Diagnostic characterization of respiratory allergies by means of a multiplex immunoassay.

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Abstract

Background: Allergic sensitization is commonly assessed in patients by performing the skin prick test (SPT) or determining specific IgE levels in blood samples with the ImmunoCAP assay, which measures each allergen and sample separately. This paper explores the possibility to investigate respiratory allergies with a high throughput method, the Meso Scale Discovery (MSD) multiplex immunoassay, measuring IgE levels in low volumes of blood. Methods: The MSD multiplex immunoassay, developed and optimized with standards and allergens from Radim, was validated against the SPT and the ImmunoCAP assay. For 18 adults (15 respiratory allergy patients and 3 controls), blood collection and the SPT were performed within the same hour. Results: Pearson correlations and Bland-Altman analysis showed high comparability of the MSD multiplex immunoassay and the ImmunoCAP assay, except for house dust mite. The sensitivity of the MSD multiplexed assay was [?]75% for most allergens compared to the SPT and ImmunoCAP assay. Additionally, the specificity of the MSD multiplex immunoassay was [?]80% - the majority showing 100% specificity. Only the rye allergen had a low specificity when compared to the SPT, probably due to cross-reactivity. The reproducibility of the MSD multiplex immunoassay, assessed as intra- and inter-assay reproducibility and biological variability between different sampling moments, showed significantly high correlations (r=0.943-1) for all tested subjects (apart from subject 13; r=0.65-0.99). Conclusion: The MSD multiplex immunoassay is a reliable method to detect specific IgE levels against respiratory allergens in a multiplexed and high throughput way, using blood samples as small as from a finger prick.

Main text (3499 words):

1. INTRODUCTION

Respiratory allergies (RA) are the most common allergies worldwide, still increasing in frequency and severity. The World Health Organization estimated patients suffering from allergic asthma (AA) at 235 million in 2013 and the numbers for allergic rhinitis (AR) at about 400 million in 2006.[1,2] Although the number of patients with RA drastically increased on a global scale, the increase is greatest in Western countries. In 2011, the European Federation of Allergy (EFA) reported that RA affected around 20-30% of the European population. More specifically, around 68 million patients suffered from AA and 113 million from AR.[3] To emphasize the increasing prevalence, The European Academy of Allergy and Clinical Immunology (EAACI) reported in 2015 that half the European population will suffer from chronic allergic diseases by 2025.[4]Alarmingly, a vast majority of individuals affected by allergies are children and young adults.[5] Although not being associated with high morbidity and mortality, RA has generated a burden on society due to both the impact on the quality of life and the cost of treatment. A quick and precise diagnosis to identify the causative allergens is of fundamental importance to establish the most adequate treatment.[6]

In addition to the history and the clinical examination, looking for IgE sensitization is an essential step in

the diagnosis of complaints compatible with allergy. Data suggests that over 50% of allergic patients are multi-sensitized, making diagnosis even more challenging.[7] Traditionally, two methods are applied:*in vivo* by executing a skin prick test (SPT) or *in vitro*by the detection of sIgE in blood by using the ImmunoCAP methodology.[8] SPT is quick and results are immediately known, but requires trained clinical personnel to perform it. Although SPT is sensitive, minimally invasive and modest in costs, *in vitro* methods like ImmunoCAP and ELISA have some advantages including direct quantitation, improved safety, and the possibility of long-term storage of specimens. Notwithstanding, the *in vitro* procedures present limitations that are an impediment for large studies and for prospective studies to be performed over several years. For instance, measurements for each allergen and sample has to be performed separately, which is time-consuming and is therefore associated with higher costs and increased possibility for technical errors. Comparing studies of the two above mentioned test methods, the SPT seems to be more sensitive (less false-negative results), while sIgE immunoassays seem to be more specific (less false-positive results).[9,10]

During the last decade, there was a gradual evolution from performing singleplex towards multiplex immunoassays. These multiplex immunoassays provide several advantages over singleplex immunoassays including increased efficiency at reduced expense, lower sample volume needed making it interesting from a pediatric standpoint, greater output (number of markers assessed) per sample volume and higher throughput predicting more detailed diagnostics, thereby facilitating personalized medicine.[11] Currently, various singleplex as well as multiplex immunoassays are available to investigate RA-associated immunological protein markers (e.g. by Luminex, Fireplex, Meso Scale Discovery (MSD)), but none provided an assay to assess sIgE levels in multiplex. In this study, we utilized the MSD multiplex immunoassays considering that this method ensures all the above-mentioned advantages, with only 25 µL of serum volume required for analysis. This particular advantage cannot be emphasized enough, since taking blood samples from children remains a demanding technique and ethically sensitive issue. By using the MSD multiplex immunoassay, a finger prick could be sufficient to collect enough sample volume to conduct the test. Likewise, biomonitoring studies could also benefit from this advantage. Additionally, the MSD multiplex immunoassay is a highly flexible method as you can interchange the allergen panel according to the individual needs of the patient. Both individual allergens and allergen mixtures could be spotted on either a complete or partial 96-well plate, making the test even more compelling for either individual diagnosis as well as for biomonitoring studies. Novel techniques like the ImmunoCAP Rapid Point-of-care, which can be performed on children and adults, still require 110µL of sample volume.[12,13] Moreover, this technique is not flexible as it operates via a fixed allergen panel.

To date, validation of multiplex immunoassays for *in vitro*testing in clinical settings is limited. In this regard, this study aimed to investigate the possibility of diagnosing respiratory allergies with the MSD multiplex immunoassay while simultaneously comparing this method to both an SPT and an ImmunoCAP assay. Statistical analysis demonstrated high comparability of the MSD multiplex immunoassay and the ImmunoCAP assay. Both tests appear reliable in terms of sensitivity and specificity. Moreover, the reproducibility of the MSD multiplex immunoassay was determined, including intra- and inter-assay variation and reproducibility over time between different sampling moments. The reproducibility parameters established significantly high Pearson correlations. Finally, a shorter version of the protocol, including 2h instead of overnight incubation with the samples, provides results within 8h after blood collection.

2. MATERIALS AND METHODS 2.1. Study population

The study population consisted of 18 adults (12 females and 6 males; age range 23-50 years; mean 34.8 ± 8.2 years). Three non-allergic controls and 15 patients, who suffered from at least one respiratory allergy, were included. Sampling was performed in August. All subjects were asked to stop with all anti-allergic medication for at least a week before sampling. Written informed consent was obtained from all individuals, the study was approved by the medical ethics committee UZA-UA (EC UZA 18/11/154).

2.2. Skin prick test (SPT)

Droplets of allergens, histamine (positive control) and a negative control were applied to the forearms of the

subjects. In each droplet, a small scratch was made into the skin. Skin reactions caused by the allergens were determined after 15 minutes by a medical doctor. The size (wheal: length and width) of redness (flare) on the skin around the allergens and controls was measured. The allergens used in this analysis are shown in Table 1.

2.3. Blood analysis

From each individual, two blood serum tubes of 10 mL were collected. One serum tube was sent to a clinical laboratory (Labo Rigo, Genk, Belgium), where the ImmunoCAP assay from Thermo Fisher Scientific (Waltham, MA, USA) was performed to measure the total IgE-level and specific IgE-levels for respiratory allergens, according to the manufacturer's protocol. The allergens analysed in this assay are shown in Table 1. The blood serum of the second tube was isolated, aliquoted and stored at -80°C for later use in the MSD multiplex immunoassay.

2.4. MSD immunoassay

The MSD multiplex immunoassay was used to measure the total IgE as well as specific IgE-levels. This method is based on the U-plex protocol of MSD (Rockville, MD, USA),[14] but was adapted specifically for IgE-measurements, using biotinylated allergens and IgE standards from Radim Diagnostics (Freiburg im Breisgau, Germany).

2.4.1 Multiplex sIgE and total IgE assay

Specific IgEs against either one particular respiratory allergen or a mixture of various respiratory allergens were tested with the MSD assay. Very frequent respiratory allergens were included, individually and in mixtures, as shown in Table 1. A food allergen mixture which can have an impact on respiratory functioning was also included (i.e. the mix for nut allergens). The assay was based on the binding of specific IgEs of the serum samples to the biotinylated allergens and the binding of the total IgE of the serum samples to a biotinylated capture antibody. The biotinylated allergens and capture antibody were supplied by Radim Diagnostics.

To perform the MSD assay, a U-plex 96-well (10-spot) plate was used. Each well contains ten individual spots and each spot can be linked to a different biomarker with specific linkers (see graphical abstract). It is possible to run a partial plate, but the protocol described below states the volumes for a full plate. First, nine specific allergens (to measure sIgEs) and an anti-IgE capture antibody (to quantify total IgE) were coupled to one of the 10 different linkers by adding 200 µL of the biotinylated allergen/capture antibody to 300 µL of the chosen linker containing a streptavidin binding site. All linker-solutions were incubated for 30 minutes at room temperature while shaking. Then 200 µL stop solution (from MSD) was added to prevent further binding and incubated for another 30 minutes. Afterwards, 600 µL of all 10 linker-allergen/antibody solutions were combined into a single tube. When less than 10 spots (allergens) were used, extra stop solution was added to get a total volume of 6 mL. Next, 50 µl of this solution was pipetted into each well to coat the spots on the plate with the appropriate allergen, followed by one hour incubation. Meanwhile, the standard curve solutions were prepared by diluting a stock of total IgE (from Radim) with diluent 43 (from MSD) to: 100 IU/mL, 33.33 IU/mL, 11.11 IU/mL, 3.70 IU/mL, 1.23 IU/mL, 0.41 IU/mL, 0.10 IU/mL and 0 IU/mL. Subsequently, the plate was washed three times with PBS-tween (PBS-T; 0.05%, 150 µL per well), and 25 µL of diluent 43 was added to each well. Then 25µL of the corresponding standard series solution or serum sample, containing specific IgEs, was added to the appropriate predetermined wells and incubated overnight at 4°C. The next day, the plate was washed and 50 µL of the custom-made sulfo-tagged detection antibody (2 µg/mL, diluted with diluent 3 from MSD) was added to each well, which completed the immunoassay 'sandwich'. This detection antibody was purchased from Radim Diagnostics and labelled with a sulfo-tag by MSD. After an hour of incubation, the plate was washed again with PBS-T and 150 µL of Read Buffer T was added to each well. Finally, the plate was read within the next 2-5 min and electrochemiluminescent (ECL) signals (at a wavelength of 620 nm) were detected with the MESO QuickPlex SQ 120 device from MSD. Using specific MSD-software, values of the ECL signals were generated as output data, which were converted to concentration values in IU/mL through calculations with the 4PL (four-parameter logistic) curve of the

total IgE, where IU/mL corresponds to kU/L (Figure S1).

2.4.2 Singleplex total IgE assay

Total IgE was also determined via a singleplex assay using a 96-well streptavidin plate (MSD). Again, the method was based on the manufacturer's protocol,[15] but adapted for IgE-measurements, using capture antibody, standards and detection antibody from Radim Diagnostics. First of all, the biotinylated anti-IgE capture antibody (from Radim Diagnostics) is diluted in diluent 100 (from MSD) to obtain a 1/17.5 ratio. The plate was washed three times with PBS-T. Then 25 µL of the solution was added to each well of the streptavidin plate, followed by incubation for an hour while shaking. Meanwhile, the standard series solutions (as described above) were prepared. After incubation, the plate was washed again and 25 µL of diluent 43 was added to each well. Subsequently, 25 µL of the corresponding standard series solution or serum sample was added to the predetermined well and incubated overnight at 4°C. The next day, the detection antibody and Read Buffer T were added (as described above) and the plate was read.

2.5. Data and statistical analyses

To analyse the results, descriptive statistics were performed. The linear correlations between the results of the clinical laboratory and the results of the MSD multiplex immunoassay were evaluated by the Pearson correlation coefficient (r). This coefficient was also used to determine the intra- and inter-assay reproducibility and the difference in incubation time of the MSD multiplex immunoassay. To assess the comparability of the ImmunoCAP assay and the MSD multiplex immunoassay, a Bland-Altman analysis was performed.[16] A Bland-Altman plot is a scatter plot of the difference between the two methods' means (y-axis) against the mean of the two methods (x-axis). The calculated mean difference is shown and the 95% confidence limits of agreement are obtained by calculating the mean difference plus or minus two times the standard deviation of the differences. The sensitivity and specificity of the MSD multiplex immunoassay were determined, both in comparison to the skin prick test and the ImmunoCAP assay of the clinical laboratory. The threshold set for a positive test in both the MSD and ImmunoCAP assay was 0.1 kU/L. When checking for outliers, subject 3 was eliminated from the analyses: positive scores were observed for all MSD multiplexed measurements (all sIgE-levels between 0.3 and 0.7 kU/L) and consistent negative scores for the other assays. Moreover, this person reported to be diagnosed with essential thrombocythemia (ET), which could explain the divergent results.

3. RESULTS & DISCUSSION

3.1 Total IgE

As mentioned in the Materials and Methods, one of the 10 spots in the MSD multiplex immunoassays was used to detect total IgE levels, which was essential to construct a standard curve. At the same time, this spot also detected total IgE levels in the wells containing individual serum samples. However, total IgE levels assessed for the individual samples in the multiplex assay were always lower compared to the ImmunoCAP measurements. An explanation for this is the range of the standard curve, which in the multiplex MSD assay went up to 100 kU/L. The total IgE levels were above this standard curve, resulting in underestimated concentrations (Table S1) when using the 4PL curve for conversion (Figure S1). As such, one could argue to omit this spot (to detect total IgE) from the wells in which serum samples are assessed. However, when this spot was left empty, it resulted in divergent levels of sIgE against the allergens in that same well, probably due to altered chemistry of the assay in those wells compared to the wells that quantified the standard curve and thus contained 10 'active' spots. Alternatively, serum samples were diluted 10x, which resulted in total IgE levels similar to ImmunoCAP, but sIgE levels against the allergens ended up below the detection limit in most cases. Diluting the samples 10x and analysing them in a parallel singleplex immunoassay allowed more accurate quantification of total IgE. These data indicate that 10x dilution of the samples is not an option for the MSD multiplex immunoassay to assess sIgE, and total IgE could not be measured accurately in the same well and is thus best analysed in a singleplex immunoassay. However, in clinical practice total IgE levels are only minimally taken into account when characterising specific allergies. Therefore, total IgE levels are not necessary to be determined in case one is interested in sIgE levels to detect specific (respiratory) allergies.

The rest of this paper will thus focus on the sIgE levels for the comparison of the various methods.

3.2. Comparison of the methods

The results of the MSD multiplex immunoassay were compared to the two most commonly used tests: the SPT and the ImmunoCAP assay, performed by a clinical laboratory. When an individual scored positive for the allergy it is shown in colour in Figure 1. When redness of the skin is observed in the SPT, it is indicated as positive. For the tests of the clinical lab and the MSD immunoassay, a threshold of 0.10 kU/L was used; a result above this threshold was considered positive. Both individual allergens and mixes of allergens were tested in parallel. The SPT measurements of subject 16 were excluded because this individual reacted to all pricks, including the negative control, which makes the results doubtful. This could be due to aspecific hyperreactivity of the skin. Most results corresponded well between the three tests, although there were some differences, as discussed later.

3.3. Correlations and Bland-Altman analysis

The linear correlations between the results of the clinical lab and the results of the MSD multiplex immunoassay are presented in Table 2. The Bland-Altman plots, which determine the comparability of these two methods, are shown in Figure 2. For both immune-assay analyses, the allergens of cow's milk, *C. herbarum* and the mixture of fungi were not included due to the lack of positives among the tested individuals (none were above 0.10 kU/L) for the MSD multiplex immunoassay (Table 2). For house dust mite no correlation was observed, and it showed also the least comparability to the ImmunoCAP assay according to the corresponding Bland-Altman plot. For the other allergens, significant correlations (r =0.829-0.978; P<0.001) were observed as well as good agreements with the SPT and ImmunoCAP assay, as shown in the corresponding Bland-Altman plots.

3.4. Sensitivity and specificity

The results of the MSD multiplex immunoassay were compared to the results of the ImmunoCAP assay and the SPT. The sensitivity and specificity of the MSD multiplex immunoassay were calculated (Table 3). Overall, the sensitivity of the MSD multiplexed assay was high for most allergens compared to the SPT and ImmunoCAP assay as references ([?]75%), however, for some sIgEs the sensitivity values were lower. As described above, dust mite allergy was not always detected using MSD (sensitivity: 60%). Furthermore, the sensitivity of the MSD assay detecting sIgE against dog epithelium and mugwort was low when using the ImmunoCAP assay as reference (20-50%), but was very good (100%) in comparison with SPT. Since the SPT is still used as the golden standard in clinical practice, the MSD multiplex immunoassay for dog and mugwort allergies was considered sensitive. It seemed that the ImmunoCAP assay was more sensitive to dog and mugwort allergens, which may have indicated that the individuals were sensitized without clinical signs of allergy. For some sIgEs, the sensitivity could not be determined, due to a lack of positive individuals for these allergies (Figure 1). In contrast to the sensitivity, the specificity of the MSD multiplex immunoassay was very high ([?]80% - with the majority showing 100% specificity). When using the data from the SPT as reference, the only sIgE with low specificity was rye (25%). This was probably due to cross-reactivity of IgE antibodies against timothy grass and rye, both species of grasses of the*Poaceae* family.[17] The cross-reactivity caused a person allergic to timothy grass, to also test positive for sIgE against rye in the MSD multiplex immunoassay and the ImmunoCAP assay. However, the SPT showed only an allergic reaction to timothy grass (Figure 1). A limitation of this study was the fact that, for the ImmunoCAP assay, only results of three mixtures of allergens were available and could be compared, and only one mixture was available for parallel tests in the SPT (Table 1).

In previous studies, the sensitivity and specificity of the ImmunoCAP assay were compared to the golden standard, the SPT.[18,19] For a select set of allergens that were analysed both in those previous studies with the ImmunoCAP assay and with the MSD multiplex immunoassay in the current study, similar sensitivity and specificity were observed (Table S2). For timothy grass, cats and dogs, the MSD multiplex immunoassay showed higher sensitivity and specificity values than the ImmunoCAP assay when compared to the SPT. The specificity values of dust mite and *Cladosporium herbarum* were also higher for the MSD multiplex

immunoassay, however, the sensitivity of dust mite and the specificity of rye were higher in the ImmunoCAP assay.

3.5. Reproducibility

3.4.1 Intra-assay variability

To assess the intra-assay variability of the results of the MSD multiplex immunoassay, each sample was added to the plate in duplicate, for both measurements of individual allergens and mixes of allergens. The correlation between the duplicate measures was calculated for all subjects (Table 4). The results show a very stable reproducibility for all samples (r =0.95-1; P<0.001).

3.4.2 Inter-assay variability

To check the variability of the same samples between two MSD multiplex immunoassays performed on different days, correlations were calculated for the individual allergens of serum samples from 7 individuals. The values in Table 4 demonstrate a good inter-assay reproducibility (r =0.97-1; P<0.001).

3.4.3 Biological variability over time

For six individuals, results were available of the MSD multiplex immunoassay from a previous blood sampling and testing, in Spring instead of Summer, one year and three months earlier (Table 4). In the prior measurements, plasma samples were used, while in the current tests, serum samples were analysed. However, previously analysis of serum and plasma samples collected at the same time showed a correlation of r =0.92 (P<0.001), indicating that both matrices show comparable sIgE-levels with the MSD multiplex immunoassay. The biological variation over time was observed to be consistent (r =0.94-1; P<0.001). The correlation for subject 13 was, however, lower (r =0.65; P<0.05), which was most probably due to seasonal effects (sampling time Spring vs. Summer).

3.4.4 Comparison incubation overnight or 2 hours

The MSD multiplex immunoassay protocol states that after adding the standard series and samples, either an incubation overnight at 4degC or for 2 hours at room temperature while shaking the plate, can be applied. To compare the two different incubation periods, the assay was performed in both ways for the individual allergens (Table 4). Correlations were very high (r =0.91-1; P<0.001), meaning that both an incubation overnight as well as for 2 hours can be applied.

4. CONCLUSION

The multiplex immunoassay from Meso Scale Discovery, combined with standards and allergens from Radim, allowed high-throughput analysis of specific IgEs against respiratory allergens in serum samples. The results were reproducible and also comparable to the commonly used, well-established methods (SPT and ImmunoCAP assay). The MSD multiplex immunoassay needs small blood sample volumes and is also very versatile, both individual allergens as well as mixes of allergens can be analysed. The choice of allergens can be adapted easily to the patients' or researchers' needs.

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

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TABLES

Table 1. Overview of the allergens used in the different tests.

Allergens
Dust mite (d1)
Cat (e1)
Dog (e5)
Cow's milk (f2)
Rye (g12)
Timothy grass (g6)
Cladosporium herbarum (m2)
Birch (t3)
Mugwort (w6)
Mix animals (ex1): cat, dog, horse and cow
Mix nuts (fx1): peanut, walnut, hazelnut, almond and coconut
Mix grasses (gx3): sweet vernal grass, perennial ryegrass, timothy grass, rye and common velvet grass
House mix (hx1): house dust, dust mite *Dermatophagoides pteronyssinus*, dust mite *Dermatophagoides farina* and cockroac
Mix fungi (mx1): *Penicillium notatum*, *Cladosporium herbarum*, *Aspergillus fumigatus* and *Alternaria alternate*
Mix trees (tx2): hazel, birch, alder and ash
Mix weeds (wx2): *Alsem ambriosa*, mugwort, daisy, dandelion and narrow-leaf plantain

Table 2. Pearson correlation coefficient (r) and its P-value between the results of the ImmunoCAP assay and the MSD multiplex immunoassay.

	Correlation *(r)*
Dust Mite	0.271
Cat	0.829**
Dog	0.978**
Rye	0.952**
Timothy grass	0.904**
Birch	0.954**
Mugwort	0.976**
Mix grasses	0.969**
Mix trees	0.929**

**P-value < 0.001 significance level.

Table 3. Sensitivity and specificity of the MSD multiplex immunoassay, comparing the MSD results to the ImmunoCAP assay and SPT. 0.10 kU/L was set as threshold for a positive test of both MSD and ImmunoCAP.

	Sensitivity (%)	**Sensitivity (%)**	**Specificity (%)**	**Specificity (%)**
	ImmunoCAP	Skin prick test	ImmunoCAP	Skin prick test
Dust mite	60	60	100	100
Cat	100	75	100	100
Dog	20	100	100	100
Cow's milk	NA	NA	100	100
Rye	100	NA	100	25
Timothy grass	100	100	100	100
C. herbarum	NA	NA	100	100
Birch	100		80	
Mugwort	50	100	100	93.3

	Sensitivity (%)	Sensitivity (%)	Specificity (%)	Specificity (%)
Mix grasses	100		100	
Mix fungi	NA		100	
Mix trees	85.7	85.7	90	100

NA: not applicable.

Table 4. Pearson correlation coefficients and their P-values for comparison of results within one assay (intra-assay reproducibility) and between assays over time (inter-assay reproducibility) as well as for biological variability over time (two different sampling times) and differences in incubation times (overnight vs. 2h incubation) of the MSD multiplex immunoassay.

Subject	Intra-assay reproducibility	Inter-assay reproducibility	Biological variability over time	Differe
1	1.000**	0.998**	0.976**	0.958**
2	0.996**	1.000**	0.994**	1.000**
3	0.994**			0.996**
4	0.997**			1.000**
5	0.999**			0.989**
6	1.000**	0.997**	0.943**	0.995**
7	0.990**	1.000**	1.000**	1.000**
8	0.986**			0.948**
9	0.999**		0.962**	0.998**
10	0.981**			0.966**
11	0.996**	0.979*		0.993**
12	1.000**			1.000**
13	0.997**	0.996**	0.652*	0.999**
14	0.992**			0.997**
15	0.996**			0.925**
16	0.999**	0.970**		0.999**
17	0.945**			0.913**
18	1.000**			0.998**

**P-value < 0.001 significance level. *P-value < 0.05 significance level.

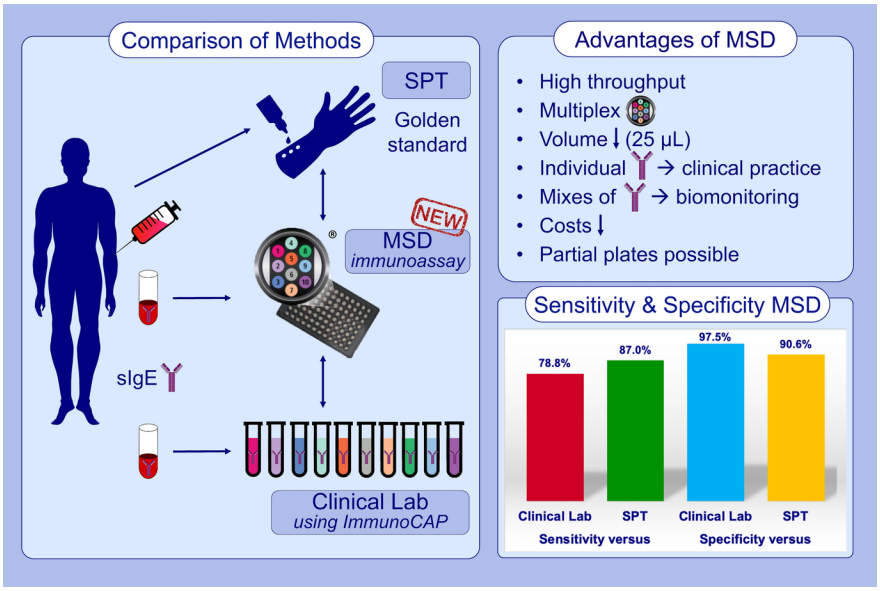

FIGURE LEGENDS

Figure 1. Results of three methods used to measure the allergy (IgE) levels in N=17 individuals. Green: positive result in the skin prick test. Yellow: positive result in the ImmunoCAP assay. Red: positive result in the MSD multiplex immunoassay. Grey: negative result.

Figure 2. Bland-Altman plots of the different allergens or mixes of allergens demonstrating the comparability of the MSD multiplex immunoassay to the ImmunoCAP assay. The difference between the two methods (y-axis) is plotted against the mean of the two assays (x-axis). The two horizontal red lines represent the 95% confidence interval of the mean difference.