multiplex IgE assays are an emerging technology that enable simultaneous measurement of IgE to several allergens in a single test
- Realize that multiplex IgE assays have certain strengths but also disadvantages compared to traditional skin testing and singleplex in vitro IgE assays

Q1. Which of the following is an example of an IgE microarray assay?

- A. ISAC
- B. IMMULITE
- C. HYTEC-288
- D. ImmunoCAP
- E. Skin prick testing

Q1 ANS: A. ISAC

Rationale:

Answers B, C and D represent clinically-approved, commonly used IgE singleplex assays. Answer E is an in vivo test. ISAC is an IgE microarray that is widely used in research applications and is in clinical use in Europe and some other countries.

References:

1. Chapman MD, Wuenschmann S, King E, Pomes A. Technological Innovations for High-Throughput Approaches to In Vitro Allergy Diagnosis. *Curr Allergy Asthma Rep.* 2015;15(7):36.
2. Hamilton RG, Hemmer W, Nopp A, Kleine-Tebbe J. Advances in IgE Testing for Diagnosis of Allergic Disease. *J Allergy Clin Immunol Pract.* 2020;8(8):2495-2504.

Q2. As compared to IgE singleplex assays, microarray assays have:

- A. A greater amount of allergen on the solid phase
- B. A smaller amount of allergen on the solid phase
- C. No allergen on the solid phase
- D. Only extracts on the solid phase
- E. Only one allergen on the solid phase

Q2 ANS: B. A smaller amount of allergen on the solid phase

Rationale:

IgE microarray assays have less allergen on the solid phase than singleplex assays. Although some microarray assays use extracts on the solid phase (eg, ALEX, Microtest), some use exclusively allergen components (eg, ISAC).

References:

1. van Hage M, Hamsten C, Valenta R. ImmunoCAP assays: Pros and cons in allergology. *J Allergy Clin Immunol*. 2017;140(4):974-977.
2. Ahlgrim C, Gutermuth J, Onell A, et al. Comparison of Molecular Multiplex and Singleplex Analysis of IgE to Grass Pollen Allergens in Untreated German Grass Pollen-Allergic Patients. *J Invest Allergol Clin Immunol*. 2015;25(3):190-195.

Q3. A reason why IgE to an allergen extract may be detected by a singleplex assay but not by microarray include(s):

- A. The relevant component is not included on the microarray
- B. The amount of allergen on the solid phase of the microarray is lower
- C. The presence of CCDs in the extract which are not present on recombinant components on the microarray
- D. The presence of blocking antibodies
- E. All of the above

Q3 ANS: E. All of the above

Rationale:

These are all reasons that a microarray may fail to detect IgE that was detected by a singleplex assay. If the IgE response is directed to a minor allergen component within an extract, and that component is not used on the microarray, then the microarray result will be negative. The lower amount of allergen on the solid phase of a microarray can cause the microarray to be less sensitive for some allergens, particularly at lower IgE levels and for those allergens that have high levels of specific IgG or IgG4. CCDs present in allergen extracts will not usually be present in allergen components that were expressed in recombinant systems. Because microarrays have lower amounts of allergen on the solid phase, low level IgE can more readily be inhibited by IgG and IgG4 blocking antibodies.

References:

1. Ansotegui IJ, Melioli G, Canonica GW, et al. IgE allergy diagnostics and other relevant tests in allergy, a World Allergy Organization position paper. *World Allergy Organ J*. 2020;13(2):100080.
2. Wollmann E, Lupinek C, Kundi M, Selb R, Niederberger V, Valenta R. Reduction in allergen-specific IgE binding as measured by microarray: A possible surrogate marker for effects of specific immunotherapy. *J Allergy Clin Immunol*. 2015;136(3):806-809 e807.

3. Hamilton RG. Microarray Technology Applied to Human Allergic Disease. Microarrays (Basel). 2017;6(1).

Q4. Measuring IgE with microarray may be more useful for monitoring success of allergy immunotherapy (AIT) than singleplex assays because of which principle(s)?

- A. There is less allergen on the solid phase of microarray
- B. IgE levels when measured with high-capacity ImmunoCAP stay relatively stable during AIT
- C. Specific IgG4 antibodies often increase during AIT
- D. Specific IgG4 block IgE binding when the amount of allergen on the assay is limiting
- E. All of the above

Q4 ANS: E. All of the Above

Rationale:

All of the listed answers are relevant to understanding why IgE microarrays could have advantages over singleplex assays for monitoring allergy immunotherapy. The key concept relates to the fact that the amount of allergen is limiting in microarray assays, which is not true in high-capacity singleplex assays where allergen is in excess.

References:

1. Wollmann E, Lupinek C, Kundi M, Selb R, Niederberger V, Valenta R. Reduction in allergen-specific IgE binding as measured by microarray: A possible surrogate marker for effects of specific immunotherapy. J Allergy Clin Immunol. 2015;136(3):806-809 e807.
2. Schmid JM, Wurtzen PA, Dahl R, Hoffmann HJ. Pretreatment IgE sensitization patterns determine the molecular profile of the IgG4 response during updosing of subcutaneous immunotherapy with timothy grass pollen extract. J Allergy Clin Immunol. 2016;137(2):562-570.

Q5. The possibility of IgE binding to a CCD is more of a problem when:

- A. The assay uses a CCD inhibitor
- B. The assay uses components that were expressed in a recombinant system
- C. The assay incorporates a known CCD, such as MUXF3, as an internal control
- D. The assay uses synthetic peptides
- E. The assay uses natural extracts

Q5 ANS: E. The assay uses natural extracts

Rationale:

Answers A, B and D relate to features that minimize the possibility that CCDs are present.

Answer C provides a control to account for the presence of CCDs. Assays that only use natural extracts are liable to be influenced by IgE binding to CCDs.

References:

1. Hamilton RG. Microarray Technology Applied to Human Allergic Disease. Microarrays (Basel). 2017;6(1).
2. Heffler E, Puggioni F, Peveri S, Montagni M, Canonica GW, Melioli G. Extended IgE profile based on an allergen macroarray: a novel tool for precision medicine in allergy diagnosis. World Allergy Organization Journal. 2018;11.
3. Stemeseder T, Klinglmayr E, Moser S, et al. Cross-sectional study on allergic sensitization of Austrian adolescents using molecule-based IgE profiling. Allergy. 2017;72(5):754-763.

1 Introduction

Prior to the discovery of IgE as the “reaginic” substance in 1967, skin testing was the primary method for determining allergen sensitization. Since that time, serum-based *in vitro* tests have become an important tool for identifying specific IgE, a hallmark of allergic disease^{1, 2}. IgE testing is now conducted routinely in clinical laboratories with high sensitivity and specificity. Over the last two decades the emergence of molecular allergology has led to further advances in the field of IgE diagnostics^{3, 4}. Historically, *in vitro* IgE testing was limited to crude allergen extracts, which represent a complex mixture of many proteins and other biomolecules. However, many tests are now available which use individual, highly-purified allergen components on the solid phase of the assay. The use of allergen components has been referred to as component-resolved diagnostics (CRD), or more recently as precision allergy molecular diagnostics (PMAD), and has helped paved the way for high-throughput multiplex assays in which IgE to several allergens components (and in some cases extracts) can be measured simultaneously in a single assay^{3, 5}. Most IgE multiplex assays use microarray technology (*ie*, microchip-based), though multiplex bead-based platforms and other technologies have also been developed. These assays have not been introduced for routine clinical use in the USA, however a number of different multiplex assays are available in Europe and Asia. Our goal here is to give an overview of IgE microarrays and other multiplex assays, with a specific emphasis on the strengths and weaknesses of using these assays for clinical and research applications. A comprehensive analysis of all studies using multiplex assays is beyond the scope of this review. Rather, our aim has been to survey the literature and highlight major studies that have evaluated this emerging technology and to summarize key findings.

Microarray and other multiplex IgE assays: the nuts and bolts

Traditional assays for measuring allergen-specific IgE, including the ImmunoCAP from Thermo Fisher, the IMMULITE from Siemens, and the HYTEC-288 from Hycor Biomedical, have relied on a “singleplex” approach in which a single allergen extract or component is conjugated to the solid phase of the assay (see Fig 1)^{6,7}. Patient serum (or plasma) is incubated with the allergen-containing “resin”, and IgE which remains bound after a wash step can be detected by means of an anti-IgE secondary antibody linked to a luminescent or fluorescent tag. This approach has become the gold standard for *in vitro* IgE detection because it affords high sensitivity and specificity and also has a quantitative read-out which can be expressed in standardized units (1 kU_A/L = 2.4 ng/mL)^{4,8,9}. Currently, the lower level of detection of ImmunoCAP and other singleplex assays is 0.1 kU_A/L, with the well-established IgE class system ranging from class 0 to class 6 (Fig 1)⁹.

Despite the strengths of the singleplex assays, there are also limitations such as cost and the volume of serum required to investigate IgE to multiple allergens. Multiplex IgE assays have recently emerged as an alternative method for measuring IgE that can help address these shortcomings. Technological advances in chip-based microarrays and molecular allergology have made it possible to simultaneously detect IgE specific for a broad array of allergens in a single test. The microarray assays uses natural and/or recombinant allergen components on the microchip, though some recent assays also include extracts (Fig 1). It has been proposed that these assays will be useful for managing patients who are poly-sensitized and/or have complex clinical histories. Generally, the assay principle in multiplex IgE tests is similar to that of singleplex IgE tests (Fig 2)¹⁰. For microarray assays several allergens, in some cases over 100, are spotted onto specific sites of a single microchip. In turn, patient serum (or plasma) is added

to the face of the microchip, and IgE which remains bound to the microchip after a wash step can be detected by means of an anti-IgE secondary antibody linked to a luminescent or fluorescent tag. Subsequently the signal from each spotted allergen is measured with the use of a microarray scanner and an internal calibration curve. The IgE read-out can be semi-quantitative or quantitative, depending on the specific assay. Bead-based and immunoblot-based multiplex assays have also been developed. These differ in some details compared to microarray, but in general substitute a different solid phase substrate in lieu of a planar microchip. Considering all multiplex assays, a major difference compared with the singleplex approach is the amount of allergen that is conjugated to the solid-phase. Whereas singleplex IgE assays use microgram levels of allergen, multiplex assays often use considerably lower levels on the solid phase¹¹⁻¹³. One of the consequences of this difference is that multiplex assays can be less sensitive for detecting low-level IgE. The explanation for this relates to the fact that specific IgG and/or IgG4 antibodies can block access to allergen when the amount of allergen on the solid phase is limited. On the other hand, multiplex assays offer a number of advantages over singleplex assays in regard to the number of specific IgE that can be tested and the amount of serum sample required. Thus, multiplex assays could be particularly well suited for situations where sample volume is limited, such as the management of pediatric patients. The question of cost is not straightforward, however it has been estimated that for investigation of sensitization to 12 or more allergens that multiplex approaches could become cost-effective compared to singleplex assays^{14, 15}. Relative strengths and weakness of singleplex and multiplex IgE assays are described in Table 1 and discussed more extensively in a recent review of molecular allergology put together by an EAACI task force⁷.

The most widely used and best characterized multiplex IgE assay is almost certainly the

Immuno solid-phase allergen chip (ISAC) from Thermo Fisher. Originally introduced in the early 2000's and currently available for use in Europe and some other overseas markets (but not the United States), the assay has undergone some minor modifications since it was first introduced^{10, 16-18}. The current assay utilizes 112 allergen components representing over 50 allergen sources. The Med-ALL assay is an offshoot of the ISAC which includes an even greater array of allergen components, particularly targeting food, and has been used for research purposes^{13, 19}. Other commercial microarrays which have been described include the Allergy Explorer (ALEX) and the Microtest. The Multiplex IgE-QBA is a bead-based array and the EUROLINE is a multiplex test that is immunoblot-based. A brief description of these assays is given in Table 2. To date, none of these tests have been approved for clinical use in the United States. It is important to note that there are several other IgE multiplex assays which have been described and may be available in countries outside of the United States. A description of all such tests is outside the scope of the current article, but examples include the AdvanSure Alloscreen (LG Life Sciences, Seoul, Korea) and the "GOLD chip" microarray.²⁰

Performance of IgE microarray assays

There are two inter-related considerations when thinking about the analytical performance of multiplex assays. First, how do the results compare to the "gold" standard of *in vitro* IgE measurement - singleplex assays⁸? Second, how do multiplex assays perform in predicting and managing allergic disease? The latter question is likely most relevant to practicing clinicians. On the other hand, most studies have focused on assessing microarray results in relation to singleplex assays. An important consideration is that performance characteristics will vary for different allergens, and thus the utility of multiplex assays may also vary depending on the clinical scenario. This could be particularly important when evaluating food allergy where subtle

differences in component sensitization profiles can significantly impact management. To facilitate the discussion we have included separate sections for each of the major multiplex assays.

Recent studies that investigated ISAC

Several reports on the ISAC IgE microarray have been published over the last two decades^{17, 21-31}. Our focus here is on relatively recent papers, in part as the assay has changed over time and in part as Westwood and colleagues carried out a systematic review of relevant literature published prior to 2015¹⁵. Huss-Marp et al compared results of ISAC (using a cut-off 0.3 ISU/mL) with results of ImmunoCAP extract assays (using a cut-off 0.35 kU_A/L) in sera from 101 adults with hayfever³². For this study and others, ISAC was considered positive for a given allergenic source if any constituent component that was included on the microarray was positive. For the 12 allergen sources investigated agreement between the assays varied between 60 and 100%. For example, percent positive agreement (PPA) was high for Timothy grass (PPA = 100%), birch (PPA = 94%) and cat (PPA = 94%) but less so for dust mite (*D. pteronyssinus*) (PPA = 64%)³². The authors concluded that the lower correlation with mite could have reflected inadequate component representation in the ISAC microchip. They also indicated that the agreement between assays for dust mite improved when considered at a higher and perhaps more clinically relevant ImmunoCAP cut-off of 1.0 kU_A/L. Both of these conclusions were supported by Koch et al., who investigated 215 dust mite allergic patients and compared findings using ISAC and the ImmunoCAP extract assay to *D. pteronyssinus*³³. Importantly, that investigation also used the ALEX microarray and ImmunoCAP component testing. They found that overall ISAC sensitivity was 88.8%, but that sensitivity increased to 97.9% when considering samples with class 3 IgE (*ie*, >3.5 kU_A/L) or greater. They also determined that ISAC, which has the components Der p 1,

Der p 2, Der p 10 on the microarray (but not Der p 23), missed 4.7% of the cases who were mono-sensitized to Der p 23³³. Önell et al compared ISAC (and also Microtest) to ImmunoCAP using serum samples from 71 asthmatic children¹⁴. Concordance of ISAC with the ImmunoCAP was generally high, with Spearman's correlation values (Rs) exceeding 0.85 for IgE to each of cat, birch, Timothy grass and peanut. They did not report results for dust mite, but did find a more modest association for cow's milk (Rs = 0.80) and egg (Rs = 0.57), though the number of positive samples were fewer and levels of IgE to cow's milk and egg using ImmunoCAP were relatively low compared to the other allergens. Our group reported a similar finding in an investigation of children at mid-childhood who were part of a birth cohort³⁴. Agreement between ImmunoCAP and ISAC was quite strong for cat, dust mite and peanut, but strikingly lower for milk and egg. A secondary analysis of that data helps to illustrate a few points which are likely applicable to other multiplex assay: i) ISAC results correspond much better with ImmunoCAP at IgE levels of class 3 or above (as compared to class 1 or 2) and ii) when restricting the analysis to low-level IgE (ie, class 1 or 2) agreement can vary markedly for different allergens (**Table 3**). There are two major explanations that are likely involved in these findings. As previously mentioned, it is possible that not all relevant allergenic components are included on the microarray. Additionally, some allergens, particularly food, are recognized by high levels of concomitant IgG and IgG4, which can block IgE binding to the small amount of allergen present on the microchip. That latter explanation is important to be aware of if using microarray assays to study or manage a disease like eosinophilic esophagitis, where IgE sensitization is common but specific IgG4 levels can be very high^{35, 36}. An investigation of nut allergy in a Spanish cohort also suggests that regional variations in allergen sensitization can impact multiplex assay performance characteristics. In this Mediterranean population, where

sensitization to lipid transfer proteins is prevalent and Ara h 9 is an important peanut allergen, ISAC results were negative in 33% of the 39 peanut allergy cases that were investigated.³⁷

Studies that have investigated ALEX Explorer

The ALEX IgE “macroarray” was recently introduced and has been the subject of recent publications. Buzzilini et al. investigated sera from 120 subjects presenting for evaluation of respiratory or food allergy³⁸. ImmunoCAP extract assays were carried out on those sera in which it was clinically indicated and ALEX was run on all 120 samples. When comparing ALEX results with samples that tested positive by ImmunoCAP extract, there was strong agreement for grass pollen (22/26 = 85%), birch (20/23 = 87%), *D. pteronyssinus* (14/16 = 88%), peach (29/29 = 100%) and walnut (18/19 = 95%), but less agreement for mugwort (2/10 = 20%), ragweed (2/14 = 14%), peanut (6/19 = 32%) and hazelnut (13/18 = 72%). The authors suggested cross-reactive carbohydrate determinants (CCD) as a possible explanation in cases with lower agreement between the assays. Of note, CCDs present in some allergen extracts and/or naturally purified allergen components can be a cause of “false positive” results³⁹. The ALEX microarray addresses this by having a CCD inhibitor (*ie*, a MUXF3 glycoprotein) and also an internal CCD reference allergen (ISAC does not use an inhibitor but does have an internal CCD reference allergen)⁴⁰. Recently Hoang et al. investigated three different multiplex platforms focusing on nut sensitization⁴¹. As part of that analysis they found strong regression coefficients between ALEX and ImmunoCAP extract assays for peanut ($R^2 = 0.89$) and walnut ($R^2 = 0.92$), but less so for almond ($R^2 = 0.70$) and hazelnut ($R^2 = 0.66$). We are also aware of two studies that compared ALEX with ISAC^{40, 42}. Generally, both studies found good agreement between the two microarrays, albeit with varying discrepancies for some allergens.

Studies that have investigated Microtest

As previously mentioned, Önell et al compared Microtest (and also ISAC) to ImmunoCAP using serum samples from 71 asthmatic children¹⁴. Concordance of Microtest with the ImmunoCAP was generally high, and, similar to the performance of ISAC, Spearman's correlation values (Rs) exceeded 0.85 for IgE to each of cat, birch, Timothy grass and peanut. In keeping with results using ISAC, for cow's milk (Rs = 0.52) and egg (Rs = 0.64) the associations were less strong. A study by Williams et al that included 103 allergic adults investigated with Microtest and ImmunoCAP found that Spearman's correlation values for dust mite = 0.80, Timothy grass = 0.93 and peanut = 0.73²⁶.

Studies that investigated other multiplex assays

Di Fraia et al. reported on the performance of the EUROLINE customized pollen multiplex immunoblot that included both extracts and components²⁸. Spearman correlations with ImmunoCAP exceeded 0.9 for Timothy grass and birch and also for most of the major component allergens. The EUROLINE was also used as a comparator in the previously mentioned study by Hoang et al⁴¹. The key finding was that the EUROLINE reported more positive sensitizations to certain allergens, including hen's egg, cow's milk and almond, than either ALEX or the ISAC-related MeDALL chip. This may well be explained by the fact that the amount of allergen on the solid phase is greater for the immunoblot-based EUROLINE than for the microarray assays²⁸. The multiplex IgE-QBA from Indoor Biotechnologies is a bead-based array that can measure IgE to up to 11 allergens and also total IgE in a single test⁶. Working with a similar system, King et al. used a six-plex bead array to investigate sera from 95 allergic subjects and found reasonable correlations with results from ELISA for Der p 1 (Rs=0.80), Der p 2 (Rs = 0.88), Bet v 1 (Rs = 0.73), Phl p 5 (Rs = 0.76) and Fel d 1 (Rs = 0.85), but less so for Can f 1 (Rs = 0.44)⁴³. The authors noted that only 9 sera were sensitized to Can f 1, which could

explain the weak correlation. Pomponi et al used a similar 16-plex bead-based array and compared results with ISAC⁴⁴. Collectively, results of the bead-based array correlated well with ISAC ($R_s = 0.87$). It is important to note that the bead-based assays reported here used much more allergen on the solid phase, ranging from 0.1 μg to over 100 μg in some cases, than microarrays⁴³.

Clinical scenarios that could favor IgE microarray

In thinking about the utility of IgE microarrays there are certain clinical scenarios where the ability to test a large panel of allergens in a single test could have real advantages. These include work-up of idiopathic anaphylaxis⁴⁵, pollen-food allergy syndrome⁴⁶, local allergy⁴⁷⁻⁵⁰ and food allergy^{51, 52}. Heaps et al investigated 110 cases labeled as idiopathic anaphylaxis based on standard evaluation and found that ISAC testing identified likely culprits in an additional 20% of these cases²⁵. The pollen-food allergy syndrome, often also referred to as the oral allergy syndrome, can present diagnostic challenges because there are many allergens in pollen and food that can cross-react with each other. The large array of PR-10 and profilin components that are included in some microarrays can help address this with a single test⁵³. Another situation where multiplex testing could be advantageous is for the diagnosis of local allergic disease, where test samples (*eg*, tears, nasal secretions) are precious and the volume that can be obtained would be insufficient for testing for IgE to multiple allergens by singleplex. A role for IgE microarray has also been proposed for monitoring allergy immunotherapy⁵⁴. Although IgE levels do not typically drop significantly during allergy immunotherapy (AIT) (as determined by ImmunoCAP studies), the amount of IgE detected will decrease when detected by microarray. Seemingly a paradox, this finding is explained by the much smaller amount of allergen on the solid phase of

the microarray as compared to singleplex. As a result, IgE binding to the microchip can more readily be inhibited by “blocking” antibodies of the IgG1 and/or IgG4 subclass which are induced by AIT, a phenomena which does not occur with the high-capacity ImmunoCAP^{55, 56}. An alternative approach to monitoring AIT would be to directly measure levels of IgG and/or IgG4, as is possible with some microarrays (*eg*, ISAC). This has been taken advantage of by groups studying AIT⁵⁷ and also in studies on the natural history of allergy development⁵⁸

Future Developments

Multiplex IgE assays have made major strides over the last 2 decades and it is likely that there will be more developments which will help address unmet needs of practicing allergists. The diagnosis and management of food allergy is complicated by the fact that IgE sensitization is relatively common on a population basis, and there are rarely ideal IgE cut-offs that have both high sensitivity and high specificity for predicting clinical reactions⁵⁹. Further understanding of which components are associated with allergic reactions will be facilitated by use of multiplex assays in research studies. In addition, microarray assays have already been utilized in research settings to assess differences in IgE binding to linear versus conformational allergen epitopes^{4, 52}, with current evidence suggesting that IgE to linear epitopes carries greater risk for clinical reactivity⁵¹. This line of work could open up novel diagnostic opportunities that could significantly improve patient care.

To date, many of the major IgE multiplex assays that have been developed and studied involve large allergen panels (*eg*, ISAC, MeDALL, ALEX). These represent powerful research tools that will undoubtedly shed novel insights into the causes of allergic disease. On the other hand, it is not clear that measurement and interpretation of 112 or an even greater number of

specificities of IgE antibodies will be practical for day-to-day allergy care. By contrast, microchip or bead-based multiplex assays that could measure IgE to a focused allergen panel could provide significant benefit. For example, a mini-array for measuring IgE to peanut and tree nut components could have great utility in the management of a patient with confirmed or suspected nut allergy. Such a tool could facilitate testing for high-risk (eg, Ara h 1 or Ara h 2) versus lower-risk peanut sensitization (eg, Ara h 8), and at the same time provide information about concomitant tree nut risk. Similarly, a mini-array of profilins and PR-10 molecules could aid in the management of a patient with suspected pollen-food allergy syndrome. There are already manufacturers who are moving into this niche, as exemplified by the EUROLINE immunoblot, and Multiplex IgE-QBA.

Conclusion

Although not currently available in the USA for clinical use, IgE microarray and other multiplex assays are emerging and offer some important advantages over traditional singleplex assays. In the short term the major utility of these assays likely resides in research studies, particularly for microarrays in cohort studies where large amounts of information can be gleaned from a limited biological sample. It will not be surprising to see further debates over the relative merits of currently available *in vitro* multiplex diagnostics, including the number of sensitizations that should be measured concurrently. However, over time we can expect to see these assays adopted as part of clinical care⁶⁰.