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The effect of soy processing on its allergenicity: Discrepancy between IgE binding and basophil stimulation tests

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ABSTRACT

Presently, many studies assess allergenicity via IgE immunoblotting and IgE binding tests; however, IgE detection does not always signal the manifestation of a clinical allergy. However, the capacity of food allergens to trigger basophils makes it possible to use the *in vitro* functional basophil activation test (BAT) to assess allergenicity. The effect of the Maillard reaction (MR) on the allergenic potential of processed soy proteins was evaluated by two IgE binding tests (Competitive ELISA and Inhibition ImmunoCAP), a Western Blot and a functional BAT; with the aim to analyze whether the sIgE binding results correspond to the functional assay results. The results between the IgE binding tests and the functional assay were in-line for 2 of the 6 studied patients. For one patient there was no correlation between any of the results. For the raw soy protein extract heated with glucose for 10 and 30 min (SH SPE + Glu and LH SPE + Glu, respectively), the results were in-line for only 3 out of the 6 patients. Thus, the present study shows a discrepancy between IgE binding tests and basophil stimulation when assessing the effect of soy processing on its allergenicity. Since IgE-binding capacity does not always correlate to IgE cross-linking capacity, the conclusions of the allergenic potential based on the IgE binding tests alone should be drawn with care and further studies on this matter would benefit from the inclusion of a functional assay such as the BAT.

1. Introduction

In the last decade the use of soy proteins in food products has increased since soy products are considered to have beneficial health effects (Cabanos, Matsuoka, & Maruyama, 2021). Moreover, soy is an inexpensive and an excellent source of quality proteins which contains all the essential amino acids (Chatterjee, Gleddie, & Xiao, 2018). Thus, it is commonly used in human food production such as infant formulas, flours, and protein concentrates but also as an emulsifier, texturizer, and protein filler (Cabanos et al., 2021). Nonetheless, soy has been classified as one of the eight most common food allergens (Savage et al., 2010; Baseggio et al., 2021). At least 28 allergenic proteins in soy have been suggested to bind to IgE (Nishinari et al., 2018; Wilson and Blaschek, 2015); of which 8 have been recognized by the WHO/IUIS Allergen Nomenclature Subcommittee (EFSA NDA Panel on Dietetic Products, Nutrition and Allergies). The major soy allergens are the Kunitz soybean trypsin inhibitor, Gly m Bd 30 K, Gly m Bd 28 K, Gly m 5 (β-conglycinin), Gly m 6 (glycinin) and Gly m 8 (2S albumin) plus the pathogenesis-related protein (PR-10) Gly m 4 (glycine max) (Wilson and Blaschek, 2015; Fukuzumi et al., 2021). Gly m 5 and Gly m 6, major soy allergens, are two highly abundant storage proteins in soy seed; importantly both are stable to heat and gastric digestion plus have been described to be responsible for anaphylactic reactions (Evrard et al., 2022; Holzhauser, 2009). Currently, relatively few products contain unprocessed or low processed soy (e.g., tofu, soy sprouts, soy milk and edamame); thus, most of the soy-based products contain highly processed soy proteins (Nishinari, Fang, Nagano, Guo, & Wang, 2018). Food processing techniques, mostly high temperature treatments, alter the structure of the protein leading to protein denaturation, degradation and several structural changes including hydrophobicity and charge. These structural changes also modify the nature of epitopes and hence the allergenicity of food proteins (Yang et al., 2011; Iwan M et al., 2011; Shi et al., 2020; Briceno et al., 2022; Bai et al., 2021). One of the reactions which occurs commonly during thermal processing of food is the Maillard Reaction

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(MR, glycation), a non-enzymatic reaction between reducing sugars and a free amino acid group of proteins or peptides (Iwan and Vissers y.m., Fiedorowicz E., Koystyra H., Kostyra E., Savelkoul H.F., Wichers H.J. , 2011; Briceno et al., 2022). The structural modifications of the proteins which are the consequences of MR affect also the allergenicity of food proteins reflected in the changes in sensitization capacity (Yang, Li, Li, & Wang, 2013; Liu et al., 2021); as well as specific IgE and IgG binding (Teodorowicz et al., 2017; Gruber et al., 2004). However, the effect of MR on food protein allergenicity is not yet clear, it seems to depend on the type of protein and its physicochemical characteristics but also the conditions of MR itself like type of sugar, temperature, pH, time of treatment and the water activity (Zhao et al., 2017). For instance, the MR can reduce the IgG/IgE binding capacity for the major cherry allergen, Pru av 1 (Zhao et al., 2017), silver carp (Nakamura, Watanabe, Ojima, Ahn, & Saeki, 2005), buckwheat allergen Fag t 3 (Yang, Li, Li, & Wang, 2013), soybean allergen ß-conglycinin (Nakamura et al., 2006), squid allergen (Bu, Lu, Zheng, & Luo, 2009), milk allergen (Taheri-Kafrani et al., 2009; Zhang et al., 2021), hazelnut allergen, Cor a 11 (Iwan et al., 2011) and shrimp tropomyosin (Gruber, Becker, & Hofmann, 2005). On the other hand, MR increased the immunoreactivity of peanut allergens, Ara h 2 (Vissers and Blanc, 2011; Vissers et al., 2011) plus Ara h 1 (Maleki and Chung, 2000; Nakamura et al., 2005) and scallop tropomyosin (Cucu, DeMeulenaer, Bridts, Devreese, & Ebo, 2012). Thus, it seems that the IgE binding changes due to the MR may differ per protein, by protein-specific epitopes, and it can also be dependent on the sIgE epitope profile of the patient (Briceno et al., 2022; Gruber et al., 2004). Moreover, the methods used to assess the changes in allergenicity varies per study. Several studies use IgE binding methods like ELISA or Western Blot (Zhao et al., 2017; Nakamura et al., 2005; Van de Lagemaat et al., 2007; Bu et al., 2009; Zhang et al., 2021; Gruber et al., 2005; Vissers and Blanc, 2011; Vissers et al., 2011; Maleki and Chung, 2000; Nakamura et al., 2005); while very few studies combine the IgE binding with functional cell-based degranulation assays to measure the capacity of the allergen to degranulate the basophils (Iwan et al., 2011; Bai et al., 2021; Maleki and Chung, 2000; Han et al., 2018). Lastly, scarcely studies address the sensitization profile of glycated proteins in *in vitro* or *in vivo* studies (Han et al., 2022; Gou et al., 2022).

Even though the analysis of IgE binding to epitopes is a good indicator of IgE-allergen complex formation and the activation of effector cells (Liu and Sathe, 2018; Santos et al., 2021), an increase in IgE binding capacity does not equal an increase in allergenicity or vice versa (Liu & Sathe, 2018). Thus, the capacity of processed food allergens to trigger basophils and mast cells should also be evaluated with *in vitro* functional assays such as a basophil activation test (BAT) (Hoffman, Santos, & Mayorga, 2015). The BAT is that assay is capable of distinguishing between sensitization but tolerant patients and those patients that are clinically allergic (Briceno et al., 2021; Gupta et al., 2018); moreover, its sensitivity is comparable to a skin prick test (SPT) and specific serum IgE (sIgE) but it is more specific when compared to sIgE tests (Schimdt-Hielltjes et al., 2017).

The aim of the study was to analyze if the effect of the MR on the allergenic potential of processed soy proteins by determination of sIgE binding differences correspond to similar differences in the functional assay results. This is done by performing three different assays and comparing the results: (1) Competitive ELISA/Inhibition ImmunoCAP, to evaluate if processed soy proteins are either more or less potent to bind IgE to Gly m 5 and Gly m 6; (2) Western Blot, to identify the most immunogenic fractions in the processed soy proteins; and (3) BAT, to analyze if processed soy proteins are more or less potent to degranulate basophils.

2. Material and methods

2.1. Patients serum

Approval for this study was obtained from the Medical Ethical Review Committee CMO Regio Arnhem-Nijmegen, The Netherlands. A total of 6 patients visiting the Outpatient Allergic Clinic in Rijnstate Hospital Arnhem were included. All patient has been previously diagnosed with soy allergy based on a positive SPT and/or sIgE in combination with a clear clinical history. Patients were randomly selected based on their medical history and were asked to complete a questionnaire plus donating a blood sample. Fresh ethylenediaminetetraacetic acid (EDTA) blood was collected to perform an ImmunoCAP inhibition test and a BAT assay. Serum was collected to determine sIgE for total soy extract, Gly m 4, Gly m 5 and Gly m 6 as the main soy allergens by means of ImmunoCAP® (Phadia AB, Uppsala, Sweden). ImmunoCAP responses were considered positive when IgE levels were $\geq$ 0.35 kU/l. Participants were asked to stop taking oral antihistamine and oral steroids 3 days and 10 days, respectively, before blood collection. Most of the patients in the present study (67 %) had a clear medical history of anaphylactic shock after the consumption of soy products; therefore, an oral food challenge was not performed.

2.2. SDS-Page

Native and modified soy proteins (raw soy protein extract (SPE), *H*10, G10, H30, G30) were separated by SDS-PAGE under reducing conditions using BioRad equipment (Herculus, CA, USA). Proteins were boiled at 95 °C for 10 min and loaded onto a 12.5 % polyacrylamide gel. After protein separation, the gel was stained using GelCode blue stain reagent (Thermo Scientific). A molecular weight marker (Precision Plus Protein dual color standards, Biorad) was included.

The treatments from this point forward will be referred to as follows:

1. Raw SPE heated at 121 °C without glucose for 10 min: short term heated (SH SPE)
2. Raw SPE heated at 121 °C with glucose for 10 min: short term heated with glucose (SH SPE + Glu)
3. Raw SPE heated at 121 °C without glucose for 30 min: long term heated (LH SPE)
4. Raw SPE heated at 121 °C with glucose for 30 min: long term heated with glucose (LH SPE + Glu)

2.3. IgE binding tests

To determine the changes in the sIgE binding to the processed soy samples two methods were used: inhibition ELISA and ImmunoCAP inhibition tests. A competitive ELISA which involves the incubation of an antigen with a primary antibody for specific binding; following incubation, this antibody-antigen mixture is then added to a plate which is coated with the corresponding antigen for the free primary antigen to bind with it. In an inhibition ImmunoCAP test the same protein source is used for both the inhibition of sIgE and sIgE measurement, which is relevant when multiple allergens are tested such is the case in the present study were multiple treatments for SPE are analyzed (Bühlmann, 2011).

2.3.1. Competitive ELISA

In the competitive ELISA the original antigen, non-treated SPE were analyzed for their IgE binding capacity upon competition with raw SPE, SH SPE, SH SPE + Glu, LH SPE and LH SPE + Glu for the six patients in the study group. The white polystyrene medium binding 96-well plates (Sigma-Aldrich, USA) were coated with raw SPE in a predetermined concentration of 10 μg/ml in coating buffer, the plates were then incubated at room temperature for 2 h. The free binding sites were blocked with 2 % BSA blocking buffer for 1.5 h. The patients' serum was pre-incubated for 30 min on a shaker with different concentrations (2500, 25, 0.25 and 0.0025 μg/ml) of the five types of competing soy proteins in a plastic dilution plate. The serum incubated without the competing soy proteins was used as the negative control (no inhibition: IgE0 %). After the blocking step, the plates were washed 4 times and the mixture of

serum and competing soy proteins were transferred into the ELISA plates. The detecting antibody polyclonal Goat Anti-Human IgE HRP (Abcam, Cat. No: ab73901) was diluted 1:10,000 in serum dilution buffer. For each well, 80 µl of OPD (o-phenylenediamine dihydrochloride) substrate was added and absorbance was measured at 492 nm on automatic plate reader within 3 min. The percentage of inhibition was calculated according to the formula:

% of Inhibition = (IgE0% - IgEX%)/IgE0% x 100.

The maximal signal was obtained by the negative control, wells with competing proteins showed decreasing levels of signal; depending on the competing protein concentration certain IgEX% inhibition took place. The Graphpad Prism software was used for calculations and statistical analysis of the results (one way ANOVA with Tukey post-hoc test).

2.3.2. ImmunoCAP inhibition

To measure allergen specific IgE in the serum, an ImmunoCAP inhibition test was performed (Bühlmann, 2011). The concentrations of inhibitor proteins (competitors) were validated in pre-test optimization assays. Raw SPE and LH SPE + Glu were mainly used as competitive proteins. The negative control (0 % inhibition) was two-fold diluted serum without inhibitor proteins. Both competitor proteins were able to decrease bound IgE levels for both Gly m 5 and Gly m 6 allergens; indicating that inhibition did occur. The soy protein samples were applied in concentrations of 1, 5 and 25 µg/ml.

The serum was diluted two-fold with 0.9 % NaCl and different treated soy protein inhibitor samples or PBS control were added; followed by 1.5 h of incubation at RT. Five soy proteins were tested as competitors: raw SPE, SH SPE, SH SPE + Glu, LH SPE and LH SPE + Glu. After incubation, the sIgE levels were measured with the Phadia250® instrument (Thermo Scientific, Germany). The percentage of inhibition was calculated with the formula:

% of inhibition= (IgE0%- IgEx%)/ IgE0% × 100.

The GraphPad Prism software was used for calculations of the EC50 value and statistical analysis of the results (one way ANOVA with Turkey post-hoc test) were performed. Additionally, the EC50 values allowed for a better comparison between the different processed samples. The EC50 of inhibitor concentrations represent the protein concentration for 50 % of inhibition to occur in which the higher the bar, the lower the IgE binding capacity.

2.4. Western blotting

The WB allowed for the identification of the individual proteins present in the various fractions which are recognized by sIgE. Additionally, the WB allowed to identify the IgE binding patterns to Gly m 5 vs Gly m 6 after heating (as shown in Fig. 5). The WB was performed with the sera of the six patients by analyzing the different forms of processed SPE as well as raw soy.

The separated proteins (SDS-PAGE) were transferred to a Whatman membrane using the Trans-blot SD Semi-Dry Transfer Electrophoretic Transfer Cell (Biorad) at 15 V for 35 min. The membrane was blocked for 1 h at room temperature (RT) with 3 % bovine serum albumin (BSA) in Tris-Buffered Saline with 0.5 % of Tween-20 (TBST). After washing, the membrane was incubated overnight at 4 °C with patient serum diluted (5 times) in 0.01 % of non-fat dry milk (NFDM) in TBST. After incubation, the membrane was washed and incubated for 1 h at RT with mouse monoclonal anti-human IgE (BD) diluted at 1:800 at 0.5 % NFDM in TBST. Thereafter, the membrane was washed again and incubated with goat anti-mouse-HRP antibodies (Dako PO447) diluted 1:1000 in 0.5 % NFDM in TBST for 30 min RT. After washing the membrane, a chemiluminescent detection was performed; the blot was incubated with ECL Western blotting detection reagent mix (from ThermoFisher) for 2 min; the blot was placed in the cassette and the film was exposed for 60 min. Finally, the film was developed, making the bands visible.

2.5. Basophil activation test (BAT)

The BAT was performed with the Flow2-CAST kit and soy protein allergens according to the manufacturer's instructions (Bühlmann Laboratories, AG, Switzerland) (Steckelbroeck et al., 2008). Basophil activation was determined by CD63 expression level of 500 basophils (FACS Canto II; BD Biosciences, San Jose USA). The values used for reporting Gly m 5 and Gly m 6 results are in accordance with the manufacturing instructions that state a positive result when there was a clear dose–response curve with the %CD63-positive basophils of > 15 % (Steckelbroeck et al., 2008). For a more accurate analysis of the BAT results, the area under the curve (AUC) was calculated. The AUC has been described not only as a reliable BAT marker of sensitivity and reactivity (Hoffman et al., 2015; Gupta et al., 2018), but since it uses several measuring points at multiple allergen concentrations, it lowers the risk of false outcomes (Gupta et al., 2018).

EDTA blood samples were freshly incubated with basophil stimulation buffer. The negative controls were sera of adult soy allergic patients with negative sIgE levels to both Gly m 5 and Gly m 6 (supplemental Table 1). The positive control (PC) were the 6 patients included in the present study all with positive results of sIgE levels to both Gly m 5 Gly m 6, obtained by stimulating the blood either with anti-FcεRI or with formyl-methionyl-leucyl-phenylalanine (fMLP). Soy protein conditions were raw, heated with glucose for 10 and 30 min, heated without glucose for 10 and 30 min, plus Gly m 5 and Gly m 6. The allergen concentrations used were 10, 300 and 1200 ng/ml as determined in optimization assays.

3. Results

In total, six adult soy allergic patients were included in this study; with a mean age of 27 years; most were female (83 %). All had positive sIgE levels for both Gly m 5, Gly m 6 and total soy extract, while only two had positive sIgE levels to Gly m 4 (as shown in in Table 1). Moreover, three patients had a much higher sIgE value to Gly m 6 compared to Gly m 5; while 3 patients had similar Gly m 5 and Gly m 6 sIgE values.

Laryngeal edema was reported by five patients and anaphylactic shock was reported by 4 patients. Oral allergy syndrome was reported by four patients. All six patients reported consuming processed soy products before developing symptoms, three patients reported additionally consuming soy milk in addition to the soy products.

3.1. Characterization of the processed soy proteins: SDS-PAGE

Upon exposing raw SPE to heat and glucose, the band intensity in the SDS-PAGE did not reduce upon short term treatment in the presence or absence of glucose (SH SPE and SH SPE + Glu) although with SH SPE an increase in high molecular weight aggregates appeared on top of the gel. Longer heat treatment (LH SPE) and especially heat treatment with glucose (LH SPE + Glu) resulted in a decrease of the density of the bands while also LH SPE + Glu resulted in more aggregates on top of the gel (as shown in Fig. 1A). Five bands (160, 130, 98, 25 and 16 kDa) were

Table 1
sIgE (kU/L) levels of Soy Allergic patients.

			ImmunoCAP results		
Patient	Age/Sex	Total Soy Extract	nGly m 4	nGly m 5	nGly m 6
1	21/female	19.5	<0.35	9.75	19.7
2	23/female	9.59	<0.35	3.58	12.3
3	32/male	2.69	<0.35	2.75	1.78
4	34/female	3.35	<0.35	2.39	2.88
5	25/female	19.8	2.51	4.86	25.5
6	26/female	2.66	4.84	1.67	2.74

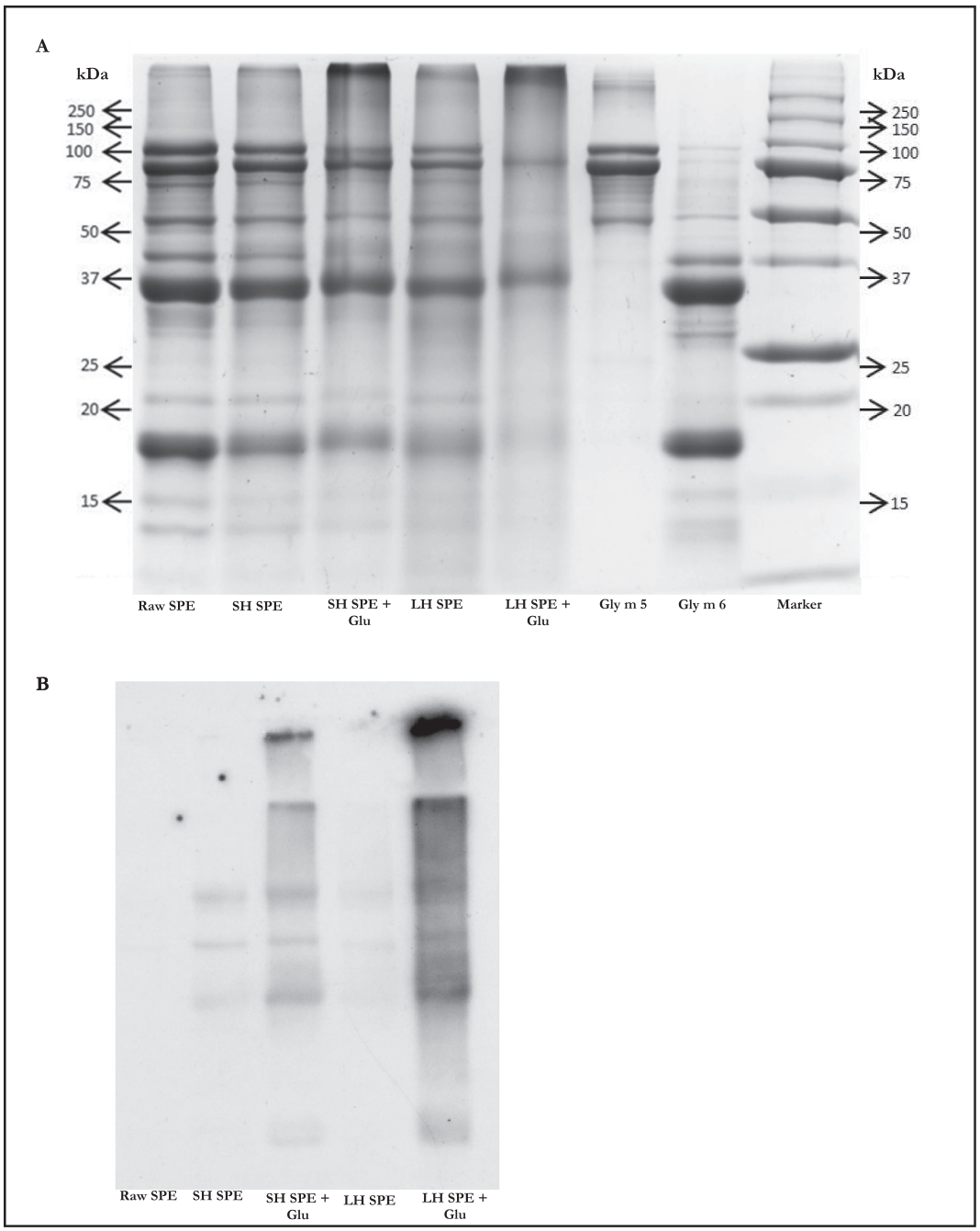

Fig. 1. A: Changes in SDS-PAGE pattern of the soy proteins upon progressive Maillard reaction and heat treatment; B: separation of the denatured soy proteins by molecular weight (in kDa). Nomenclature of the soy specific bands according to WHO/IUIS Allergen Nomenclature Sub-committee [**Error! Reference source not found.**].

present in SH SPE but were not detectable upon SH SPE + Glu, indicating that the structure was changed due to the MR. Seven bands (65, 55, 50, 40, 29, 27 and 20 kDa) remain present with LH SPE but not in LH SPE + Glu, indicating that their structure changed due to the MR and not due to heat treatment alone (as shown in supplemental Table 1). Treatment of raw SPE changed the protein to the highest degree in the LH SPE + Glu. Moreover, proteins formed agglomerates in the glycated samples and even more agglomerates of > 250 kDa (as shown in Fig. 1B) were formed in the glycated samples compared to the heated samples. Of the total protein fractions present, a fraction consisted of agglomerates with a molecular weight > 250 kDa; for SH SPE it represented 16 % of the total observed fractions, for the SH SPE + Glu these agglomerates represented 27 %; for the conditions LH SPE and LH SPE + Glu the percentage was 31 % and 38 %, respectively.

3.2. *Quantitative assays: IgE binding tests*

In the present study, the outcomes of both IgE binding tests (Competitive ELISA and ImmmunoCAP Inhibition tests) are comparable. ELISA results (as shown in Fig. 2) with extract were comparable with ImmunoCAP results (as shown in Supplement Fig. 1) with purified proteins (Gly m 5 and Gly m 5 in the present study). In general, there is a tendency per patient for binding of all processed forms of SPEs, with an increased IgE binding observed in four patients and a decreased IgE binding for the other two patients. For most patients, a difference between the different treatments of the allergens is hard to observe. For

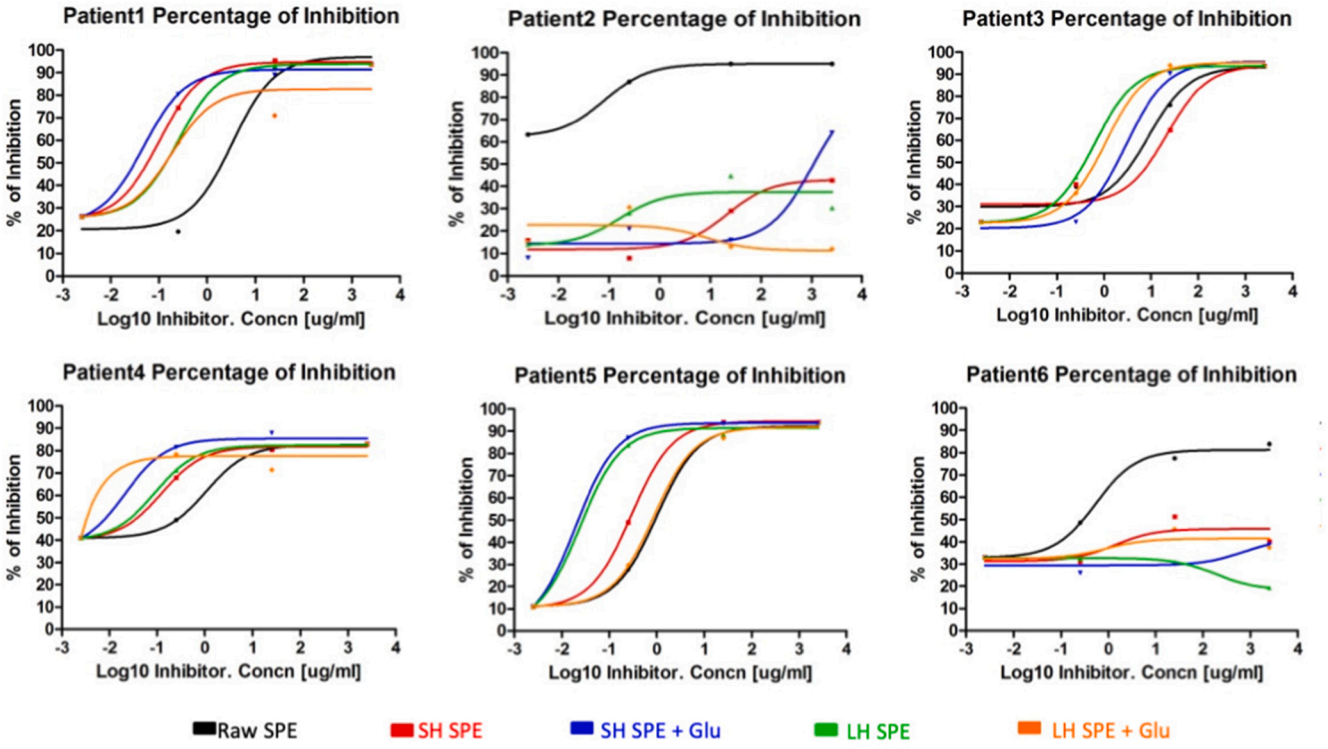

Fig. 2. Competitive ELISA results with ^{10}log inhibitor concentration (in μg/ml) and the observed % of Inhibition without competitor (100 %) for the different sample preparations as indicated by the colors.

most of the patients (four out of six patients), the glycated proteins were capable to increase the sIgE binding to a higher degree than the only heated proteins (as shown in Fig. 3). Since the ImmunoCAP inhibition requires lower allergen concentrations compared to ELISA, with this assay the influence of the MR on the analyzed soy allergens (Gly m 5 and Gly m 6) can be observed. The results are not only in line with the competitive ELISA results, but the outcomes are also aligned for both allergens per patient (as shown in supplementary Fig. 1).

3.3. *Qualitative assay: Western Blot (WB)*

For most patients, the WB analysis showed IgE binding to glycated

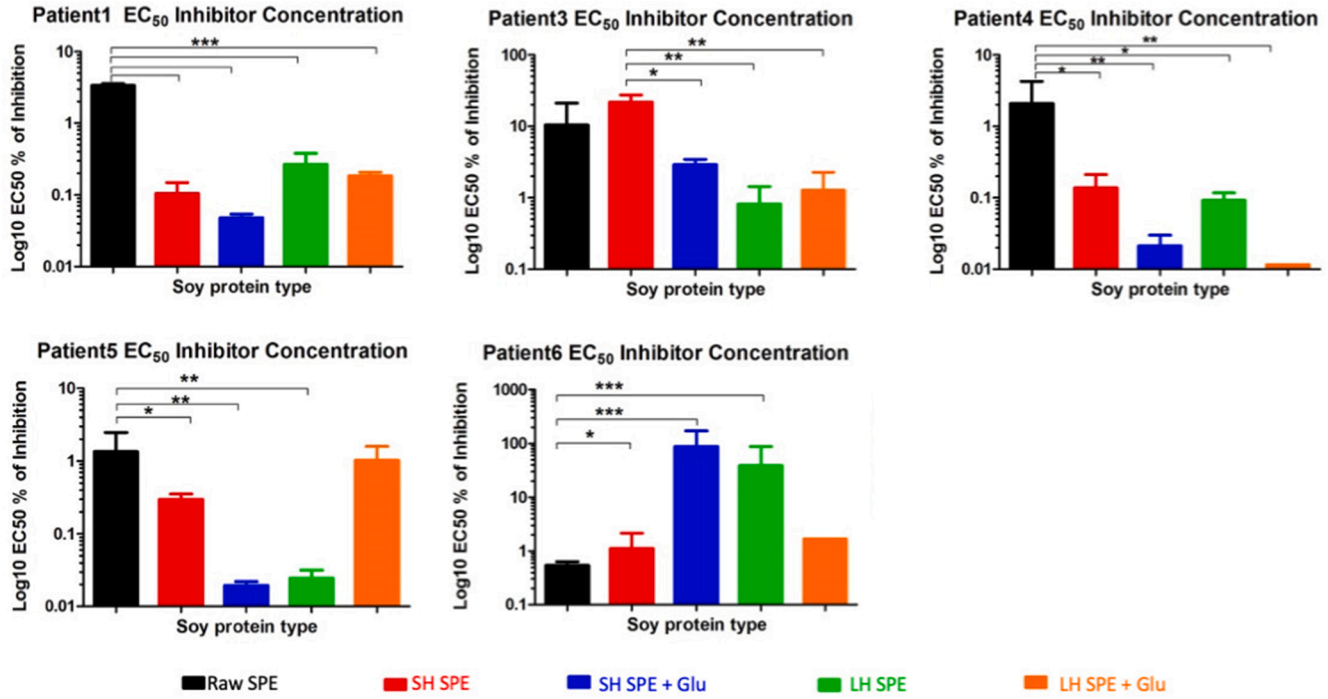

Fig. 3. ELISA EC_{50} of Inhibition Concentration for the different sample preparations as indicated by the colors. The results for patient #2 are not represented because no inhibition was observed with the treated SPEs (EC50 could not be calculated).

proteins (as shown in Fig. 4B). In addition, the high molecular fractions observed on the top of the gel of these patients (except for patient #3) can be categorized as large aggregates which contain advanced glycation end products (AGEs) that are known to be immunogenic (as shown in Fig. 4A and Fig. 4B). It is possible that when these aggregates are visible (except patient 6), the presence of these bands could point to the involvement of Gly m 5 and Gly m 6 present in the raw SPE band profile. Aggregates in the glycated samples were present in five out of six patients; thus, showing that IgE binding could be potentially increased by glycation. The WB results are in line with the IgE binding tests, where inhibition was strong for all the processed proteins.

3.4. Functional assay: Basophil activation test (BAT)

When analyzing the role of glycation by comparing the heated SPEs we observed that in the SH SPE + Glu, the AUC decreased in five out of the six patients (Fig. 5). For the SH SPE samples no discernable pattern was observed. For the 30-minute processed LH SPEs, either glycated or heated, the AUC increased when compared to raw SPEs in three out of six patients, indicating that this change occurred due to either glycation or temperature alone. Five out of six patients showed a higher AUC for the LH SPE + Glu when compared to the SH SPE + Glu, while this pattern was not seen for the heated SPEs samples (as shown in Fig. 5).

3.5. Comparison between IgE binding test (Competitive ELISA) vs functional assay (BAT)

The correspondence between the results from the Competitive ELISA and the BAT was evaluated (as shown in Table 2). For two patients all results were in-line for the IgE binding test and the functional assay, in the case of patient #1 all treated SPEs increased the IgE capacity as well as the basophil degranulation, while in the case of patient #2 there was an overall decrease. On the other hand, for one patient (patient #5) there was no correlation between any of the results (as shown in Table 2). For the SH SPE, the results were most consistent in the evaluated patients, with 5 out 6 patients (83 %) obtaining similar results (as shown in supplemental Table 2). For the two glycated samples (SH SPE + Glu and LH SPE + Glu), only 50 % of the evaluated patients showed results in which the competitive ELISA and the BAT were in-line. Lastly, for the LH SPE sample, 4 out of 6 patients (67 %) showed similar results between the two types of assays.

4. Discussion

The present shows the value of adding a functional assay to the assessment of the effect of food processing on soy allergenicity since the reported results between the IgE binding and the basophil stimulation tests were incompatible. The correspondence between IgE binding test (Competitive ELISA) and the BAT assay for the processed SPEs was low, namely only for 2 out of the 6 patients. Therefore, clinicians and researchers assessing allergenicity with only IgE binding tests, should view conclusions from individual assays with care. This is mainly due to these tests not revealing information regarding the functional properties of the allergenic components upon interaction with effector cells like mast cells and basophils (Iwan et al., 2011; Maleki and Chung, 2000; Han et al., 2018). IgE binding tests indicate sensitization to a particular allergen and do not always indicate the manifestation of clinical symptoms of an allergy therefore providing a limited overview when assessing food allergenicity (Jimenez-Saiz, et al., 2011). Regardless of this drawback, presently most studies assess allergenicity via IgE immunoblotting and IgE binding test, which are both dependent on the affinity between IgE and the corresponding allergens. The results observed in the present study outline the value of adding a functional assay when assessing the effect of food processing on allergenicity (Iwan et al., 2011; Gruber et al., 2004; Han et al., 2018; Gupta et al., 2018). The capacity of food allergens to trigger basophils has the potential to become an important *in vitro* assay to diagnose allergenicity, as basophils have an important role in IgE-mediated food allergies, like mast cells, but have the advantage to be accessible by obtaining peripheral blood for further analysis *ex vivo* (Hoffman et al., 2015). Additionally, the results of the BAT are not only dependent on the amount of IgE alone but also on IgE affinity and avidity to relevant epitopes and the possibility of IgE crosslinking (Gupta et al., 2018).

The MR can modulate the binding potential of specific IgE antibodies to food allergens via (i) disruption of the conformational and linear epitopes accompanied with changes in the secondary and tertiary structures that in turn impair the IgE binding potential of the protein; (ii) formation of new IgE binding epitopes and (iii) formation of agglomerates carrying high number of IgE binding epitopes (Briceno et al., 2022; Gruber et al., 2004; Zhang et al., 2021; Rouvinen et al., 2010). The fact that high molecular weight material was observed in five out of six patients in the WB under glycation conditions argues for an increased formation of aggregates from glycated proteins compared to only heated proteins that can be recognized by sIgE antibodies. Further research is needed to separate heat-induced aggregation from heat plus glycation induced aggregation of soy allergen.

Regarding soy protein glycation and changes in allergenicity, currently there are no *in vivo* reports (Santos et al., 2021). It was previously suggested that the MR of a soy protein extract in combination with fructose and fructooligosaccharides resulted in a reduced allergenicity of Gly m 5, Gly m 6 and Gly m Bd 30 K when analyzed by SDS-PAGE (Bu et al., 2009; Santos et al., 2021; Lehmann et al., 2006). However, Walter et al attempted to determine the effect of a limited and controlled Maillard-induced glycation on the allergenicity of soy protein and reported that limited Maillard-induced glycation could either reduce or increase the immunoreactivity of soy protein hydrolysate (SPH), depending on the individual patient serum used (Lehmann et al., 2006). Therefore, not only the extent of alteration of potential epitopes of most soy allergens remains unknown but the current studies suggest that some patients are sensitized against processed food rather than raw (Gruber et al., 2004; Liu and Sathe, 2018). Upon treatment, some fractions of Gly m 5 and Gly m 6 disappeared on the SDS-PAGE, which indicates that not all fractions in these major soy allergens are heat stable. Moreover, in all patients we found that both IgE inhibition tests showed that Gly m 5 and Gly m 6 allergens were affected in a similar manner while obtaining processed SPEs. Bu et al described that glycation reduced the IgE-binding activity of Gly m 6 compared with both the native and heated form due to changes in the allergen structure (Taheri-Kafrani, Gaudin, & Rabesona, 2009), concurring with our findings. In the present study, the BAT results showed that glycation was responsible for masking epitopes, since in five out of six patients the AUC decreased in the SH SPE + Glu when compared to heated SPEs. Additionally, five out of six patients showed a higher AUC for the LH SPE + Glu when compared to SH SPE + Glu. In this case there is a possibility that glycation for 10 min halts the MR at an initial stage, thus producing very few Maillard Reactions Products (MRPs) and therefore inflicts less structural changes to the soy protein. However, when continuing the MR for a longer time (30 min in the present study), more MRPs will be induced and more structural changes to the protein occur with the possible formation of new allergic epitopes, potentially reflecting higher allergenicity. The SH SPE 10 resulted in the highest correspondence between the results of the IgE binding test and the BAT assay for five out of the 6 patients, while for the two glycated samples (SH SPE + Glu and LH SPE + Glu) the correspondence was 50 %. Vissers et al reported a reduced IgE capacity but an increase in the degranulation capacity of Ara h 1, which was attributed to Ara h 1 aggregates presenting a large surface containing multiple copies of the same IgE epitopes: thereby possibly enhancing the cross-linking capacity of the protein (Maleki & Chung, 2000). Therefore, it is likely that aggregated structures are more effective in enhancing the degranulation capacity (Maleki and Chung, 2000; Usui and Tamura, 2004). Similar findings were reported by Lehmann et al regarding Ara h 2 and Ara h 6, where the functional assay

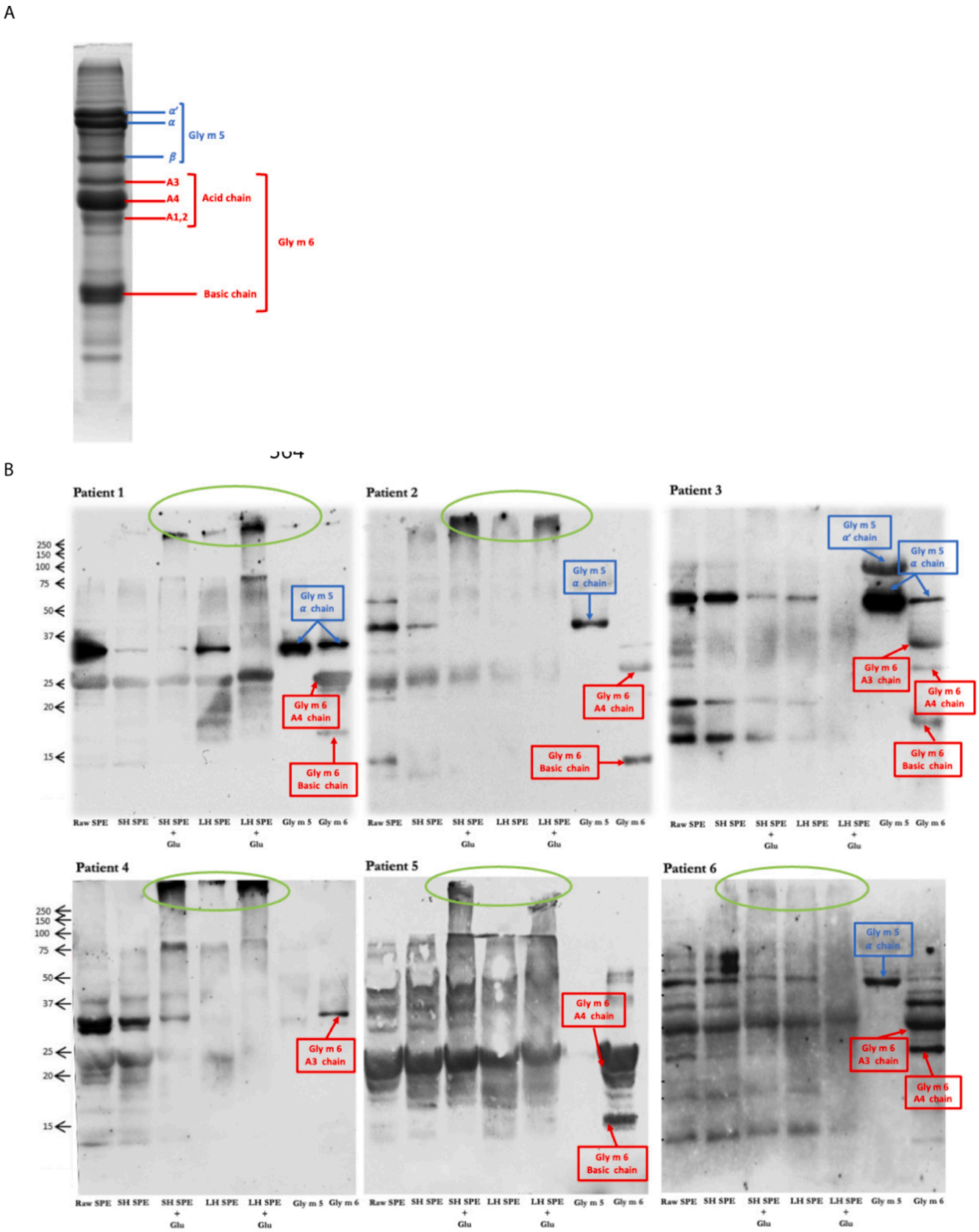

Fig. 4. A: Separation of the denatured soy proteins by molecular weight; B: Individual Western Blot results for the different sample preparations per patient.

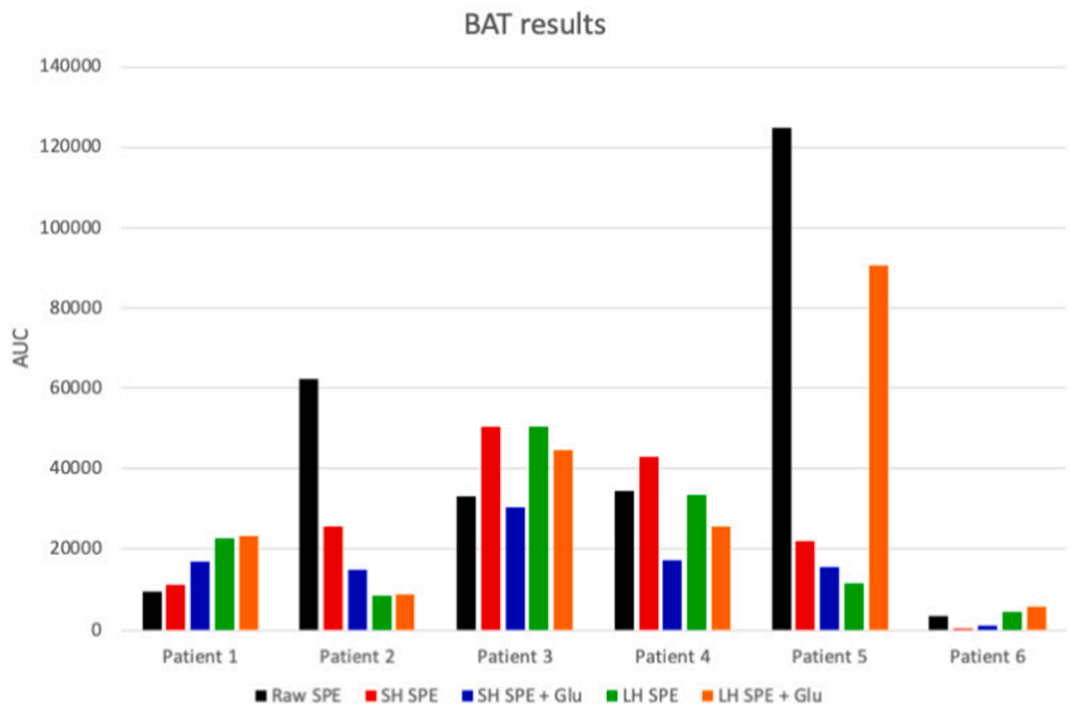

Fig. 5. Results for the ratio of the area under curve (AUC) for the basophil activation test (BAT) from the Gly m 5/Gly m 6. Panel A shows different sample binding profile per patient where the colors indicate the different sample preparations tested. Panel B shows the difference in binding by comparing combinations of samples for the individual patients tested.

Table 2
IgE binding increase or decrease for all SPE treatments when compared to raw SPE for competitive ELISA vs the BAT assay. The red upwards arrows indicate the results reflect increase in IgE binding or in basophil activation; the green downwards arrow indicate the results reflect decrease in IgE binding or in basophil activation.

	SH SPE		SH SPE + Glucose		LH SPE		LH SPE + Glucose	
	Competitive ELISA	BAT	Competitive ELISA	BAT	Competitive ELISA	BAT	Competitive ELISA	BAT
1	↑	↑	↑	↑	↑	↑	↑	↑
2	↓	↓	↓	↓	↓	↓	↓	↓
3	↑	↑	↑	↓	↑	↑	↑	↑
4	↑	↑	↑	↓	↓	↓	↑	↓
5	↑	↓	↑	↓	↑	↓	↑	↓
6	↓	↓	↓	↓	↓	↑	↓	↑

(mediator release from a functional equivalent of basophils, the humanized RBL cells) showed that the reduction in IgE-binding capacity did not necessarily translate into a reduced allergenic potency (Breiteneder & Mills, 2015). The findings of Iwan et al suggested also that aggregation of the proteins because of the MR may be responsible for the observed decrease in IgE binding properties while an increase was found in the degranulation capacity of Cor a 11 at 60 °C and 145 °C (Iwan et al., 2011). In the present study, the formation of immunoreactive large aggregates are visible in the WB from most patients, particularly in the glycated samples. Breiteneder et al reported that 11S Globulins, such as Gly m 5 and Gly m 6, are highly thermostable with the cupin barrel remaining intact, while the unfolding of other regions results in a structure loss and thus facilitating formation of large aggregates (De Leon et al., 2005). While previous data suggest that aggregates formed by heating or glycation can have an impact on IgE binding capacity; the capacity of these aggregates to elicit basophil degranulation, which is a measure if functional biological activity and thus more indicative of a potential reduced or increased allergenic potency *in vivo*, has been scantly studied and remains unclear. Importantly, there might be multiple IgE epitopes present on the aggregates permitting more efficient cross-linking of the surface bound IgE although the impact of digestion on these aggregates remains unknown. Therefore, further studies *in vivo* are needed to confirm the potential clinical role for these aggregates. Additionally, De Leon et al suggested that activation of the effector cells by cross-reactive IgE antibodies may be affected by allergen abundance as well as the affinity of the IgE antibodies for the relevant allergens; thus, high allergen concentrations may be required to trigger basophil through low-affinity IgE antibody interactions (Valenta & Kraft, 2001). This discrepancy between IgE binding and effector cell activation has previously been reported for other allergens (Iwan et al., 2011; Maleki and Chung, 2000; Breiteneder and Mills, 2015; Zuidmeer et al., 2008).

A possible limitation of the present study is that the small number of subjects included reduces the statistical power; however, this low number of individuals is not an uncommon feature of soy allergy studies due partly to the low prevalence of this food allergy in the general population, well below 1 % regardless of the age group (Farjami et al.,

2021). Due to the small sample size, the results of the present statically can't prove that there are different IgE-binding profiles to the different processed soy products compared to untreated soy, which correspond to a particular degree of allergic reaction as shown by the BAT. Nonetheless, the results observed in this study in a group of well-characterized soy allergic patients, strongly indicate that the increase or decrease of IgE binding does not correspond unambiguously to clinical reactivity against four different processed soy products. Moreover, the correct selection of patients (clinically relevant soy allergy) was necessary for properly assessing the impact of food processing on allergenicity (Han et al., 2018). Additionally, since this is the first time to our knowledge that a comparison between IgE binding and functional assays results in the assessment of soy allergenicity has been done, the present study can be viewed as a forebear for future studies to perform this comparison not only for soy allergic patients but for other allergens as well.

In conclusion, the results in the present study show a lack of correspondence between the IgE binding test and the functional assay, reinforcing the view that further structure–function studies are necessary. Moreover, conclusions on the allergenic potential based on the IgE binding tests alone should be drawn with care since altered IgE binding capacity in glycated samples could also be due to the formation of new epitopes or by glucose favored recognition of IgE antibodies [54]. Thus, further studies on this matter would benefit from the inclusion of a functional assay such as the BAT.

5. Data availability

Data for this trial will be available from the corresponding authors on reasonable request.

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Ethics Statement

This clinical trial has been registered as part of "Immunological assays as useful tools in the study of soy allergy" with the registration number 2013/235, NL nr. 44545.091.13 after approval by the research ethics committee of the CMO Regio Arnhem-Nijmegen and followed the Declaration of Helsinki.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jff.2023.105477.

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