Soy Allergy:

A rising problem and a diagnostic challenge

Daniela Briceno Noriega

Propositions

1) The basophil activation test (BAT) differentiates between co-sensitization and cross-reactivity in patients with multiple food allergens thus improving the quality of food allergy diagnosis. *(This thesis)*

2) For soy proteins, the Maillard reaction (MR) modulates the binding potential of specific IgE antibodies due to the formation of agglomerates; thus, increasing allergenicity in most patients. *(This thesis)*

3) Climate change increases the exposure to antigens therefore increasing the development and symptoms of infectious and inflammatory diseases.

4) Vitamin D deficiency modulates immune responses which leads to sensitization and clinical signs of inflammatory diseases.

5) Dietary modulation of immune function in early life improves immune protection upon infection.

6) The rapid spread of misinformation during the COVID-19 pandemic demonstrated that the scientific community needs to drastically improve their communication approach to the general public.

7) Presently, we haven't achieved true gender equality in science; thus, we need to do more to encourage the participation of women in science.

8) The newly hybrid working environment improves the work-family balance, particularly for women.

Propositions belonging to the thesis, entitled

'Soy Allergy: A rising problem and a diagnostic challenge'.

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Thesis

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Chapter 1

General Introduction

GENERAL INTRODUCTION

In recent decades, the incidence of food allergies has been continuously rising, particularly in the developed world, with a prevalence of approximately 6.5% among the general population [1-3]. Almost 90% of food allergies are caused by only eight foods, nicknamed 'The Big Eight', one of which is soy [4]. Soy consumption may represent how recent changes in western diets possibly impact food allergy incidence [5]. The consumption of soy has risen significantly in the developed world, not only because soy is a popular protein alternative to animal protein and therefore, many processed foods contain soy but also it is an important source of nutrition for patients with milk allergy [4,5]. The availability of soy in a variety of processed products results that the dietary advice of total soy avoidance becomes very problematic for a patient diagnosed with soy allergy [1]. Moreover, it remains a challenge for clinicians to differentiate between soy specific IgE concentrations (sensitization) and a clinical soy allergy. Furthermore, as many other food products, soy is frequently subjected to various food processing techniques including thermal treatments such as boiling, frying or roasting, and more novel processes such as high-pressure treatment, extrusion or ultrasound [6]. These processes can cause conformational changes in the processed proteins which include numerous products of the Maillard reaction (MR) [6,7]. The MR is one of the best-known food processing reactions, known also as glycation occurring between heated proteins and sugar [7]. Therefore, there is an interest in analyzing the effects of food processing techniques on protein allergenicity, because food processing has the potential to modulate protein immunogenicity [6-9].

1. Risk factors for Food Allergy Development

Food allergy, like all chronic diseases, is influenced by genetics, environment and genome-environment interactions which include epigenetic effects [3]. Several genetic factors can influence the risk to develop sensitization or food allergy; however, since its increase in prevalence has occurred over the last few decades this rise cannot be explained by genetic changes alone which tend to develop over longer periods of time [8]. Thus, it is possibly that environmental exposures or lack thereof, could possibly induce epigenetic changes which then interrupt the immunological state of tolerance to foods (as shown in *Figure 1*) [8,9].

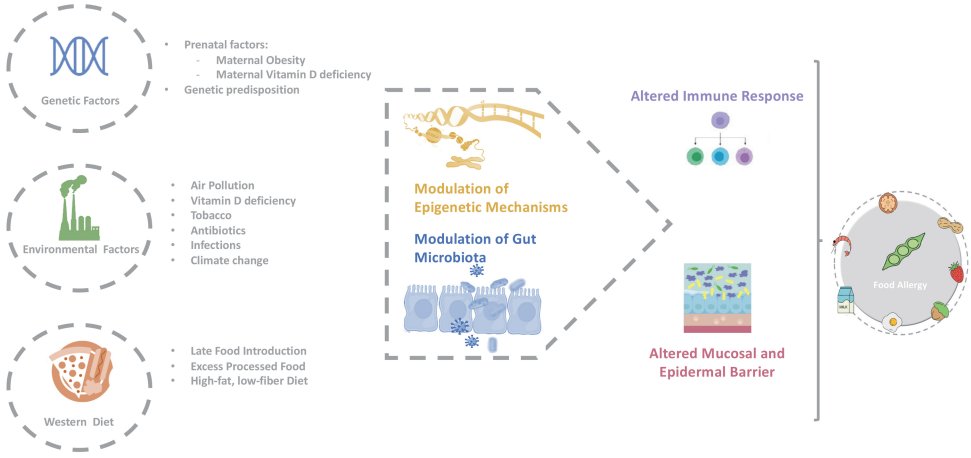

Figure 1. Schematic representation of the current theories for food allergy development

1.1 Genetic Factors

There is no doubt that the interplay between genetics and the environment is a complex interplay with several studies establishing the strong risk a family history of food allergy represents [10-21]. Hourihane et al reported that a child has a five-fold increase in the risk of peanut allergy when they have a sibling with a peanut allergy [12]. Additionally, Sicherer et al

reported that among identical twins the concordance rate of peanut allergy was significantly higher (64.3%) than that among dizygotic twins (6.8%), with the heritability of peanut allergy was 81.6% [15]. Therefore, sensitization to common allergens is influenced by both genetic and environmental factors [10,14]. A review, more than 10 genes associated with food allergy or food sensitization were identified, several of them associated with allergen presentation, a Th2-skewed immune system or both [10].

1.1.1 Environmental Factors

Presently, it is thought that a low exposure to microorganisms and the decrease in infections during early childhood are a risk factor for allergy development by creating an imbalance of the immune response favoring a Th2 lymphocyte profile rather than a Th1, known as the hygiene hypothesis [17]. An allergic rather than a tolerogenic response is favored by environmental exposure to food allergens in early childhood through an altered skin barrier allowing skin exposure in the absence of the tolerogenic signals delivered from gut following food ingestion [18]. A “window of opportunity” has been identified, from intrauterine development to the first two years of life, which is the critical period where the individual’s susceptibility to develop allergies can be established [17-19]. Additionally, the altered microbiota composition is another described risk factor for the development for food allergy; since it has been suggested that some bacterial strains and microbial diversity support T reg lymphocyte maturation that favor tolerance to food antigens [17]. Moreover, the overly indiscriminate use of antibiotics facilitates the onset of allergies by destroying microbiota diversity [20].

1.1.2 Dietary Factors

Dietary exposure *in utero* and during infancy have been regarded as important factors in the development of allergic diseases [21]. Currently, the evidence to suggest that dietary restrictions during pregnancy have a role in sensitization *in utero* remains debatable; however, it appears that the maternal diet during breastfeeding has an impact in food allergy development [9]. A clear example of the importance of a balanced maternal diet during breastfeeding can be found when the levels of vitamin D are evaluated. When a breastfeeding mother has deficient serum levels of vitamin D, the breastmilk will also contain low levels of vitamin D and since breastmilk is the only source of vitamin D for the infant, since their sun exposure is extremely limited due to skin sensitivity; thus the infant will also be vitamin D deficient [21]. Consistently, vitamin D deficiency has been linked to the risk of developing allergies [21-24]. Vitamin D inhibits maturation of dendritic cells (DCs), increases IL-10 secretion in Antigen presenting cells (APCs) and forkhead box P3+ (FOXP3) T regulatory cells plus reduces T cell activity [21]. Moreover, correcting the vitamin D status by oral supplementation inhibits the plasma levels of TNF-α resulting in an increase of the expression levels of the vitamin D receptor (VDR) and the factor prohibitin, both of which are involved in reducing the allergic inflammation in the skin and airways [25]. Additionally, vitamin D can increase anti-microbial immune defense; thus, strengthening immune regulation ensuring peripheral tolerance at mucosal tissues [21].

In recent years, dietary recommendations and practical guidelines for food allergy prevention have been updated considering the evidence that demonstrates that the practice of excluding food allergens from children's

diet has contributed to the increase in food allergy over the last years [17,26-28]. Presently, it is recommended regular consumption of food antigens in early childhood since it provides a protective sustained immune response; therefore, a sustained consumption of major allergenic food is now recommended from the first year of life [17].

1.1.3 Epigenetic Factors

Epigenetics is defined as changes in gene expression patterns which can be inherited and independent of changes in DNA sequences. These epigenetic alterations, such as DNA methylation and chromatin modification, occur mainly prenatally, shortly after birth and during developmental periods; thus, influencing gene expression that can last an entire lifespan [10]. Currently, research suggests that epigenetics plays a role in T helper cell differentiation and cytokine gene expression, which are both important pathways in the development of FA [29,30]. For example, Nadeau et al demonstrated that there was an increased in FOXP3+ DNA methylation in regulatory T cells in the blood of asthmatic children from highly polluted areas compared with asthmatic children from less polluted ones [31]. Moreover, Martino et al reported that methylation biomarkers outperformed allergen specific IgE (sIgE) and skin prick test (SPT) for predicting oral food challenge (OFC) outcomes; thus, genome-wide DNA methylation (DNAm) biomarkers could be used for novel diagnostic testing in patients with food allergy [32].

2. IgE-Mediated Food Allergy: Soy Allergy

Food allergies are defined as an adverse immune response to the ingestion of food proteins which consist of IgE-mediated immediate hypersensitivity reactions, non-IgE reactions and mixed IgE-mediated with cell-mediated immune reactions [33,34].

Antibody IgE-mediated reactions are the most common allergic reaction which induce a variety of symptoms, usually of rapid onset compromising several organs in the human body [34,35]. The most common range from urticaria, nausea and/or vomiting, abdominal pain, mild to severe bronchospasm and respiratory distress, hypotension, tachycardia with the most severe and life-threating symptom anaphylaxis [34,36]. The first contact or initial phase with the allergen mostly occurring orally is referred to as allergic sensitization, on which the initial immunological response is determined leading to the breaking of tolerance followed by the production of food specific IgE antibodies (as shown in *Figure 2*) [37]. These antibodies then bind with their Fc fragment to the high-affinity Fce receptor on the membrane of the mast cells and basophils; thus, sensitizing these cells [38]. Upon a second contact with the same food allergen, these allergens bind to their specific IgE antibodies triggering the release of mediators such as histamine, prostaglandins, and leukotrienes in as these two cells degranulate [39]. Following cell degranulation, the production of other immunological mediators occurs which includes platelet activation factor (PAF), leukotrienes and cytokines such as interleukin (IL)-4, IL-5, and IL-13, together they all contribute to the allergic inflammation [17]. Thus, the initial phase mainly involves histamine and PAF and is under control of regulatory T lymphocytes (Treg). Nonetheless, sensitization can be present without clinical reactivity,

with specific IgE (sIgE) to a food present in the patient sera but no reaction occurring upon food exposure [37,38,40]. Additionally, to this immediate phase, an IgE-mediated allergic reaction also has a late phase [40]. During the early phase reaction, several chemotactic mediators released in the tissues attract additional inflammatory effector cells which activate and through the production of further inflammatory mediators cause the inflammation to become chronic [41]. The late phase reaction occurs 4 to 6 hours after the disappearance of the initial symptoms, it can last for several days or weeks [37]. Moreover, while the symptoms of the early phase are acute and rapidly reversible; the symptoms in the late phase are more slowly reversible. Since in the late phase the allergen has entered the bloodstream, activation pathways that can also be activated by basophils and neutrophils with specific inflammatory cytokines (tumor necrosis factor (TNF)-a, IL-9, IL-31 and IL-33) mediate late tissue inflammation [37,40,41]. Finally, following repeated exposure to the specific food allergen, the allergic inflammation is perpetuated thus mast cells increase in tissues therefore forming the background of persistent clinical symptoms such as gastrointestinal manifestations (as shown in *Figure 2*) [37].

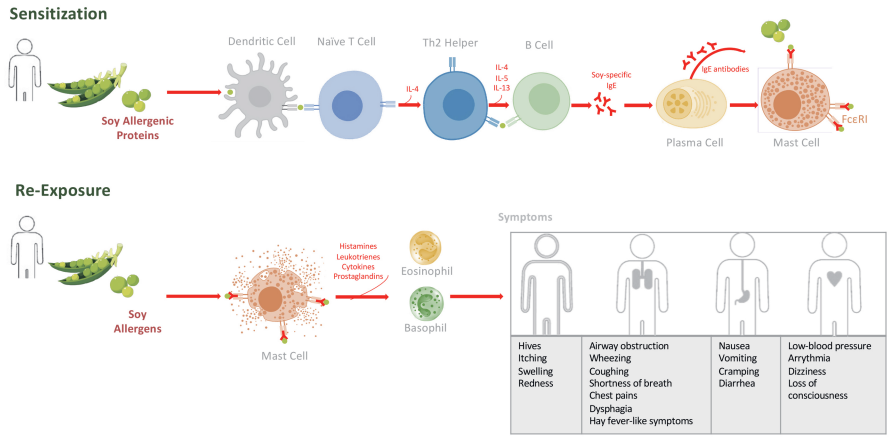

Figure 2. Mechanisms of IgE-mediated food allergy

Generally, soybean contains 35-40% protein, 30% carbohydrate, 20% lipids, 9% dietary fiber and 9% moisture based on its dry weight; these values can vary slightly depending on the location and climate where the soybean has been cultivated plus the soybean variety [42].Presently, at least 28 allergenic proteins have been suggested to present IgE-binding, but only 8 have been recognized by the World Health Organization/International Union of Immunological Societies Allergen Nomenclature Subcommittee [42,43]. The major soybean allergens are the Gly m 3, Gly m 4 (pathogenesis-related protein, PR-10), Gly m 5 (β-conglycinin), Gly m 6 (glycinin), Gly m 8 (2S albumin), Gly m Bd 28 K, Gly m Bd 30 K and Kunitz soybean trypsin inhibitor (as shown in *Table 1*) [44,45].

Table 1. Characteristics of the identified major soy allergens plus birch pollen and peanut homologous proteins

Major Soy Allergens			
Allergen	**Protein Family**	**Molecular Mass (kD)**	**Important Homologous Proteins**
Gly m 3			
Gly m 4	PR-10	17	Bet v 1
Gly m 5	ß-conglycinin (7S globulin)	140-180	Ara h 1
Gly m 6	Glycinin (11S globulin)	320-360	Ara h 3
Gly m 8	2S albumin	28	Ara h 2 and Ara h 6
Gly m Bd 28 k	Vicilin-like glycoprotein	28	
Gly m Bd 30 k	Thiol proteinases of papain family	34	
Kunitz trypsin inhibitor	Trypsin inhibitors	20	

It has been reported that in soy allergy there is a significant relationship between soy IgE levels and the rate of soy allergy resolution, with the most increased soy IgE levels corresponding with a more persistent soy allergy [1]. Moreover, Savage et al suggested two types of soy allergy phenotypes; the first and most common, early-onset soy allergy; the second, late-onset soy allergy [1]. This late-onset phenotype is possibly related to either birch pollen cross-reactivity or persistent peanut allergy [1,5,46-48]. This concurs with the results from an international multicenter study that reported that a relevant proportion of soy allergy in Europe is associated with peanut allergy; additionally, there is a strong association between soy and birch pollen allergy [5]. Presently, it has been proposed that even though cross-sensitization is soy allergic individuals is common, most do not translate into true clinical allergy. However, the high degree of cross-reactivity between peanut allergens (Ara h 1, Ara h 2 and Ara h 3) and soy allergens (Gly m 5, Gly m 6 and Gly m 8, respectively) has been scantly studied [46,49]. Cross-

reactivity between Gly m 4 and Bet v 1 has been reported repeatedly showing 53% amino acid sequence identity plus almost identical three-dimensional structure of both allergens; thus, suggesting a possible casual association between soy allergy and exposure to birch pollen [5,47,50,51]. Presently, it has been suggested that sequence homology is the key regarding the relevance of cross-reactivity; however, there are few studies regarding the clinical relevance of cross-reactivity and its impact in food allergy diagnosis [46]. Moreover, detection of IgE antibodies, or sensitization (as shown in *Figure 2*) does not always translate into a clinical allergy, with many factors determining clinical outcomes [46,50]. To give optimal health care for food allergies, avoid unnecessary elimination diets and avert exposure to potential allergenic foods, clinicians need to comprehend the risks of clinical cross-reactivity, correctly interpret clinical irrelevant cross-sensitization and consider the influence of cross-reactivity in food allergy diagnosis [40,46,50].

3. Diagnosis Soy Allergy

Currently, the ability to estimate the risk of severe reaction is one of the most significant gaps when diagnosing food allergies [52]. Although, an OFC is currently the gold standard for food allergy diagnosis, it has many pitfalls (e.g., can only be performed by a trained specialist who can treat allergic reactions, it is costly and time consuming plus it carries inherent risk of severe reactions); thus, it is infrequently used [3,53,54]. A global survey of 89 countries reported that only 10% had a prevalence data based on OFC with most relaying on other markers such as self-reported clinical history, skin prick test (SPT) or serum specific IgE (sIgE) [55]. However, these surrogates have a

low sensitivity and specificity; thus, the rates of false positives can be high which leads to an overestimation in food allergy diagnosis [52,56,57].

Recently, interest in developing better diagnostic techniques with a higher sensitivity and specificity that can predict clinical severity has grown remarkably. A promising diagnostic approach is component-resolved diagnostic tests (CRD), were sIgE antibodies are measured against individual allergenic molecules [58]. CRD testing has the potential of improving the specificity of serum IgE testing and can be performed either by single tests or by testing a range of purified allergens simultaneously in a microarray [3,58]. The BAT is a functional assay that measures the degree of basophil degranulation following stimulation with an allergen or control by flow cytometry [53]. Thus, the BAT assesses IgE cross-linking providing a more precise readout than measuring the concentration of allergen sIgE [53]. Nevertheless, the BAT still requires analytical and clinical validation as well as standardization of procedures plus quality assurance to ensure its results are not only reliable but also reproducible [53,59]. Therefore, research is needed to validate results obtained with the BAT assays and strengthen its reliability in food allergy diagnosis.

4. Food Processing on Soy Allergenicity

Since, as previously mentioned, soy is rarely consumed raw it is generally processed before consumption using different food processing technologies which can alter the protein structure and thus, impact allergenicity [6,7]. Recently, the MR, a non-enzymatic browning reaction that occurs between reducing sugars and a free amino acid group of proteins, peptides, or free amino acids has gained interest as a food processing technique that can have

an impact in food allergenicity [60-62]. Evidence suggests that the MR induces conformational and chemical modification of food proteins on the level of IgG/IgE recognition; additionally, it increases the interaction and recognition of these modified proteins by antigen presenting cells (APCs); thus, affecting their biological properties which include allergenicity [7,63-65]. Moreover, the neo-allergens formed due to the MR during soy processing have shown to trigger stronger basophil stimulation than raw soy proteins in soy allergic patients showing [8]. Nonetheless, the relationship between the chemical and physical modification of food proteins, and their potential effects on allergenicity is very complex. Walter et al reported on the variation among IgE specific to different epitopes obtained to different individuals, proving that what can be hypoallergenic for on patient is not so for another (as shown in *Figure 3*) [66]. Thus, showing how limited the knowledge is regarding the methodologies to produce a hypoallergenic protein ingredient while at the same time maintaining its functionality, flavor and nutritional quality. Since soy is a known allergen widely used in the food industry, it is important to understand the impact of heating and glycation on soy allergenicity which will in turn allow for the optimization of these processing conditions.

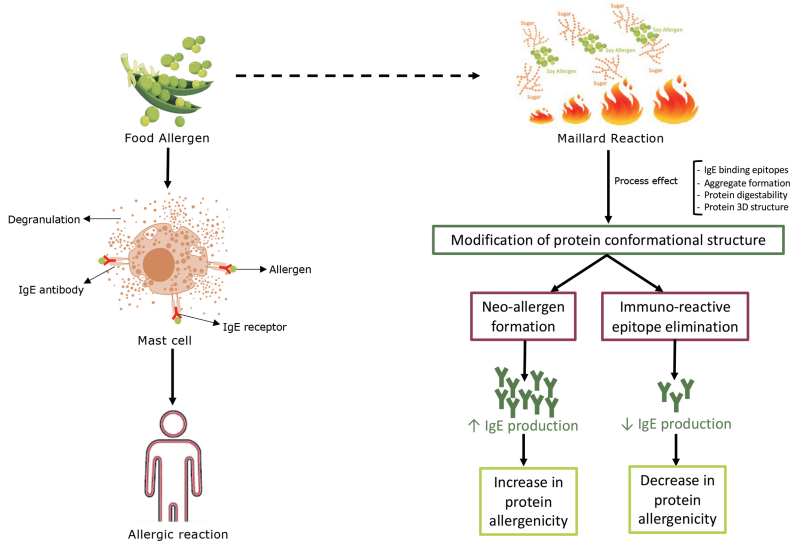

Figure 3. Possible effects of the Maillard Reaction (MR) on soy allergenicity [64]

The aim of this thesis is to analyze the current challenges in diagnosing soy allergy in adults paying special attention to the sensitization patterns to different soy allergens in accordance with their cross-reactive allergen homologous allergens. Moreover, the value of adding a basophil activation test (BAT) in the diagnostic accuracy of clinical soy allergy will be examined. Furthermore, since soy is rarely consumed raw, the effects of food processing techniques, exemplified by the MR, in soy allergenicity will be addressed. For these purposes, this thesis has been divided into two sections; *Section 1* entitled 'Clinical Soy Allergy: a diagnostic challenge' and *Section 2* entitled 'The Maillard Reaction: the impact of food processing on soy allergenicity'. *Section 1* starts with *Chapter 2*; a review where the current diagnostic pitfalls in food allergy diagnosis are discussed and the BAT is evaluated as viable option for improvement in the future diagnosis and clinical management of food allergies. Next is *Chapter 3;* a diagnostic study which aims to evaluate the value of Gly m 8 sensitization while taking into consideration the major

soy allergen homologous Bet v 1, Ara h 1, Ara h 2 and Ara h 3. Last in this section is *Chapter 4*; which describes the influence of birch pollen sensitization in the basophil activation in a patient with soy allergy and pollinosis, who experiences worsening of gastrointestinal (GI) symptoms during the birch pollen season even though the eliciting food factor does not cross-react with birch pollen allergens and their homologous (e.g., Bet v 1 and Gly m 4). *Section 2* begins with *Chapter 5;* where a comprehensive review of the effects on innate and adaptative immunity of the advanced glycation end products (AGEs) receptors formed during the MR is presented, with special focus in the relevance for food allergies. Next is *Chapter 6;* a diagnostic study of the effects of the MR on soy proteins where the biochemical characteristic and formation of MRPs/AGEs in soy glycated proteins at different heating time points was analyzed, these structural changes were then related to functional properties measured by antioxidant capacity, binding potential to sRAGE and ability to stimulate the immune cells to secrete pro-inflammatory cytokines. Last in this section is *Chapter 7*; a study that compares IgE binding tests and a functional assay (BAT) when assessing the effects of the MR in soy allergenicity. Finally, in *Chapter 8* the main findings of the present thesis will be discussed in the context of their potential clinical and immunological implications.

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Section 1

Clinical Soy Allergy: a diagnostic challenge

Chapter 2

The basophil Activation Test for Clinical Management of Food Allergies: recent advances and future directions

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Abstract

The basophil activation test (BAT) is an *ex vivo* functional assay that measures by flow cytometry the degree of basophil degranulation after stimulation with an allergen. In recent years there has been an increased interest in the diagnostic value of the BAT as it has the potential to mimic the clinical phenotype of sIgE sensitized patients, in contrast to allergen specific IgE levels. This diagnostic potential would be of particular interest for food allergies present early in life such as peanut, cow's milk and eggs which require an expensive, time-consuming, and patient unfriendly oral food challenge (OFC) for diagnosis. However, routine applications of the BAT for clinical use are not yet feasible due to the lack of standardized protocols and large clinical validation studies.

This review will summarize the current data regarding the application of the BAT in food allergy (FA) for cow's milk, egg, and peanut, being the most common causes of FA in children. Additionally, it will discuss the hurdles for widespread clinical use of the BAT and possible future directions for this diagnostic procedure.

1. Introduction

Most food allergies (FA) are diagnosed in early life, nevertheless the diagnosis can be made in older children or even adults. Currently there are fourteen foods listed as recognized food allergens in the European Union [1], of which eight are responsible for approximately 95% of FA cases: cow's milk, eggs, fish, peanuts, shellfish, soy, tree nuts and wheat [2]. In the US, approximately 9% of adults have a FA diagnosis [3]; meanwhile in Europe it stands at about 6% among adults [4]. In children below the age of five the prevalence of FA ranges between 4% and 10% worldwide [5,6].

An oral food challenge (OFC) is the current gold-standard for FA diagnosis; however, this method is labour intense, costly and implies the risk of a severe and life threating anaphylaxis. Determination of the prevalence of food allergies is dependent on confirmation by this gold standard; however, since OFCs are not only time-consuming but carry an inherent risk, they might not be acceptable to all study participants [7].

FA varies in aetiology, allergen type and severity which contributes to a challenging diagnosis. Adding complexity to this diagnosis there is also the cross-reactivity between allergens, the concomitant presence of multiple food allergens and food intolerances [8]. The symptoms of a FA and a food intolerance can overlap; therefore, a proper differential diagnosis is crucial since a FA triggers an immune response which can end in a live threating event known as anaphylaxis. The current cornerstone of FA diagnosis is the clinical history aided in most cases by specific IgE levels in blood (sIgE) and/or skin prick test (SPT). Both tests have a high sensitivity but low specificity; both detect sIgE sensitization which is not the same as a food allergy; in other words, sensitization does not necessarily lead to the development of

symptoms. Additionally, both assays currently lack reliable threshold values as they are dependent on factors like the type of allergen/food and study population (e.g., age, other diseases). The next step for food allergy diagnosis is an OFC; particularly the double-blind placebo-controlled food challenge (DBPCFC). Because of the drawbacks of these diagnostic challenges, FA diagnosis is trending towards the development of screening tests reducing the indications for OFCs. This approach, however, requires the establishment of thresholds values for SPT and sIgE that can predict the likelihood of a clinical reaction [9]. The burden of a FA misdiagnosis manifests in increased anxiety and diminished quality of life for patients plus potential nutritional deficiencies due to dietary restrictions [10]. Furthermore, the economic cost of FA misdiagnosis to society are not insignificant with many resources being dedicated to further medical evaluation, additional testing, and unnecessary prescriptions [11].

Under this scenario, the interest in the basophil activation test (BAT) has increased as a potential tool which simulates an allergic reaction *ex vivo*. The BAT is a flow cytometric assay that detects the functional ability of IgE to activate basophils which are stimulated due to allergen exposure (see *Figure 1*). Specifically, the BAT measures the expression of activation markers (mainly CD63 or CD203c) on the basophil cell membrane following cross-linking of IgE antibodies caused by an allergen [14,15]. Even though basophils are scarce (they form less than 3% of peripheral white blood cells) they are easily accessible cells and their activation is quantifiable. However, there is still knowledge lacking regarding degranulation metrics and implementation guidelines to guarantee universality in the execution of the test and

interpretation of the results [12,13]. Nevertheless, the BAT is still a promising biomarker for the diagnosis of a food allergy and can replace the OFC.

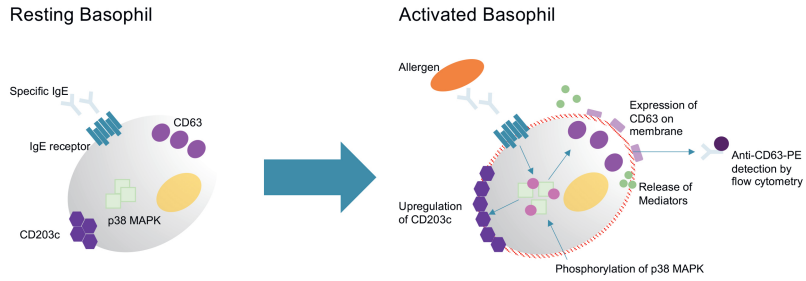

Figure 1. Basophil activation test principle**.** At a resting mode, the activation marker CD203c is expressed at low levels, but upon activation it is rapidly up regulated. In addition, when the basophil is in a resting mode the activation marker CD63 is mainly present inside the cell granules. Upon activation, after exposure to an allergen, the granules fuse with the cell membrane and CD63 is exposed on the cell surface and can be detected by labelled antibodies with subsequent flow cytometry. Therefore, the expression of CD63 is closely associated with degranulation.

The BAT has been validated for different IgE-mediated food allergies and showed a high sensitivity and specificity [16,17]. The accuracy of the BAT has been shown to be higher than tests for IgE sensitization (sIgE/SPT) [12,17] and the BAT has been able to differentiate clinically allergic patients from those who were sensitized but tolerant, with a specificity ranging from 75% to 100% and a sensitivity between 77% to 98% [12,15-17]. This specificity and sensitivity profile has positioned the BAT as a potential tool in reducing the number of OFCs [12] contributing to the interest in further developing this technique for FA diagnosis.

One reason for this improved diagnostic power can be attributed to the fact that the BAT is a functional assay, therefore the results are not

dependent on the amount of IgE alone but also on its characteristics such as affinity, avidity and even on antibodies of other isotypes (*e.g.* IgG4) [18].

Kits for performing the BAT have become more readily available for any clinical or research laboratory with a flow cytometry facility [19]. Nonetheless, standardization and harmonization of the BAT technique plus interpretation of results are still lacking and not yet defined. Many questions remain regarding how feasible it is to standardize the BAT and its methodology while adopting a protocol that allows for comparison of results between different centres. Simultaneously, universal threshold values should be established and data for specific allergens validated in different allergic populations whilst evaluating the cost-effectiveness of this technique. It is important to keep in mind when validating the BAT in the context of cross-reactivity syndromes that the performance of the test will be influenced by the control group, a healthy control group will likely overestimate the specificity of the test and therefore also the diagnostic accuracy of the reference test [13].

2. The BAT and Food Allergy Diagnosis

Food allergies that begin at an early age such as: cow's milk, egg, and peanut, present a higher diagnostic challenge as performing an OFC in children generates significant anxiety in parents and clinical staff. Furthermore an OFC in young children can be difficult to perform (*e.g.* willingness of the patient to eat) and interpret since the majority of the symptoms are subjective and the test is often not continued until objective symptoms are reached [20,21]. Both milk and egg allergies start early in life and the majority becomes naturally tolerant over time whereas peanut

allergy starts later in life and is usually lifelong. Therefore, in this subgroup of FA, the BAT as a tool would represent a significant diagnostic improvement in FA diagnosis in the clinical practice as it has the potential to reduce the need for an OFC [22]. Furthermore, most of the clinical validation data currently available for the BAT are obtained with egg, milk, and peanut; therefore, these data are used to deduce and sum up the current clinical application of the BAT. Although most articles regarding the BAT focus on diagnosis there is pertinent data available regarding the role of the BAT in monitoring the effect of immunotherapy as well [23].

The BAT mimics the clinical phenotype of patients while other allergy tests can only detect the presence of allergen specific IgE. Additionally, the BAT can differentiate sensitization from a true food allergy and thus segregate between allergy and tolerance, particularly in children with peanut or egg allergy [24].

2.1 Peanut Allergy

Peanut allergy (PA) is one of the most common food allergies worldwide accounting for 0.5% to 1.5% of the population in Western countries [25]. PA usually starts around 18 months of age but can begin later in life, either as stand-alone allergy or as part of the pollen-food allergy syndrome [26]. PA, along with tree nut allergies, is the most common cause for life threatening anaphylactic reactions. Even though only a small percentage of US children are clinically allergic to peanut (currently 1.4%), approximately 10% are sensitized to it; consequently, the proper diagnosis in this group is vital for the quality of life of the patients and their families [27]. In FA diagnosis, it is becoming critical to be able to identify the individual

disease phenotype to provide the correct individualized treatment; thus, techniques that can separate cross-reactivity with other allergen families and pollen allergen are gaining importance [28]. For example, Arachis hypogea 2 (Ara h 2) specific IgE (reflecting sensitization) has been shown to be a useful predictor of clinical reactivity [29]. In children with suspected PA, the BAT diagnosed PA with 98% specificity and 75% sensitivity. Moreover, the BAT was the best biomarker for severity, identifying severe reactions with 97% specificity and 100% sensitivity [30]. Thus, the BAT outperformed the level Ara h 2 specific-IgE, level of peanut specific-IgE and IgG4/IgE ratio, only to be surpassed by the SPT [30]. Therefore, the BAT is capable of predicting the allergic clinical status to peanut in children and could reduce the need for OFCs [12,30].

It has been observed that a higher proportion of activated basophils is associated with more severe reactions and a lower threshold of activation, raising the question if severity is linked to threshold. For example; in adults that suffer from severe peanut allergy similar associations have been identified using the BAT, where basophil reactivity to peanut was significantly higher in patients who had a history of severe allergy to peanuts when compared to patients who were sensitized to peanuts ($p<.001$) [31]. However further validation is necessary before applying this result more broadly including to other food allergens and to other patient populations [28].

Moreover, single peanut or tree-nut allergic patients are often sensitized to other tree-nuts requiring multiple OFC for diagnosis since the main challenge with these patients is distinguishing between sensitization and allergy. The BAT has shown to be capable to discriminate between allergic and nonallergic children, to the respective nut/seed when tested

against peanut, hazelnut, cashew nut, sesame, and almond [32]. Moreover, this study reported that the use of the BAT as a second step in the diagnostic process reduced the number of OFC by 5% to 15% after equivocal SPT and IgE to extracts and components. This notion was later reinforced by further studies showing that separately the SPT and the BAT were limited in their capacity to distinguished allergy from tolerance; however, when used in conjunction as part of the diagnostic kit their ability to identify allergic and tolerant patients improved [33]. Moreover, the same study also concluded that this approach could potentially reduce the need for OFCs with 78.2% in walnut/pecan cases and with 76.6% in cashew/pistachio cases [33]. Additionally, the BAT has been useful to differentiate between the allergenicity of different allergen extracts in hazelnut allergic patients. In 132 hazelnut allergic patients, sensitization was confirmed by SPT and sIgE against hazelnut [34]; following a hazelnut free diet, a DBPCFC was performed with increasing amounts of native and roasted hazelnut. The BAT was measured before and after provocation and it showed that significantly higher concentrations of the allergen extract were needed (roasted>native) to induce 50% basophil activation. Therefore, the researchers concluded that the BAT was useful in determining the reactivity of an allergen extract [34].

In PA patients, oral immunotherapy (OIT) can significantly shift the threshold dose of peanut that can be ingested without generating symptoms. Sustained protection during and after OIT has been reported in association with lower levels of basophil activation at 13 weeks after active OIT [35]. Hence, patients with a low basophil responsiveness after OIT were more likely to achieve treatment success [36]. Using the Ara h 2 as a predictor of clinical reactivity, the BAT could provide a functional surrogate of efficacy

since studies have demonstrated that basophil sensitivity to Ara h 2 is a useful biomarker for long time efficacy of peanut OIT [37]. Therefore, there is increasing data that the BAT provides additional value in monitoring the response to immunotherapy [38].

2.2 Cow's Milk Allergy

Cow's milk allergy (CMA) is the most common childhood allergy with a prevalence of approximately 2.5% worldwide [20]. The onset of this allergy is generally related to the introduction of cow's milk based infant formula and currently it affects 1 in every 50 infants under the age of one with most of the patients outgrowing their allergy during childhood or pre-puberty [39]. CMA is highly variable with allergens involved in the allergic response. Previously, ß-lactoglobulin, the most abundant whey protein, was thought to be the most important allergen in CMA since it is not present in human milk and up to 76% of the patients react to this protein [40]. Nowadays, it is known that other proteins, including □-lactalbumin and caseins, are critically involved in the disease. Low levels of ß-lactoglobulin and casein allergen-specific (known as Bos d 8) sIgE concentrations have been described as predictive for the resolution of CMA [41].

The lack of specific clinical manifestations can often lead to a misdiagnosis; the current diagnostic tests include sIgE (sensitivity 87%, specificity 48%) and SPT (sensitivity 88%, specificity 68%) while the current gold standard for CMA diagnosis is the double-blind placebo-control food challenge (DBPCFC) [41,42]. The difficult diagnostic scenario has resulted in an over-diagnosis of CMA, possibly undermining breast-feeding rates and leading to unnecessary elimination diets with negative nutritional impact

[43]. For example, a meta-analysis showed that the self-reported prevalence of CMA was approximately 6%, however the prevalence fell to 0.6% when CMA was confirmed by DBPCFC [41]. The BAT has been described as highly efficient in confirming CMA in children [44] with a sensitivity of 89% and a specificity of 83% for cow's milk extract [45], positive predictive value of 81% and negative predictive value of 96% in detecting persistently allergic patients [46]. For safety and commercial reasons, milk is processed by using various treatments (heat and other physio-chemical) that can alter the allergenic potency of milk proteins. The BAT can help to distinguish between patients that can tolerate heated forms of cow's milk from those who are not able to. Therefore, the BAT can provide important information which has implications for the prognosis of CMA patients as patients who persistently do not tolerate heated milk will have a higher chance to develop a more persistent form of CMA allergy [47,48].

Even though most patients diagnosed with CMA will outgrow this allergy in the first years of life, the prognosis for those who do not is worse; with cow milk IgE levels reported being highly predictive for this outcome [20].

Therefore, in FA that are commonly outgrown with time such as CMA, the BAT can be useful in assessing the natural resolution of food allergies and in deciding when to reintroduce cow's milk to the patient's diet. The BAT has been described as highly effective in improving the diagnostic accuracy in CMA since it can mimic the acute degree of CMA against cow's milk and human milk allergens as well as assist in monitoring the development of CMA [49]; offering the possibility that the BAT could be a

reliable and cost effective diagnostic tool when clinicians suspect an IgE mediated CMA which could in turn diminish the need for DBPCFCs [50].

In recent years, many studies have demonstrated the efficacy of OIT for CMA ranging from 67% tolerance at 18 weeks [51] to 90% showing complete desensitization after 1 year [52]. Nevertheless, follow-up studies have found that full milk tolerance decreases dramatically over time, which suggests that protection is more difficult to maintain than previously described [53]. Current recommendations suggest treatment of CMA should wait until the child is 3 years old; however, recent findings suggest that OIT is a promising strategy for CMA even in young children. OIT has demonstrated to achieve full tolerance in a high percentage of children with mild side effects that can be easily managed by slowing the desensitization [54]. At the end of oral food allergy desensitization, a significant decrease in specific IgE levels and increase in specific IgG4 levels is described in the literature, highly possible due to a switch from a Th2 to a Th1 response [55]; therefore by analysing the up-regulation of allergen-induced CD63 with flow cytometry it is possible to monitor the progression of clinical tolerance by OIT in FA. There is little information regarding the use of the BAT for monitoring the clinical tolerance induced by OIT in CMA; however, a reduction of cow's milk protein-induced CD63 expression levels at the end of the desensitization protocol has been observed and the BAT has been described as highly sensitive and closely correlated with clinical tolerance [56].

2.3 Egg Allergy

Egg allergies are common IgE mediated food allergies in children with a prevalence ranging from 1.3% to 10.1% [20], with most cases presenting

during the first year of life. The prognosis is generally good with the majority of children outgrowing their allergy at school age. A proper diagnosis of egg allergy is crucial for several reasons, which can also be valid for other food allergies, (1) it can cause severe allergic reactions in sensitized children, (2) unnecessary avoidance due to a misdiagnosis leads to significant dietary restrictions and the possibility of nutritional deficiencies, (3) early sensitization to egg is a known marker of later sensitization to aeroallergens and the possible future development of asthma and (4) many vaccines that are administered during childhood contain egg therefore an egg allergy diagnosis is crucial to determine which vaccines are safe to administer [57].

Most of the allergenic proteins are found in the egg white: ovomucoid (Gal d 1), ovalbumin (Gal d 2), ovotransferrin (Gal d 3), egg white lysozyme (Gal d 4) and ovomucin; with Gal d 2 ovalbumin being the most abundant protein in egg white [58]. Currently the BAT for egg allergy is described with a sensitivity of 63% and a specificity of 96% for CD203c expression and a sensitivity of 77% and a specificity of 100% for CD63 expression using ovalbumin [17]. An important diagnostic conundrum for clinicians is the distinction between allergic and sensitized children (clinically tolerant but demonstrate a positive sIgE and/or SPT). Studies have attempted to evaluate if the BAT can play role in addressing this diagnostic problem, which when presented requires further evaluation with an OFC. At the moment, the data suggests that most egg-sensitized but tolerant children are unable to elicit basophil activation upon allergen challenge [59]. Therefore, the BAT might be a handy tool to complement conventional tests in this group of patients allowing for a better differentiation between allergic and non-allergic patients [60].

Generally, individuals with egg allergy can tolerate cooked products that contain egg which would imply that the allergic response is dependent on epitope configuration. The ovalbumin proteins are heat labile while the ovomucoid epitopes do not seem to be affected by heating. Some heat labile allergenic proteins change their configuration during the cooking process and therefore their immunogenic potential is either blunted or minimized [61]. Alternatively, partially unfolded proteins could expose existing hidden epitopes and increase their allergenic activity. So, net effects are hard to predict without pre-existing knowledge or careful analysis of epitopes present in the allergens and severe allergic reactions can occur with a single bite of cooked egg (about 70mg of egg protein). Patients that are diagnosed with an egg allergy are placed on egg-free diets, but total egg avoidance is very challenging both for patients and caregivers. Therefore, new treatment strategies are being explored. Food oral immunotherapy can induce tolerance or desensitize patients that are allergic to egg, and it is associated with a median success rate of over 80% [62]. The aim of the OIT in the treatment for a FA, as previously indicated, is to achieve tolerance to the usual or certain doses of the allergenic food to prevent reactions in case of ingestion of small quantities of said food. This is mainly attributed to several immunological responses, mainly a decrease in specific IgE, increase in specific IgG4 antibodies, formation of specific regulatory T cells and changes in the basophil response [63].

Research has shown that in OIT in egg allergic children produces a reduction in basophil activation after allergen stimulation; a significant decrease in percentage of CD203c+ cells ($p = .04$) and a lower percentage of CD63+ cells ($p = .07$) over time after stimulation with 0.01 µg/ml anti-

ovomucoid [64]. This could be caused by changes in circulating levels and surface bound IgE on the basophils and in the amount of IgE receptors on the basophil surface. Therefore, using the BAT to test the safety and efficacy of a new immunotherapy such as low allergenic hydrolyzed egg is a handy tool for researchers and clinicians to circumvent expensive OFCs.

3. Future directions of the Basophil Activation Test

The BAT is currently not widely used in FA diagnosis, although it possess a high diagnostic potential, it lacks validation and standardization to allow results comparisons between different laboratories and protocols that can be universally applied need to be formulated. This should process should include, not only the proper reference test that would be used to validate and standardize the BAT but also the considerations of the possible drawbacks of utilizing such a reference and additionally a protocol for the allergens used and their importance in each subgroup of patients, as well as which activation markers are better suited for a specific patient population. These are among the main factors that need to be clearly established before the BAT can be universally used for clinical diagnosis or research (see *Table 1*).

3.1 BAT Validation and Standardization, the reference test

Standardisation and validation of a new diagnostic test starts with the choice of the reference test(s). Currently, OFCs outcomes are used as the gold standard for FA diagnosis. A DBPCFC would be the best test as it removes patient and observer bias [65], therefore it should be the reference test for validation and standardization of a newer diagnostic tool such as the BAT. However, even this gold standard has limitations as 3% false-positive, 3%

false-negative results and 10% dubious outcomes occur [66-68]. In addition, there are several other points of concern like differences in dosing scheme and type of food matrixes. Uniformity concerning these issues has not been obtained yet. Furthermore, reproducibility of the OFC has not been extensively tested. However, Glaumann et al. found that that the threshold of the OFC showed a much higher variation and lower reproducibility than the sensitivity of the BAT (CD-sens) [69]. Finally, although there are guidelines for the stop criteria of an OFC (i.e., Practall [70]) the question remains whether subjective symptoms are similar to objective symptoms in predicting the degree of severity and avoidance of food allergen traces. Thus, even though the OFC is the best diagnostic tool currently available, uncertainties remain about its value in clinical practise. Therefore, when validating the BAT by using the OFC, the limitations of the reference test need to be considered. This also urges us to rethink the applications of the OFC and new diagnostic tools, with their own drawbacks, in the optimisation of food allergic diagnostics. Nevertheless, like the gold standard, a new diagnostic tool should be extensively validated in robustness and applicability. For the BAT it means that much research is still required concerning the source and type of allergens used, the optimal readout-parameters and the determination of reliable and safe cut-off values.

Table 1. Technical Considerations for Widespread Use of the BAT

Validation and Standardization	
Current Challenge	How to use the proper test to validate and standardize a new tool such as the BAT
Proposal	DBPCFC best current option - Gold standard for FA diagnosis - Removes patient and observer bias
Pitfalls	DBPCFC has limitations - 3 % false positives, 3% false negatives and 10% dubious outcomes
Allergen Preparation	
Current Challenge	Factors impact the basophil surface activation markers dose-response curve: affinity of the antigen for the cell-bound IgE antibodies, density of the epitope-specific IgE antibodies and epitope spreading of the IgE antibody
Proposal	Include a broad range of allergen concentrations to better evaluate the effect of the allergen in the basophil response
Pitfalls	Most FA diagnostic tools include mainly water-soluble allergens, thus lipophilic allergens are missing Which processed allergens should be used in the BAT still require research
Basophil Identification/Activation Markers	
Current Challenge	For a valid interpretation of the results, the precise identification of the basophil population is crucial Since several markers can be measured, the BAT is a valuable tool as it can assess various immunological pathways providing valuable insights into immune mechanisms of allergic disease
Proposal	Studies that compare different identification and activation markers in the same population of patients are needed
Pitfalls	Researchers need to be aware that the current data suggests that depending on the allergen and the cohort used, there might be variability in the sensitivity and specificity of the basophil activation markers.
Read-out Parameters	
Current Challenge	Establish the most effective way to measure basophil activity. At the moment, it is advised to measure to first measure basophil reactivity and then measure basophil sensitivity.
Proposal	The area under the dose-response curve (AUC), a marker of both reactivity and sensitivity is gaining attention since it measures at multiple allergen concentrations lowering the risk of false outcomes
Pitfalls	“Basophil anergy”, a non-responding basophil to IgE receptor-mediated signalling after stimulation with one or more types of allergen remains a problem in approximately 10% of cases.

3.2. Allergen Selection

The BAT is a functional assay that evaluates the activation state of basophil cells before and after stimulation with allergens. The structure and availability of IgE-binding epitopes of the allergens in the food is influenced by food matrix composition as well as food processing steps before exposure to the patient. For food diagnostic purposes, raw allergen preparations are often used while individuals are mostly exposed to processed foods, e.g., in the case of soy or peanut proteins. Therefore, the use of raw extracts can compromise the diagnostic procedure and interpretation of the efficacy of tolerance induction; the BAT gives the possibility to test various allergen preparations which gives it a diagnostic advantage over other techniques, like the OFC.

The expected response in an allergic patient in a BAT test is a bell shape dose-response curve. Nonetheless the complexity of antigens and the affinity of different profiles of epitope-specific IgE can vary the shape of the dose-response curve [71]. Several factors have an impact on the basophil surface activation markers dose-response curve, amongst them: affinity of the antigen for the cell-bound IgE antibodies, density of the epitope-specific IgE antibodies and epitope spreading of the IgE antibody [71]. All of these factors determine the optimal allergen concentration for basophil activation and might vary significantly among subjects. To account for this issue, it is advised to include a broad range of allergen concentrations to better evaluate the effect of the allergen in the basophil response.

Currently food allergy diagnostic tools mainly include water-soluble allergens and thus lipophilic allergens are missing. Using purified mixtures of Ara h 10/11 and Ara h 14/15 (i.e., peanut lipophilic oleosins) the BAT showed

that the rate of peanut oleosin sensitization among peanut allergic patients was 65% [72]. This shows that sensitization to peanut oleosins is clinically relevant and therefore oleosins are major allergens in peanut allergy. Nevertheless, in order to evaluate the predictive value of these results they need to be compared with the outcome of the OFC and other peanut allergens with additional multicentre studies [73].

Peanut allergenic proteins are significantly affected by thermal processing which is caused by the interaction between the proteins and other food compounds such as sugars. An example of such interaction is the Maillard reaction, which involves the formation of neo-allergens by promoting the aggregation and formation of new epitopes. These neo-allergens may increase the severity of the allergic reaction in some individuals who are sensitized against processed and not raw allergens; therefore, providing a need to include processed food in the diagnosis of FA, which normally use raw extract for PA diagnosis. Moreover, it is known that thermal processing affects the degranulation capacity differently, increasing in Ara h 1 while decreasing in Ara h 2/6 [74]; granting the BAT an important role in the assessment of food processing on protein allergenicity. As shown by Vissers et al it is important to include degranulation assays in addition to the commonly studied IgE reactivity to have an inside in the clinical relevance of an allergen [74]. The authors showed that the IgE-binding capacity of Ara h 1 roasted in the presence of sugar was decreased 9000-fold compared with native Ara h 1, while the capacity to elicit mediator release was increased. In this area, the BAT has been described as helpful to determine the effect of thermal processing on the allergenicity of peanut proteins. A study which used both IgE immunoblotting and the BAT for raw and roasted peanuts did

not find a correlation between the two techniques, highlighting the fact that IgE binding studies do not predict the potential of an allergen to trigger cell degranulation. The authors explained the discrepancy between a positive immunoblotting result and a negative BAT as an occurrence due to sensitization to heat-resistant cross-reactive carbohydrate determinants, which is a main cause of positive IgE results that have no clinical significance [75]. Consequently, the BAT provides important additional information to the results yielding from the IgE binding techniques.

In addition to proving valuable information regarding the allergenicity of peanuts and hazelnut, the BAT has yielded meaningful information about the non-specific lipid transfer proteins that are involved in allergies to fresh and processed fruits. The BAT showed that only severe heat treatment of Mal d 3 from apple peel caused significant decrease in its allergenicity, suggesting that the sugar in fruit may contribute to the thermostability of the allergenic activity [76]. Hence, the BAT is a suitable diagnostic tool to assess the allergenicity of processed allergens. Nonetheless, which processed allergens should be used in the BAT to reflect the *in vivo* situation still needs much research.

3.3. Determination of the degree of basophil activation

3.3.1 Activation Markers

Following allergen stimulation, human basophils exhibit different degranulation patterns releasing various mediators and expressing particular activation markers. The BAT detects phenotype changes of allergen induced basophil degranulation; for this different protocols are in place using CCR3, CD123, CRTH2, CD203c or anti-IgE to identify basophils. All these markers are

expressed on the basophil membrane but secondary makers are necessary to exclude CRTH2 + T cells or CD123+ plasmacytoid dendritic cells. Additionally, it has been suggested that CD203c is the marker that reveals the purest basophil cell population when compared to other markers ($CCR3^+$, $CRTH2^+/CD3^-$, $CCR3^+/CD3^-$, IgE^+, among others) and that anti-IgE should be used when a basophil population of very high purity is needed [77]. For a valid interpretation of the results, the precise identification of the population of basophils is crucial [15]. As long as these markers are not compared in one population it is difficult to determine which markers perform best; hence studies that compare different identification and activation markers in the same population are needed.

Moreover, degranulation is detected by surface expression of CD63, which is only expressed on the inner side of the granule membrane of the resting basophil [78]. Since the development of the BAT, several activation markers have been studied and compared. In the mid 1990's the CD63 activation marker was discovered and currently is the most favoured activation marker because it is directly related to histamine release and it is easily accessible since it is expressed in a distinct positive population [48,77,79]. The other valuable marker is CD203c [80]; however, it does not form a distinct positive population and is a more general basophil marker which can be used both as an identification and as an activation marker [48]. CD63 and CD203c are upregulated after IgE receptor aggregation but follow partially different metabolic pathways.

Presently, there is no standardization of CD63 or CD203c detection and the determination of the positive threshold value [24]. Furthermore, at several allergen concentrations, some studies have shown, that CD63 and

CD203c expression differed between allergic or sensitized patients [77]. For example, in hazelnut allergy CD203c expression showed a better discrimination capacity when compared to CD63 and therefore was more accurately able to distinguish between sensitized and allergic patients [80]. This could be explained by the differences in kinetics between CD63 and CD203c. While the maximal up-regulation for CD63 is between 25-30 minutes, for CD203c it is at 10-20 minutes; in this study the stimulation time used averaged 15 minutes to capture both markers [80]. In general, expression of CD203c reach their peak quickly and starts to decline after approximately 20 minutes and both CD63 and CD203c disappear after 4 to 5 hours of incubation.

A possible new marker is CD300, a surface receptor on basophils. This receptor was shown to increase during IgE mediated basophil degranulation, but it is not necessarily elevated as a result of an allergen-mediated degranulation. The CD300 marker is correlated to enhanced degradation and its expression levels correlated with the severity of symptoms particularly in children with a severe CMA symptomatology; thereby suggesting that CD300c has a role in the clinical manifestation of CMA by decreasing the activation threshold of basophils [81]. An additional marker of interest is CD300a, an inhibitory receptor that is rapidly up regulated in response to IgE/FcεRI (high affinity receptor for IgE) and inhibits anaphylactic basophil degranulation [82]. Furthermore, the expression of CD300a, similar to CD63, remained in a plateau for approximately 2 hours. CD300a is associated with inhibitory ITIM-mediated signalling (immunoreceptor tyrosine-based inhibitory motifs) which may hamper activation processes [82]. Moreover with an inhibitory net observed effect, several studies have reported a CD300a dominance over

CD300c [81,83]; thus more precise tools are needed to characterize the functional input from CD300a and CD300c.

The current data suggest that depending on the allergen and the cohort used, there might be a variability in the sensitivity and specificity of the basophil activation markers. On the other hand, as several markers can be measured, the BAT is a valuable tool for researchers as it can assess various immunological pathways providing valuable insights into immune mechanisms of allergic diseases [22].

3.3.2. Result Analysis and Interpretation

Since the analysis and interpretation of the results of the BAT can be intensive and laborious, recently a data driven programmatic approach has been proposed to analyze the flow cytometric results in a more reproductible, unbiased and productive way. By using the R Bioconductor package, flowCore, researchers were able to analyze 269 basophil activation tests from a clinical trial in a quick and efficient way, representing a net saving of 1340 minutes of labour by a skilled operator and only 2% of the basophil activation results differ significantly from manual gating [84]. This data-driven approach could provide a platform for the BAT data to be analysed in a more transparent and reproducible way with better quality control and additionally providing an adequate way for data sharing among clinicians and researchers.

Commonly there are two ways to measure basophil activity; by the number of basophil that respond to a stimulus (basophil reactivity) or by the allergen concentration at which half of all reactive basophils respond (basophil sensitivity); once reactivity is confirmed it may useful to evaluate sensitivity [48]. Basophil reactivity can be expressed as %CD63+ basophils at

a given allergen concentration and it refers to the proportion of basophils that express CD63 compared to the negative control [85]. Basophil reactivity can also be expressed as the ratio of %CD63+ to allergen IgE-mediated positive control (anti-IgE or anti- FcεRI) and recently two studies reported an association between basophil reactivity and symptom severity in PA patients [30,85]. To measure sensitivity the reactivity at 6-8 allergen concentrations is measured, then the graded response is fitted to a curve of reactivity vs allergen concentration and the eliciting concentration at which 50% of basophils (EC50) is determined [71]. From this parameter, CD-sens can also be determined as $1/EC_{50}$ x 100. Basophil sensitivity has been reported to be helpful in the diagnosis of food allergy [12,71]. Both basophil reactivity and sensitivity seem to be distinct parameters of activation; nonetheless both are regulated by *Syk* and appear to be independent [71].

Recently, the area under the dose-response curve (AUC) is gaining interest since it is a marker of sensitivity and reactivity [48,71] and it that it can be calculated in cases where responses do not fit well to a typical dose-response curve [48]. Furthermore, it uses several measuring points, at multiple allergen concentrations, which lowers the risk of false outcomes.

Therefore, basophil reactivity and basophil sensitivity plus read-out parameter such as CD-sens, EC50 and AUC have been reported as sensitive biomarkers that reflect the clinical severity of anaphylactic reactions, the clinical thresholds for eliciting symptoms and OIT particularly in FA patients [86]. A result of increased basophil reactivity and sensitivity reported in thresholds of BAT parameters, can help a clinician decide whether or not to perform an oral provocation test [86].

3.4. Automation of the Basophil Activation Test

Presently, the BAT is regarded as a good *ex vivo* test that holds many diagnostic advantages in FA which include high sensitivity/specificity, low risk profile, potential to predict symptom severity and importantly the potential to discriminate between sensitized asymptomatic and truly allergic patients [17,87]; nevertheless, as mentioned before, there are still some hurdles to fully embrace the capabilities of this test broadly in the clinical practice.

It is not only the lack of standardization that is currently a challenge, but there are also issues concerning some of the procedural limitations such as the needed flow cytometry expertise, cost, technical operational and maintenance aspects, the challenging pre-processing, and pre-labelling of samples and the ponderous of existing workflows. Difficulties like this means that currently the BAT remains limited to specialized laboratories.

The BAT is performed in a limited number of laboratories as blood sampling and storage for this type of test requires special conditions to preserve cell viability and functionality (*e.g.* sample needs to remain refrigerated) and should be used within 8 hours after obtaining the sample, although some researchers currently suggest it can be as long as 24 hours [88,89], since at room temperature IgE mediated reactions decrease faster [59]. Currently, there are several time limits mentioned in the literature so there is no real consensus and this poses a problem because presently the inclination is to obtain the sample and perform the test so quickly that it is a challenge [15].

In order to reduce hands-on time efforts have been made to develop a simplified and standardized whole-blood based BAT prototype procedure to increase the automation of this test. Arif-Lusson et al proposed a whole

blood based and simplified procedure for the BAT which relies on a dry antibody formulation technology that can be transposed in a 96 well plate format [90]. Moreover, a novel microfluidic-based immunoaffinity BAT (miBAT) has been introduced to simplify the cumbersome BAT processes and therefore makes it more accessible to clinical practice. This microfluidic device is coated with anti-CD203c and designed to capture basophils from whole blood directly which are then activated by anti-FcεRI antibody and followed by optical detection of CD63 expression [91]. Blood collected from allergic patients and healthy controls was analysed with the miBAT with the expression of CD63 percentage significantly higher after allergen activation when compared to the negative control ($p<.001$) and miBAT data were comparable to flow cytometry [92]. This technique however still needs validation in larger patient populations to assess its performance.

In addition to the storage time of the sample, there is a lack of consensus regarding which of the existing BAT protocols should be applied to harmonise results and therefore be able to compare them between laboratories. Behrends et al recently published a simplified protocol with automation of sample preparation, measurement, and data analysis plus lengthening of the time between blood collection and sample processing [87]. The researchers created a novel gating strategy with 3 antibodies (FcεRIα, CD203c, CD63) which was compared to their previous protocol that used 12 antibodies; the results found no differences in sensitivity and specificity between the two protocols or between the automated and the manually analysed samples, which saved 90% of labor time. Moreover, this new protocol by Behrends considerably extended the time frame for performing a BAT after blood donation to 7 days for whole blood storage at

room temperature and 17 days at 4°C prior to BAT preparation and measurement. The researchers confirmed their results via a nationwide ring trial that showed a robust and applicable BAT protocol in a variety of flow cytometers [87]. Agyemang et al took a different approach to preserve basophil activation stability and therefore expand the use of the BAT [93]. Agyemang and colleagues evaluated a novel peanut-BAT (P-BAT) as a diagnostic method of peanut allergy; in this pilot study basophils in whole blood were stimulated with six peanut concentrations (0.0001-10 mg/ml) within 3 to 4 hours of sample acquisition and activation was measured by CD63+ and CD203c+ expression via flow cytometry on days 0, 1, 3 and 5. Findings showed basophil activation at each peanut concentration with the P-BAT method on day 0, and it was sustained on days 1, 3 and 5 without further stimulation; therefore by using the P-BAT method researchers eliminated the immediate need for sample processing by a simple activating basophils in a simple way using whole blood, which can be easily performed in the first 4 hours of sample collection [93].

3.5 Passive BAT

Directly exposing basophils from fresh whole blood or isolated peripheral blood mononuclear cells to various allergen concentrations is a technique known as “direct BAT” [14]. This technique has certain limitations; for example: the recommendation that the test has to be performed within 8 to 24 hours after collection of the blood sample as basophil reactivity decreases over time [89] and a key problem known as “anergy”, which means non-responding basophils to IgE receptor-mediated signalling after stimulation with one or more types of allergen or anti- FcεRI IgE receptor

stimulation, a problem that presents in about 10% of the individuals [15]. This "basophil anergy" appears to be associated with a down-regulation of basophil *Syk* expression and function plus an apparent reduction in the incidence of allergic rhinitis [94].

Research has shown that it is possible to reproduce passive sensitization of basophils with IgE *ex vivo*, this technology can be applied to allergic diseases as well. In the "passive" (also called "indirect") BAT (iBAT) isolated basophils from pooled healthy donor blood are stripped from receptor bound IgE and passively sensitized with sIgE from an allergic patient's serum. Subsequently, these basophils can be activated by exposing them to the allergens of interest. This allows the storage of large amounts of patient serum and to determine the allergic status at once at a later point in time, solving one of the crucial performance issues with the BAT [14]. Moreover, by using pooled blood from several donors the risk for "basophil anergy" is reduced. This technique demands more time and is more labour intensive when compared to the direct BAT, but the advantages it presents for researchers and clinicians for exploring immunological allergy mechanisms as well as optimizing allergy diagnosis and monitoring treatment efficacy shouldn't be overlooked [14]. A different approach to combat the issue of non-responding basophils was taken by Santos et al, by researching if the ability to elicit peanut-induced cell activation could be transferred by passive sensitization of LAD2 mast cells with patients' plasma 85. The mast cell activation test (MAT) to peanut strongly correlated with the BAT (R_S = 0.808, $P < .001$) plus the MAT gave a conclusive results for participants with non-responding basophils; however, the BAT showed a greater diagnostic accuracy due to its higher sensitivity [95].

Additionally, it is important to note that by using a passive BAT the influence of the patient basophils are not taken into account which might be an important factor. However, a study regarding peanut allergy and the use of the iBAT predicted a peanut allergy which is comparable to studies that used the direct BAT [17]. Nevertheless, it is unknown whether intrinsic basophil characteristics can play a minor role in some patients.

4. Conclusions

The current challenge in FA diagnosis is to develop a technique which is both accessible and reliable plus could replace the expensive, time-consuming, and patient-unfriendly OFCs. The key messages from this review are summarized in *Figure 2*. Currently, the BAT is a promising *ex vivo* diagnostic tool in food allergy diagnosis. Nonetheless, several methodological aspects need to be investigated before a standardized protocol is available which can be universally applied making comparisons between results of different studies justified, which are summarized in *Table 1*. Besides the fact that the diagnostic accuracy of the reference test, an OFC, is crucial the type of allergen used in the BAT is a main point to consider as the BAT doesn't reflect food processing and digestion. In addition, it is also highly necessary to standardize the basophil identification and activation markers plus procedures for detection of activation basophils. In our opinion, the way conquering this challenges might be possible by collaboration between clinicians and laboratory technicians in multi-centre studies. Furthermore, the role and applicability of current diagnostic tools, such as OFC, should be discussed in order to be able to determine the diagnostic power and place of the BAT in the current diagnostic work-up for a food allergy.

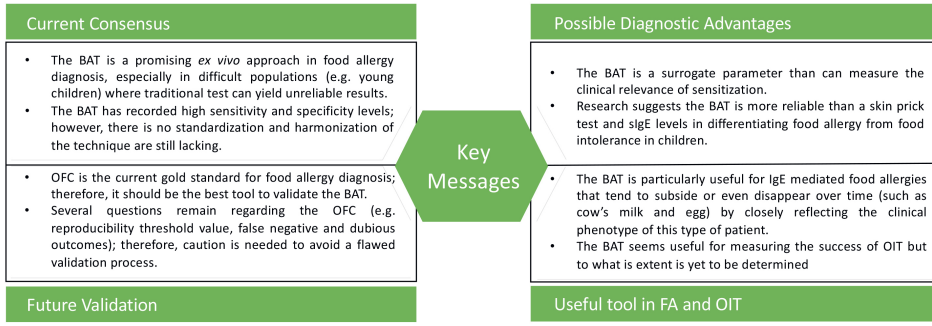

Figure 2. Summary of the key messages of the current status of applying BAT in allergy diagnostics and immunotherapy

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Chapter 3

Soy Gly m 8 sIgE has limited value in the diagnosis of Soy Allergy in Peanut Ara h 2 Sensitized Adults

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Abstract

Recently, specific IgE (sIgE) sensitization against Gly m 8 (soy 2S albumin) has been described as a good diagnostic marker for soy allergy (SA). The aim of this study is to evaluate the diagnostic value of Gly m 8 by determining the sensitization profiles based on the homologues soy allergens Bet v 1, Ara h 1, Ara h 2 and Ara h 3. Thirty soy allergic adults were included, sIgE to total soy extract, Gly m 8, Gly m 4, Gly m 5, Gly m 6, Bet v 1, Ara h 1, Ara h 2 and Ara h 3 were determined. Sensitization patterns were analyzed and determined. The clinical relevance of sIgE of Gly m 8 sensitization was measured by assessing its capacity to degranulate basophils in Gly m8 sensitized patients by an indirect Basophil Activation Test (iBAT). Based on the sIgE patterns of sensitization two groups of SA patients were identified. (i) Peanut-associated SA group (all patients were sensitized to one or more of the peanut compounds) and (ii) Non-Peanut/PR-10-associated SA group (22 patients were sensitized to Gly m 4 and Bet v 1 but not to any of the peanut compounds). A high and significant correlation between total soy extract and Gly m 6 (R^2 =0.97), Gly m 5 (R^2 =0.85) and Gly m 8 (R^2 =0.78) was observed. A non-significant correlation was observed between the levels of sIgE of Gly m 8 vs Ara h2. The iBAT results showed that Gly m 8 did not induce basophil degranulation in any of the Peanut-associated patients; indicating that the Gly m8 sensitizations were not clinically relevant. Gly m 8 was not a major allergen in the selected soy allergic population. The iBAT results indicated that Gly m 8 was not able to induce basophil degranulation in sIgE Gly m 8 sensitized soy allergic patients. Thus, Gly m 8 would have no added value in the diagnosis of SA in the present study population.

1. Introduction

In Europe, prevalence of self-reported soy allergy (SA) is approximately 1.5%, dropping to 0.3% when diagnosed with an oral food challenge (OFC) [1]. This variation is not uncommon in food allergy (FA) diagnosis, which currently relies on a skin prick test (SPT) and specific IgE (sIgE) levels [1-3]. However, an SPT and sIgE with soy extract while specific (87.3% and 93.8%, respectively) are not sensitive enough (44.4% and 33.3%, respectively) for diagnosing IgE-mediated SA [4]. Nonetheless, an OFC is not frequently performed since it is time consuming, costly and carries inherent health risks [5-7].

Presently, most studies describe sIgE to Gly m 5 and Gly m 6 as reliable diagnostic markers and both have shown to contribute to a more severe form of SA [8-10]. Both have shown a higher sensitivity than soy extract, 63% for Gly m 5 and 68% for Gly m 6 but a lower specificity, 73% for both allergens [11]. Gly m 4, known as a pathogenesis-related protein (PR-10), due to cross-reactivity with the birch pollen allergen Bet v 1, has been identified as the most common allergen in SA patients with birch pollinosis with a sensitivity of 81% and a specificity of 78% [12]. Additionally, high levels of sIgE to Gly m 4 have been reported in patients with anaphylactic reactions to soy drinks [13,14]. Recently, interest is growing in Gly m 8 (2S albumin) as a diagnostic marker of SA; however, its value as a diagnostic marker in SA has not been settled. Previous studies reported that Gly m 8 had the best accuracy in diagnosing SA when compared to Gly m 4, Gly m 5 and Gly m 6 but performed equally when compared to SPT and sIgE of soy extract [11,15]. Furthermore, Ebisawa et al reported Gly m 8 as the best-known predictor of severe SA in

children [16]. However, Lin et al reported that 2S albumins were major allergen in SA patients [17].

Peanut allergy has been suggested to be the most common co-existing allergy in SA patients [18-22]. It was reported that of 133 SA children, 103 were allergic to peanut (88%) [18]. Conversely, in a study on peanut-sensitized adults, 87% were identified to be sensitized to soy [19]. Because of the homologous proteins in peanut and soy it is not uncommon to encounter patients that have sIgE antibodies to these two food-based allergens without necessarily being clinically reactive to both [20]. It has been described that peanut allergens Ara h 1, Ara h 2 and Ara h 3 show similarities to soy allergens Gly m 5, Gly m 8 and Gly m 6, respectively [21,22]. Nonetheless, the influence of cross-reactive allergens on the IgE profile to soy allergens with respect to peanut has been poorly considered when studying SA [21]; with few studies reporting the influence of peanut allergy on soy allergen sensitization profiles and thus on the clinical relevance of these sensitizations when SA is suspected [23-25]. Cross-reactivity between Gly m 8 and Ara h 2 has been reported by several studies [20-24]. Since there are homologous proteins in peanut and soy, it is not uncommon to encounter positive IgE antibody tests to both of these foods in individuals who are clinically reactive to one of these allergens.

The present study aims to evaluate the value of Gly m 8 sensitization in the diagnosis of SA considering sIgE to the major cross-reactive allergens of soy (peanut components Ara h 1, Ara h 2, Ara h 3 plus birch component Bet v 1) and by analysing the individual clinical allergic reactivity to Gly m 8 and Ara h 2 (2S albumins) with an indirect Basophil Activation Test (iBAT) since this

functional assay mimics an *in vivo* allergic reaction; thus, the determination of the clinical relevance of positive Gly m 8 sIgE [26-28].

2. Materials and Methods

2.1 Study Population

The study was approved by the Medical Ethical Review Committee CMO Regio Arnhem-Nijmegen, The Netherlands. A total of 30 adult patients visiting the Outpatient Allergy Clinic in Rijnstate Hospital Arnhem were selected based solely on a SA diagnosis determined by clinical evaluation in combination with sIgE measurements. All patients signed an informed consent and filled a questionnaire regarding allergy symptoms, severity of symptoms and the specific soy products consumed upon the onset of symptoms as well as the presence of co-existing allergies and related symptoms. Before blood collection, patients were requested to stop the use of oral antihistamines for 3 days and steroid medications for 10 days.

An OFC was not performed since a high number of patients enrolled in this study had a clinical history of anaphylactic shock (AS) (64%).

2.2. Determination of Specific IgE

Specific IgE to total soy extract, native Gly m 8 (nGly m 8), recombinant Gly m 4 (rGly m 4), nGly m 5, nGly m 6, rBet v 1, rAra h 1, rAra h 2 and rAra h 3 were determined using the ImmunoCAP250 with allergen caps (nGly m8 was a gift by Thermofischer, Uppsala, Sweden) according to the manufacturer's instructions. Patients with sIgE of ≥0.35 kU/L were considered as IgE sensitized.

2.3. Indirect Basophil Activation Test (iBAT)

A 4-mL aliquot of fresh EDTA-anticoagulated blood from eight adult non-allergic healthy blood donors with the blood group O was centrifuged for 10 min at 2200 g at room temperature within 24h of collection. Buffy coats were collected and combined, then washed with physiological salt and resuspended in a total volume of 2 mL (leucocyte count between 12.5–15 × 10^9/L). The resuspended buffy coat was centrifuged for 5 min at 1000 g and 11°C after which 2 mL of cold stripping buffer (0.15 M H4NaO5P and 0.005 M KCl, pH 3.55) was added to the buffy coat and the centrifuge protocol was repeated. After the stripping procedure the buffy coat was washed with Basophil Stimulation Buffer (BSB) which contained calcium, heparin, and IL-3 (Bühlmann, Basel, Switzerland). A 500 µL aliquot of buffy coat was incubated with 130 µL of undiluted serum from the tested patient for 16h at 37°C. A BAT was performed with the resensitized donor basophils which were separately stimulated with nAra h2 (Bühlmann Laboratories AG, Schönenbuch, Switzerland) or rGly m8 (Abcam plc, Cambridge, UK).

The iBAT was performed using a Flow2-CAST kit (Bühlmann) according to the manufacturer's instructions [29]. Basophil activation was determined by the CD63 expression level of 500 basophils measured using flow cytometry (FACS Canto II; BD Biosciences, San Jose, USA). The stimulation of basophils using an anti-FcRI high-affinity IgE receptor antibody was used as a positive control (a <20% difference between the positive and negative control values indicates a putative non-responder).

Dose-response curves were performed using nAra h 2 or rGly m 8 using a final concentration range of 0.00125–18 ng/mL for Ara h 2 and 100-2000 ng/ml for Gly m 8 to reach a plateau phase in the dose-response curve for

most patients. Basophil reactivity was expressed as the %CD63+ basophils upon stimulation with the allergen adjusted for the negative control [28-30]. Presently, the cut-offs for BAT positivity are not clearly established and the ones defined in one population are not necessarily directly transferable to another one [31]. However, the manufacturer suggest a range from 6% to 15% [29]. Thus, for the present study the cut-off value was set at 15% CD63+ cells for Gly m 8 and 10% CD63+ cells for Ara h 2. Dose-response curves were fitted in GraphPad Prism (version 8.0.2, GraphPad Software, San Diego, USA) using a three-parameter logistic curve fit (hill slope 1).

2.4. Dot Blot

In the iBAT assay, rGly m 8 (Abcam plc, Cambridge, UK) was used. To confirm that the sIgE from patients' sera recognized rGly m 8, a Dot Blot was performed. Three patients were selected: patient #6, patient #8 and patient #14 as a negative control.

3. Results

3.1 Sensitization profiles of studied population

3.1.1. Sensitization pattern against soy proteins

From the total study population (n=30, *Table 1*), sIgE to soy extract was detected in 14 patients (47%), Gly m 4 in 26 patients (87%), Gly m 5 in 7 patients (23%), Gly m 6 in 6 patients (27%), and Gly m 8 in 11 patients (33%).

Table 1. Demographic Data plus the sIgE profiles for soy and soy allergens Gly m 4, Gly m 5, Gly m 6 and Gly m 8 plus potential cross-allergens: Bet v 1, Ara h 1, Ara h 2 and Ara h 3. Pt. = patient

Pt.	Age/ Sex	ImmunoCAP IgE (kU/l)								
		Soybean	nGly m 4	nGly m 5	nGly m 6	nGly m 8	rBet v 1	rAra h 1	rAra h 2	rAra h 3
1	26/F	2.66	4.84	1.67	2.74	0,38	13.5	19.0	92,90	10.7
2	23/F	9.59	<0.35	3.58	12.3	<0,35	<0.35	81.1	>100	28.3
3	32/M	2.69	<0.35	2.75	1.78	0,97	<0.35	19.5	36,00	2.93
4	55/M	1.63	0.81	0.36	0.86	<0,35	14.5	<0.35	1,64	<0.35
5	40/F	1.96	20.4	<0.35	1.32	0,41	45.0	3.84	5,71	1.38
6	21/F	19,5	<0.35	9,75	19,7	3,76	<0.35	68,4	>100	71
7	34/F	3,35	<0.35	2,39	2,88	0,47	<0.35	63,4	71,5	12,8
8	25/F	19,8	2,51	4,86	25,5	5,59	>100	63,3	>100	67,3
9	56/F	<0.35	1.24	<0.35	<0.35	<0,35	8.62	<0.35	<0,35	<0.35
10	32/F	2.23	2.00	<0.35	<0.35	0,60	26.1	<0.35	<0,35	<0.35
11	43/F	<0.35	8.28	<0.35	<0.35	<0,35	28.5	<0.35	<0,35	<0.35
12	26/F	0.93	28.0	<0.35	<0.35	<0,35	64.8	<0.35	<0,35	<0.35
13	65/F	<0.35	1.84	<0.35	<0.35	<0,35	9.88	<0.35	<0,35	<0.35
14	46/F	<0.35	4.98	<0.35	<0.35	<0,35	12.8	<0.35	<0,35	<0.35
15	51/F	<0.35	7.2	<0.35	<0.35	<0,35	14.7	<0.35	<0,35	<0.35
16	36/M	<0.35	1.43	<0.35	<0.35	<0,35	9.55	<0.35	<0,35	<0.35
17	68/F	0.83	8.67	<0.35	<0.35	0,66	45.5	<0.35	<0,35	<0.35
18	50/F	0.36	5.16	<0.35	<0.35	<0,35	15.6	<0.35	<0,35	<0.35
19	21/M	0.58	11.3	<0.35	<0.35	<0,35	78.1	<0.35	<0,35	<0.35
20	52/F	<0.35	0.63	<0.35	<0.35	<0,35	2.84	<0.35	<0,35	<0.35
21	69/F	<0.35	10.8	<0.35	<0.35	<0,35	25.4	<0.35	<0,35	<0.35
22	67/M	<0.35	8.12	<0.35	<0.35	1,00	37.0	<0.35	<0,35	<0.35
23	60/F	<0.35	0.95	<0.35	<0.35	<0,35	8.25	<0.35	<0,35	<0.35
24	52/F	<0.35	5.78	<0.35	<0.35	<0,35	36.4	<0.35	<0,35	<0.35
25	58/F	<0.35	0.99	<0.35	<0.35	<0,35	4.93	<0.35	<0,35	<0.35
26	58/F	<0.35	11.8	<0.35	<0.35	<0,35	45.1	<0.35	<0,35	<0.35
27	46/M	<0.35	1.05	<0.35	<0.35	<0,35	4.59	<0.35	<0,35	<0.35
28	54/M	1.97	3.27	<0.35	<0.35	0,50	19.4	<0.35	<0,35	<0.35
29	26/F	<0.35	4.34	<0.35	<0.35	<0,35	16.3	<0.35	<0,35	<0.35
30	20/F	<0.35	37.8	<0.35	<0.35	0,51	69.5	<0.35	<0,35	<0.35

A high and significant correlation was observed between sIgE to soy extract and Gly m 5 (R^2 = 0.85, *Figure 1A*), Gly m 6 (R^2 =0.97, *Figure 1B*), and Gly m 8 (R^2 =0.78, *Figure 1C*); while no significant correlation was found between soy extract sIgE and Gly m 4 (*Figure 1D*), possibly because soy extract in the reagent used in the diagnostic platform contains little Gly m 4 due to the pre-treatment procedures.

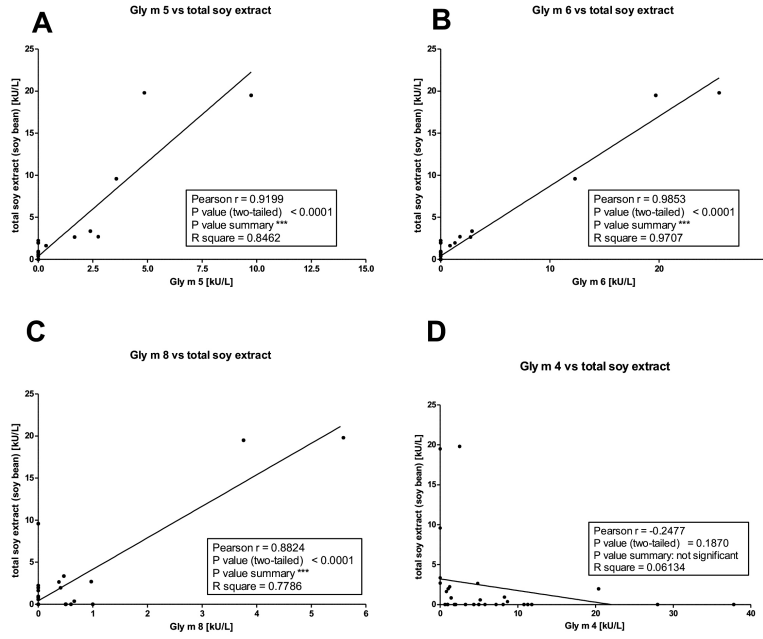

Figure 1. Correlation between sIgE levels against total soy extract and specific soy components: Gly m 4, Gly m 5, Gly m 6 and Gly m 8 detected in the sera of 30 soy allergic patients.

3.1.2. Sensitization Profiles Identified: Peanut-Associated and PR-Associated SA

Based on the individual sIgE sensitization patterns, two profiles were identified:

(i) Peanut-Associated SA group (26%) - represented by eight patients all sensitized to peanut components Ara h 1, Ara h 2 and Ara h 3 (as shown in *Table 1*, represented by light green). All patients in this group were sensitized to Gly m 6 (100%), 87% to Gly m 5 and 75% to Gly m 8; 50% were sensitized to Gly m 4 all of whom were also sensitized to Bet v 1 (as shown in *Table 1* and *Figure 2*).

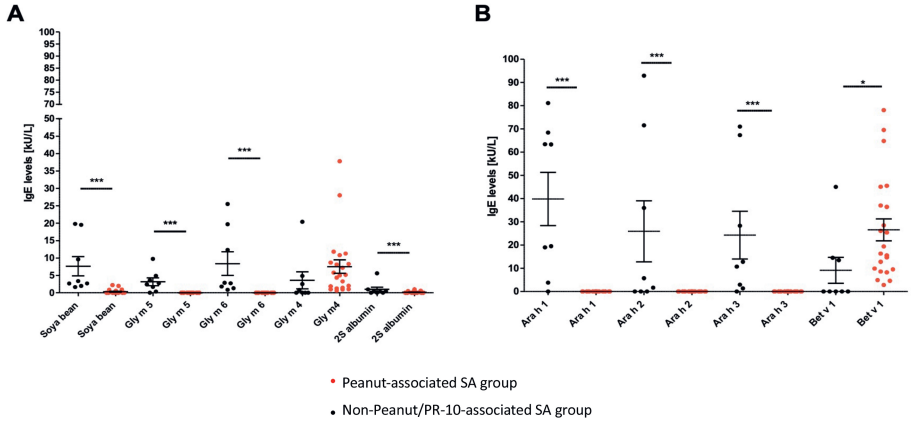

Figure 2. The sIgE distribution in the two sensitization profiles observed in the studied population (n=30); Black – Peanut associated SA; Red- Non-Peanut/PR-10 associated SA; (A) sIgE levels against soy compounds and (B) peanut compounds (Ara h 1, Ara h 2 and Ara h 3) and birch pollen compound (Bet v 1).

Correlation between sIgE levels against specific soy components and their homologue allergens in peanut and birch were analyzed. A strong positive correlation between sIgE to Gly m 6 and Ara h 3 (R^2=0.94, *Figure 3A*) was observed; while non-significant correlations were observed between sIgE to Gly m 5 and Ara h 1, as well as Gly m 8 and Ara h 2 (*Figure 3B* and *3C*).

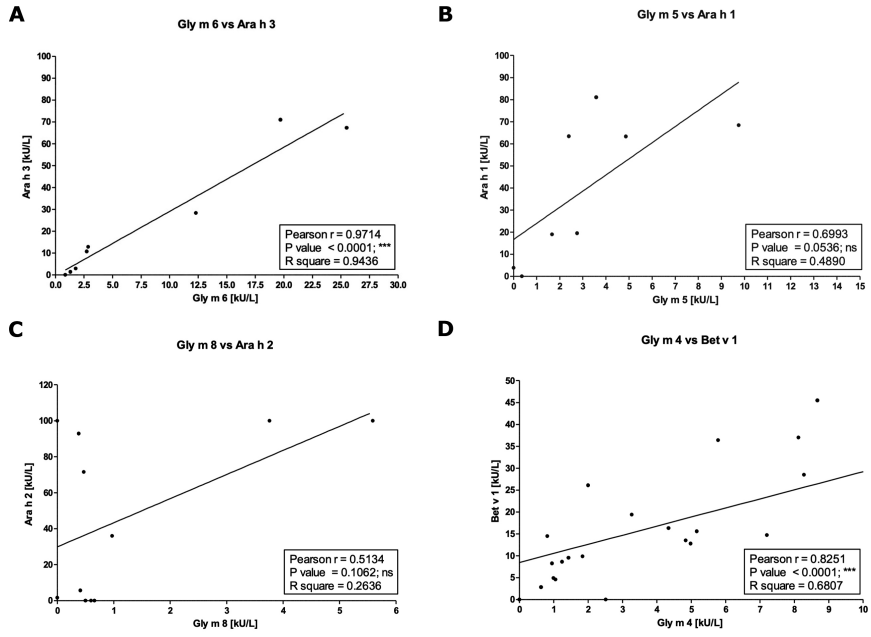

Figure 3. Correlation between sIgE levels against specific soy components: Gly m 6, Gly m 5, Gly m 8 and Gly m 4 with their homologues allergens: Ara h 3, Ara h 1, Ara h 2 and Bet v 1, respectively.

All eight patients reported allergic complaints after consumption of processed soy products such as soy sauce, beansprouts and soy flour, 4 after consumption of soy milk (50%), with 2 patients indicating they intentionally avoided consuming soy milk (*Table 2*, represented by light green). The most common symptom in this group was laryngeal edema (LE) (87%); followed by AS (62%). Oral allergy *syndrome (OAS) was described by four patients (50%) (Figure 4A).*

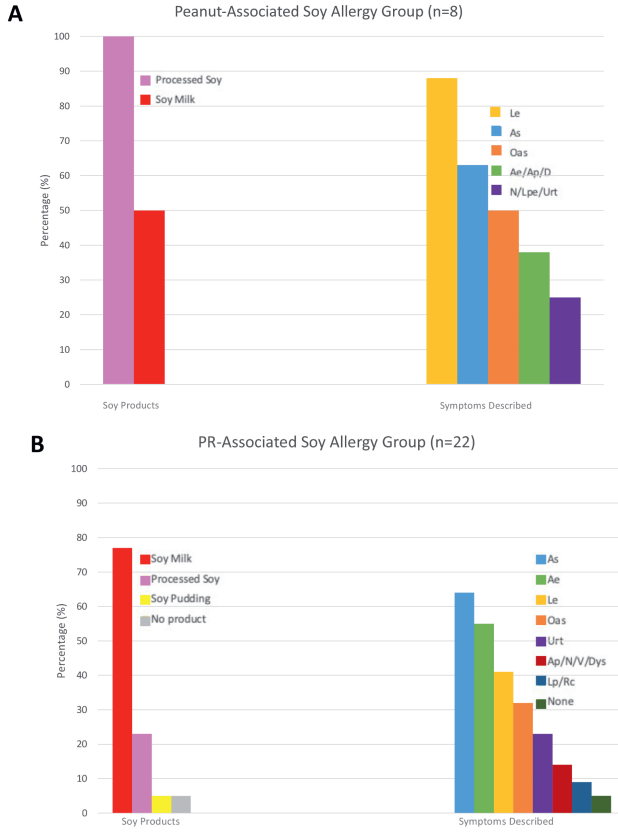

Figure 4. *Symptoms and soy products consumed according to co-sensitization profile. (A) Peanut-Associated SA group, 100% indicated consumption of processed soy and 50% indicated consumption of soy milk; (B) Non-Peanut/PR-Associated SA group, 77% consumed soy milk before symptoms, indicating the clinical importance of cross-reactivity between Bet v 1 and Gly m 4 for dietary implications, and 23% consumed processed soy products.*

Table 2. Symptoms described by patients after the ingestion of indicated soy products; *Oas=oral allergy symptoms; as=anaphylactic shock; ap=abdominal pain; d=diarrhea; urt=urticaria; n=nausea; le=laryngeal edema; ae=angioedema ; v=vomiting; dys=dyspnoea; lp=low blood pressure; rc=rhinoconjunctivitis; lpe=lip edema; pt = patient*

Pt	Symptoms	Soy products
1	oas, as, ap, d	Processed soy (tauge/soy sauce)
2	as, urt, n, d, le	Processed soy (tauge/soy sauce) and soy milk
3	oas, as, le	Processed soy (chicken pate/soy sauce) and soy milk
4	as, n, le, ae	Processed soy (soy sauce/flour)
5	le, ap, ae	Processed soy (large amounts) and soy milk
6	le, lpe, as	Processed soy (ketchap/tomato sauce/others) - avoidance of raw soy and soy milk
7	oas, le, ae, urt, lpe, ap, d	Processed soy and soy milk
8	le, oas	Processed soy (soy sauces/asian dishes/brands of meat) – avoidance of soy milk
9	as, le, ae	Alpro Soy milk
10	as, oas, ae, ap	Processed soy (soy sauce/tofu/tauge) and soy milk
11	as, le, n, d, v	Processed soy (soy sauce/soy yoghurt/soy flour)
12	oas, as, ae	Processed soy (soy sauce/soy dessert)
13	as	Processed soy and Alpro soy milk
14	as, le, dys	Alpro Soy milk
15	as, n, v, le, lp	Alpro Soy milk
16	as, ae, le, rc	Alpro Soy milk
17	as, le, urt, ae	Soy pudding
18	le, urt	Soy milk
19	ap, ae	Soy milk
20	as, ae, lp	Alpro Soy milk
21	oas, le, ae	Alpro Soy milk
22	as, ae	Alpro Soy milk
23	ae, dys, ap	Processed soy (tofu/soy sauce/tauge)
24	oas, as, rc, dys, urt, ae	Soy milk
25	as, urt	Alpro Soy milk
26	as, ae, le, n, v	Alpro Soy milk
27	oas	Soy milk
28	None	None
29	oas, urt	Alpro Soy milk
30	oas	Alpro Soy milk

(ii) Non-Peanut/PR-Associated SA group (74%) - represented by the remaining 22 patients all sensitized to Gly m 4 and co-sensitized to Bet v 1 (*Table 1*, represented by light grey), none were sensitized to any of the peanut components nor to Gly m 5 or Gly m 6 and sIgE to Gly m 8 was detected in only five patients (23%) (*Figure 2B*). A significant positive correlation was observed between sIgE Gly m 4 and Bet v 1 (R^2 =0.68) (*Figure 3D*).

Most patients (77%) pointed to soy milk as the product consumed before symptom onset, five patients (23%) reported that symptoms occurred after the consumption of processed soy products in addition to soy milk (*Table 2*, represented by light grey). The most common reaction was AS (64%), angioedema was described by twelve patients (55%), LE by nine patients (41%) and OAS by seven patients (32%) (*Figure 4B*).

3.2. Clinical relevance of IgE Gly m 8 soy sensitization

To evaluate the clinical relevance of Gly m 8 sensitizations in the diagnosis of SA, an iBAT was performed with sera of the patients from the Peanut-Associated SA group (n = 8), since all patients had elevated IgE levels to the Gly m 8 (2S Albumin). Moreover, to analyse the validity of this assay an iBAT for Ara h 2 was performed in this group as well. The reliability of the iBAT Gly m 8 results are strengthened by the fact that the iBAT Ara h 2 outcomes correspond with the recorded medical history.

For Gly m 8, there was no increase in basophil activation, with an average of %CD63 activation of 3.68 at an allergen concentration of 300 ng/mL, rising to 5.5 when the allergen concentration was 1000 ng/ml (*Figure 5A*). Thus, Gly m 8 did not induce basophil degranulation in the iBAT assay. For Ara h 2 (n=8), dose-response curves reached positive IBAT results in seven

patients (*Figure 5B*). Patient #4 had a negative iBAT to Ara h 2, which corresponded to the absence of a recorded allergic reaction to peanut or other type of nuts. The seven positive iBAT results also correlate well to the clinical history of the patients, with all describing a clear medical history of peanut allergy that started at an early age and a recorded medical episode of anaphylactic shock after peanut consumption.

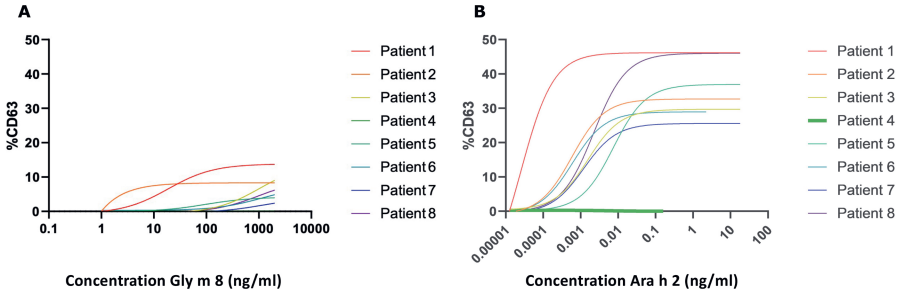

Figure 5. Non-linear fitted curves for CD63% basophil activation (%) for patients in the Peanut-associated SA group; (A) Different (final) concentrations of rGly m 8; (B) Different (final) concentrations of nAra h 2.

3.3. Dot Blot

Following pre-incubation of patient's sera with raw peanut extract to block binding of cross-reactive IgG antibodies, a positive signal was observed for patient #6 and #8 (Figure 6A), thereby confirming that rGly m 8 is recognised by sIgE. The binding for patient #6 was much lower when compared to the binding shown for patient #8 (as show in *Figure 6B*), possibly because the total IgE binding is mostly based on Gly m 8 plus the cross-reactive peanut allergens, Gly m 4 and Bet v 1; while patient #6 shows IgE partially from Gly m 8 and the cross-reactive peanut allergens but not from Gly m 4 nor Bet v 1. The negative control, patient #14, showed insignificant

binding to rGly m 8 in the Dot Blot, showing IgE binding to Gly m 4 and partially from cross-reactive Bet v 1 (*Figure 6A* and *Figure 6B*).

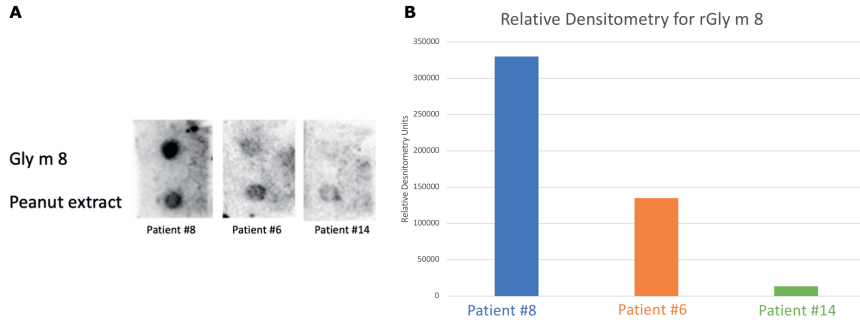

Figure 6. Dot Blot results. (A) Dot Blot images for rGly m 8 and peanut extract for patients #6, #8 and #14; (B) Relative densitometry Histogram for rGly m 8 for patient #6, #8 and #14 (relative densitometry values are corrected for the Dot Blot background reading).

4. Discussion

Currently the diagnostic capacity of Gly m 8 remains unclear, the present study analysed the diagnostic value of Gly m 8 within a SA population by determining the sensitization profiles based on the homologues soy allergens Bet v 1, Ara h 1, Ara h 2 and Ara h 3 [16,18]. Moreover, since sensitization does not equal allergy, in contrast to previous studies, the clinical relevance of sIgE Gly m 8 sensitization was analyzed using the iBAT.

The results of the present study showed two different sensitization profiles in the selected soy allergic population based on homologues soy allergens present in peanut and birch: Peanut-Associated (26%) and Non-Peanut/PR-10-Associated (74%) SA groups. These sensitization patterns are in-line with a soy-allergy phenotype described by Savage et al. possibly related to either birch pollen cross-reactivity or persistent peanut allergy [18]. In the Non-Peanut/PR-10-associated SA group, sIgE against Gly m 4 and Bet v

1 was identified in all patients; reinforcing the well-known and relevant cross-reactivity between Gly m 4 and Bet v 1 [21,25]. It has been suggested that SA individuals with pollen-food syndrome could possibly be sensitized to other legumes, particularly peanuts [21,25,32]. This association was observed in the present study in four patients who were sIgE positive to Gly m 4 and Bet v 1 as well as one or more of the peanut allergen components (Ara h 1, Ara h 2 and/or Ara h 3). This association is mainly because many food allergies are acquired due to cross-reactivity between aero-allergens and food allergens, mainly pollen; thus pollen-allergic patients can suffer from many different plant food allergens, including peanut [33,34].

In the total study population (n=30), sIgE against Gly m 8 was only found in about a third of the patients (33%). In the Non-Peanut/PR-Associated SA group, a low number of patients (23%) had a positive sIgE against Gly m 8 compared to the Peanut-Associated SA group were most of the patients (75%) had a positive sIgE against Gly m 8. This prevalence of Gly m 8 sensitization differs from previous studies that have evaluated the accuracy of sIgE to Gly m 8 for the diagnosis of SA [11,15]. However, previous studies had a high prevalence of peanut co-sensitization in their study population, ranging from 57% to 89% [11,15], which differs from the present study population, 23%. This concomitant high prevalence of peanut allergy could have influenced the diagnostic value of Gly m 8, possibly due to cross-reactivity to Ara h 2 [11]. Thus, sIgE to Gly m 8 appears to not be a very frequent soy marker when the soy allergic population is not highly sensitized to peanut. The cross-reactivity between Gly m 8 and Ara h 2, both δ-conglutin proteins, has been previously described [20-24]. However, data regarding the clinical implications of their cross-reactivity is lacking [35]. 2S albumins, such as Ara h 2 and Ara h 6, have

been described as the most important allergens of peanut and their structure is likely to contribute to the cross-reactivity between peanuts and other foods, such as soy [36]. Clinically relevant cross-reactivity of 2S albumins of different species has been reported as uncommon [37], probably due to the fact that sequence homology between peanut and soy 2S albumins is too low, i.e. approximately 40% [23,38], while a sequence homology of greater than 70% is associated with a clinically relevant cross-reactivity [39]. The homology of Gly m 8 to Ara h 2 is higher than to Ara h 6, thus a clinically relevant cross-reactivity between Gly m 8 and Ara h 6 is not expected [20,21,40].

The clinical relevance of sIgE Gly m 8 sensitization can only be confirmed with a functional assay. To our knowledge, this is the first time the iBAT has been used to assess the clinical relevance of sIgE Gly m 8. The observed results showed that sIgE sensitization to Gly m 8 was not clinically relevant in all studied patients, compared to Ara h 2 which showed positive iBAT results with adequate correlation with clinical symptoms. Therefore, studies in which sIgE sensitization profiles are correlated with clinical outcome would benefit from the inclusion of an *in vitro* functional assays, such as the iBAT. The results of the present study show the reliability and possible future uses for this type of assay in SA diagnosis, even though the iBAT for Gly m 8 needs to be validated in larger populations.

The discrepancy between the present study and previous reports regarding the diagnostic value of Gly m 8, may be due to criteria for population selection, geographical reasons and/or different 2S albumin preparations. In the present study population selection appears to be similar to previous studies, namely based on a SA diagnosis [11,12,15,16]. However, the main difference of the present study population is that the rate of peanut

co-sensitization is considerably lower, i.e., 23% vs 89% [15]. There is no obvious reason for this co-sensitization difference in study populations with the exception that there is an age discrepancy with Kattan et al since the study was performed in a paediatric out-patient setting [15]. Therefore, it is possible that the high prevalence of peanut co-sensitization might have previously overestimated the diagnostic value of Gly m 8. On the other hand, Lin et al concluded that soy 2S albumins were not major allergens in their study population, results in-line with the present study [17]. Additionally, the selected study population of Lin et al reported a 37% peanut co-sensitization, similar to the rate in the present study [17]. Even though regional variations have been reported both for soy allergy as well as for cross-sensitization and clinical-reactivity patterns for different legumes, these differences would not justify the different results since the studied population is mainly European, including different European regions [11,17]. This described differences might be reminiscent of variability in diet, including cross-reactive pollen-fruit allergies that are common in Europe, together with a changing food allergy pattern in Asia [41]. The allergen sources, the handling of these preparations, and the main products derivatives consumed could all contribute to the difference in results.

A Dot-Blot experiment was carried out to test the IgE binding ability of serum of patients reflecting the different profiles to recombinant Gly m8 allergen. The Dot Blot results confirmed that sIgE from the sera of the selected patients was recognized by the utilized form of rGly m 8 in the iBAT. Moreover, recombinant allergen preparations have been evaluated and showed results that are highly specific plus avoided false positives by elimination of cross-reactive allergens [42]. Therefore, even though this is the

first time the iBAT has been used with rGly m 8, the CD63-based iBAT has been validated for the use of recombinant allergens for the detection of sensitization to foods before [43].

In conclusion, the results of the present study show that Gly m 8 was not clinically relevant, as demonstrated by the negative iBAT results despite sIgE sensitization. In line with other studies, 2S albumins from soybean, Gly m 8, was not a major allergen in the selected soy allergic population. Hence Gly m 8 would have no added value in the diagnosis of SA in the present study population. The present study highlights why it is crucial to analyze co-sensitization patterns in allergic populations and verify the clinical relevance of sensitization with the use of functional assays such as the BAT to avoid over- or underestimating the diagnostic value of sIgE sensitizations.

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Chapter 4

Pollen sensitization can increase the allergic reaction to non-cross-reactive allergens in a soy allergic patient

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Abstract

During and after the pollen season, an increase in food-triggered allergic symptoms has been observed in pollen-food syndrome patients, possibly due to season boosting of pollen-IgE levels. It has been suggested that consumption of birch-pollen related foods plays a role in seasonal allergenic inflammation. However, whether this increased pollen sensitization during the pollen season can also affect the allergenicity of allergens that are non-cross-reactive with birch pollen remains in question.

This study presents the case of a patient with soy allergy and pollinosis, who experiences worsening of gastrointestinal (GI) symptoms during the birch pollen season even though the eliciting food factor does not cross-react with birch pollen allergens and their homologous (e.g., Bet v 1 and Gly m 4). The results showed a notable increase for sIgE for Gly m 4 (3.3-fold) and Bet v 1 (2.6-fold) during the birch pollen season compared to outside the birch pollen season; while Gly m 5 and Gly m 6 showed only a slight increase (1.5-fold). The basophil activation test (BAT) showed that in this patient Gly m 5 and Gly m 6 are clinically relevant soy allergens, which correlates to the reported clinical symptoms to processed soy. Moreover, the BAT against raw soy shows an increase in basophil activation during the birch pollen and a negative basophil activation result outside the birch pollen season. Thus, the worsening of GI symptoms could possibly be due to an increase in IgE receptors, an over-reactive immune system and/or significant intestinal allergic inflammation. This case highlights the importance of including allergens that do not cross-react with birch pollen and using a functional assay such as the BAT to evaluate clinical relevance when assessing birch pollen seasonal influence on soy allergenicity.

Introduction

Legume proteins share homology; however, they are not necessarily similarly allergenic. Presently the most prevalent legume allergies are peanut, soy, lupine, chickpea, lentil, and pea [1,2]. In Europe, soy comprises one of the eight most common food allergens [3-5], with eight soybean allergens recognized by the WHO-IUIS Allergen Nomenclature Subcommittee [6]. Soy allergy is commonly characterized by IgE sensitization to the storage proteins Gly m 5 (vicilin, 7S globulin) and Gly m 6 (legumin, 11S globulin), which constitute 65% to 80% of the total seed protein and can provoke severe allergic reactions to all kinds of dietary soy including processed foods [7-9]. Moreover, these allergens have been reported to be stable to heat and gastric digestion plus may be associated with food allergies primary associated with sensitization through the gastrointestinal tract [8]. In the Netherlands, most soy allergic patients appear to be sensitized to Gly m 5 and Gly m 6, known as 'conventional' soy allergy, according to recent report [3]. Another form of soy allergy is linked to Gly m 4 (a pathogenesis-related protein (PR-10), which has been identified as the most common allergen in soy patients with birch pollen allergy [8,10]. Moreover, it is not uncommon that patients who are allergic to birch pollen, show allergic symptoms after the ingestions of certain fruits and vegetables due to the cross-reactivity of IgE antibodies induced by sensitization to the major birch pollen allergen Bet v 1 with homologous food allergens, Gly m 4 in the case for soy allergy [11]. In contrast to 'conventional' soy allergy, Gly m 4 has an elevated sensitivity to heat and pepsin digestion; thus, Gly m 4 allergy seems to be associated with immediate reactions known as pollen-food syndrome (PFS), a clinical group of signs and symptoms which result from cross-reactivity between pollen and plant food allergens [12]. In

the case of Gly m 4, PFS is linked to the consumption of unprocessed soy, often soy milk [13-17]. Furthermore, symptoms can be severe as anaphylactic reactions which occur in approximately 10% of birch allergic patients who present a cross-allergic reaction to soy [18,19]. Soy-based drinks, such as soy milk, can also cause systemic allergic reactions, which suggests that drinks contain a high amount of soy protein that has not been thoroughly thermally processed and as liquids can partially bypass gastric digestion thus quickly reaching the intestine [13,18,20]. IgE-mediated food allergies, such as soy, may result from sensitization through the gastrointestinal tract or through a less well-recognized form via the respiratory tract which results in primary sensitization to homologous pollen allergens, causing reactivity to cross-reactive food allergens [21]. An example of this form of sensitization is evidenced by the fact that in Europe plant food allergy is strongly influenced by sensitization to birch pollen proteins [21,22]. Moreover, it has been reported that approximately 60% of food allergies in adolescents and adults are linked with an inhalant allergy [13]. This association is further supported by the fact that during and after the pollen season, an increase in food-triggered allergic symptoms has been observed in pollen-food syndrome patients, possibly due to season boosting of pollen-IgE levels [15,23,24]. Nonetheless, clinical evidence in support of seasonal variation of serum IgE (sIgE) levels has been quite limited so far. The levels of birch pollen have not only risen in recent decades, but the period of exposure has increased due to climate change which has resulted in a rise in the prevalence of birch pollen sensitization, further research on this topic is warranted [24]. In Northern Europe, birch is the major pollen-allergen-producing tree, the main flowering period starting at the end of March with pollen values peaking in May and

duration which is remarkably temperature-dependent varies can last as much as 8 weeks [25]. Moreover, birch has the greatest allergenic potential of the allergenic trees, with a positivity skin prick test (SPT) to birch allergens of 5% in the Dutch population [24,25]. Additionally, a high rate of patients, 50% to 80%, with a respiratory allergy may be sensitized and clinically allergic to other allergens, known as polysensitization, with patients tending to gain sensitizations over time [26-29]. Polysensitization includes cross-reactivity, when the same IgE binds to several different allergens with common structural features and co-sensitization, when different IgEs bind to allergens that may not necessarily have common structural features [29]. Additionally, Magnusson et al reported that birch pollen allergic patients show signs of elevated numbers of eosinophils, IgE positive cells, CD3+ T cells and CD11c dendritic cells during the pollen season consistent with increased severity of allergic symptoms [30]. Furthermore, elevated IgE levels to most PR-10 proteins have been observed during the pollen season, particularly in patients), which indicates that cross-reactions between birch pollen related foods may play a role [31]. Previous studies have reported an increase in pollen sensitivity as the pollen season progresses [31-35]. Together these findings support the hypothesis that during the birch pollen season, it is possible that consumption of birch-pollen related foods (e.g., soy) plays an important role in allergenic inflammation which increases during the pollen season [30-32,35]. However, whether this increased pollen sensitization during the pollen season can also affect the allergenicity of allergens that are non-cross-reactive with birch pollen remains in question.

The aim of this study is to present the case of a soy allergic patient with clinical symptoms after consumption of processed soy and whose

symptoms increase during the birch pollen season, which suggests a pollen-soy related allergy. The sIgE to the major birch allergen and soy allergens are measured in and outside the birch pollen season. Additionally, as sensitization does not always correlate to clinical symptoms, a basophil activation test (BAT) was used to evaluate the clinical relevance of sIgE sensitization to the major relevant soy allergens Gly m 5 and Gly m 5 plus assessing the patient's capacity to degranulate basophils outside, at the start and during the peak of the birch pollen season [36-38]. An ImmunoCAP Inhibition test was performed to confirm that the patient's sIgE binds to processed soy allergens, since her symptoms occur upon consumption of processed soy products.

Case Presentation

A 26-year-old Dutch woman presented to the Allergy Centre Rijnstate Hospital in Arnhem, the Netherlands, with a chronic history of multiple food allergies; including a peanut allergy begun at an early age and has recorded episodes anaphylactic shock (AS). Patient developed PFS, abdominal pain, diarrhea and AS after the ingestion of soy products; patient indicates that she has never consumed soymilk or soymilk products.

Recently, she reported a difference in soy tolerance during and outside the birch pollen season, she is capable of tolerating small amounts of processed soy outside the birch pollen season without experiencing any symptoms but must maintain a strict diet during the birch pollen season (*Table 1*). During the birch pollen season, consumption of even small amounts of processed soy will lead to the development of PFS, gastrointestinal symptoms and in two occasions, AS (*Table 1*).

Additionally, the patient reports a history of hay fever with moderate to severe symptoms including sneezing, runny nose, itchy eyes, nose, and throat; and persistent asthma aggravated by the exposure to furry animals and respiratory irritants.

In the general physical examination discoid eczema patches were observed in the head and neck region. The rest of the physical examination was normal. The only medication prescribed is Budesonide/formoterol (Symbicort®) daily. Emergency medication is desloratadine, salmeterol, prednisone and carries an EpiPen.

Table 1. Patient sIgE values measured out and during the peak of the birch pollen season plus soy diet restrictions and reported patient symptoms**;** specific IgE (sIgE) values tested using the ImmunoCAP (Thermo Fisher Scientific®), manufacturer's recommendations were followed with a positive result when IgE levels were ≥ 0.35 kU/L; Oas=oral allergy symptoms; As=Anaphylactic shock; Ap=abdominal pain; D=diarrhea

	ImmunoCAP IgE (kU/L)*					Soy Diet	Symptoms
	Soybean	Gly m 4	Gly m 5	Gly m 6	Bet v 1		
Out of the Birch Pollen Season	0.63	2.13	0.91	1.42	6.3	Tolerance to processed soy	-
Peak of the Birch Pollen Season	1.15	7.12	1.33	2.14	16.3	Strict diet	Oas, As, Ap, D

- **SPT and sIgE results**

The SPTs showed a slightly positive reaction to soy, strong reaction to peanut, apple, hazelnut and a weak reaction to celery, tomato, nutmeg, and pea. It has been reported that in patients with sensitive skin or dermatographism can alter SPT results; thus, the results of the SPT to soy may have been influenced by the patient's atopic eczema [39].

During the peak of the birch pollen season, the patient showed positive sIgE values against whole soybean and multiple soy allergens: Gly m 4, Gly m

5 and Gly m 6 and a positive value to the major birch pollen allergen Bet v 1 (*Table 1*). Furthermore, sIgE to Gly m 4 and Bet v 1 notably increases, 3.3-fold and 2.6-fold, respectively, during the birch pollen season compared to the measured sIgE values out of the birch pollen season (*Table 1*). However, sIgE for Gly m 5 and Gly m 6 a slight increase across the different season is observed, 1.5-fold for both allergens (*Table 1*).

Additionally, molecular allergy diagnostic was used to evaluate patient's sensitization to various inhalant allergens and peanut allergens, the results of the defined partial allergen diagnostics (DPA-Dx) which showed the patient was positive to Bet v 1 and negative to Bet v 2, Bet v 4 and Bet v 6 (*Table 2)*.

Because the patient had an unequivocal and convincing clinical history to soy plus a positive sIgE result in addition to a history of AS, an oral food challenge was deferred due to the high probability of a reaction [40].

Table 2. Birch and peanut allergens measured by DPA-Dx, cut off for positive values >0.35 kU/l.

DPA-Dx (Ku/l)			
	kU/l*		kU/l*
Birch	38.5	**Peanut**	98.1
rBet v 1	43.8	rAra h 1	0.7
rBet v 2	<0.35	rAra h 2	11.4
rBet v 4	<0.35	rAra h 3	2
rBet v 6	<0.35	rAra h 6	37.2
		rAra h 7	21.1
		rAra h 5	<0.35
		rAra h 9	<0.35

- **Basophil Activation Test (BAT)**

Since IgE sensitization does not equal the clinical manifestation of an allergy, a direct basophil activation test (BAT) was performed [38]. The BAT was performed on whole blood using the Flow CAST® kit (Bühlmann, Basel, Switzerland) following the manufacturer instructions [40]. Basophil activation was expressed as the %CD63 positive basophils (CD63%+), the cut-off value for positive basophil activation was set at >15% CD63%+ [8,36]. The patient was instructed to stop any medication a week prior to blood collection. EDTA blood samples were freshly incubated with basophil stimulation buffer (BSB) with the negative control being BSB without allergen. The allergen concentrations used was 1, 10, 100 and 300 ng/ml for Gly m 5 as well as for Gly m 6 and 5, 10, 20, and 40 ng/ml for Ara h 2.

The BAT showed positive results during the pollen season for Gly m 5 and Gly m 6 (*Figure 1A*) as well as for the major peanut allergen Ara h 2 (*Figure 1B*). The positive BAT result for Ara h 2 correlates well to the clinical history of the patient, who describes a severe form of peanut allergy that started at an early age. The BAT results outside, at the beginning and during the peak of the birch pollen season show a season-dependent change in basophil stimulation, remaining negative outside the birch pollen season, starting to rise at the beginning of the birch season and increasing during the peak of the birch pollen season (*Figure 1C*). These results correlate with the medical history where the patient reports that outside the birch pollen season consumption of small amounts of processed soy is possible without experiencing any symptoms. However, during the birch pollen season a strict diet must be maintained and consumption of even small amounts of

processed soy can lead to clinical symptoms such as oral allergy syndrome (OAS) , abdominal pain plus diarrhea and in two occasions even AS (*Table 1*).

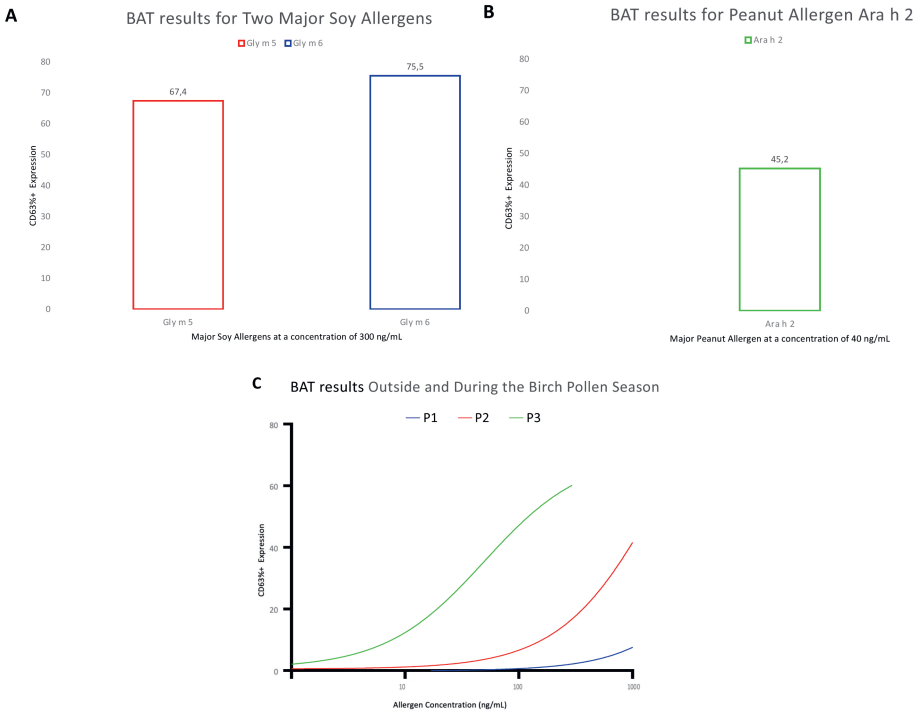

Figure 1. BAT results for soy and peanut allergens plus measurements out, at the start and in the peak of the birch pollen season. (A) BAT results for soy allergens Gly m 5 and Gly m 6 at an allergen concentration of 300 ng/ml, (B) BAT results for peanut allergen Ara h 2 at an allergen concentration of 40 ng/ml, (C) BAT results against raw soy extract at an allergen concentration of 300 ng/mL; Period 1: December, out of the birch pollen season (P1- color blue), Period 2: March, at the start of the birch pollen season (P2- color red) and Period 3: May, at the peak of the birch pollen season (P3- color green).

- **ImmunoCAP Inhibition Assay**

Since the patient's complaints arose with the consumption of processed soy, an ImmunoCAP Inhibition test was performed to measure the binding capacity of sIgE to processed allergens [44]. Raw soy protein extract (SPE) was

used as inhibitor proteins, two times the diluted serum with no inhibitor protein (0% inhibition) was used as the negative control and changes to the IgE binding capacity were measured in raw soy and two forms of processed soy: (i) processed soy type 1 (raw soy protein extract heated at 121°C without glucose for 10 minutes) and (ii) processed soy type 2 (raw soy protein extract heated at 121°C with glucose for 10 minutes)

To evaluate which soy allergens were more susceptible to changes caused by processing techniques (heating and glycation), an inhibition of sIgE against Gly m 4, Gly m 5 and Gly m 6 were measured and compared. The final concentration used were 1, 5 and 25 ug/ml; using the sIgE levels were measured with the Phadia250® instrument (Thermo Scientific, Germany) and the percentage of inhibition was calculated with the formula:

$$\% \text{ Of inhibition} = (IgEo\% - IgEx\%)/IgEo\% \times 100$$

The results showed no inhibition for Gly m 4 for raw SPE and the two forms of processed soy (*Figure 2A*); therefore, Gly m 4 was either not present or at a very low concentration in the soy extracts. For Gly m 5 and Gly m 6, a strong inhibition signal was observed (*Figure 2B and 2C*), indicating that the positive BAT results with soy extract are due to the patient's reactivity to Gly m 5 and Gly m 6.

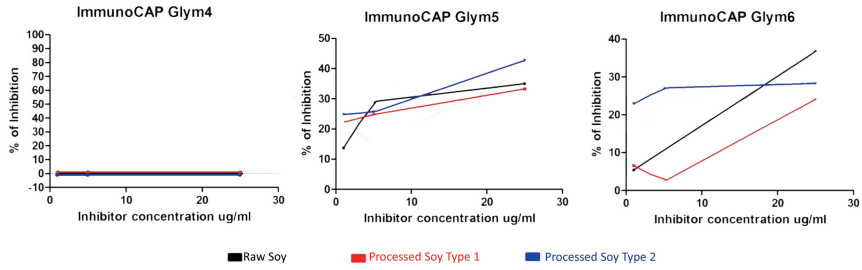

Figure 2. ImmunoCAP Inhibition test results for raw soy extract, processed soy Type 1 and processed soy Type 2. (A) Percentage of Inhibition against Gly m 4, (B) Percentage of Inhibition against Gly m 5, (C)Percentage of Inhibition against Gly m 6.

Discussion

Presently, it has been reported that there is a high prevalence of sIgE to Gly m 4 among Bet v 1 sensitized patient which usually indicates that birch PFS may be clinically relevant after the ingestion of soy products, usually soymilk [14,15,18]. However, the present study presents the case of a patient mainly sensitized against soy storage proteins Gly m 5 and Gly m 6 (sIgE and BAT results), who doesn't consume soymilk or soymilk products but reacts to processed soy (ImmunoCAP Inhibition) and still experiences PFS plus gastrointestinal symptoms and a difference in soy tolerance in and out of the birch pollen season.

In the present study, a BAT against Gly m 4 was not performed; thus, the patient's clinical reactivity to Gly m 4 was not analyzed. To our knowledge, a BAT with Gly m 4 has been reported only once and in a patient with Gly m 4-exclusive soy allergy, which resulted in no degranulation for the CD63 marker for the all the allergen concentration used except 67.5 ng/ml [8]. In the present study, no inhibition was observed for Gly m 4 for either raw SPE or the two forms of processed soy. Mittag et al reported that the content of

Gly m 4 in soy products is highly variable, ranging from 0 to 70 mg/kg and depends strongly on the degree of food processing [45]. Moreover, Gly m 4 degrades during heating, with Vissers et al reporting that after 30 minutes to 4 hours of heating no Gly m 4 could be detected [46]. Thus, allergenicity of Gly m 4 in soy products strongly depends on food processing conditions [45-48]. Therefore, the no inhibition results observed in our study might be explained by the absence or by very low concentration of Gly m 4 in the soy extracts. Moreover, commercial extracts currently available generally contain very low amounts of Gly m 4, 0.01–0.1% in total soy crude protein soy extracts; thus, tests for allergen-specific IgE tend to be not sensitive enough in cases of suspected Gly m 4-induced soy allergy [47,48].

Outside and during the birch pollen season, the patient shows sIgE differences for Gly m 4 and Bet v 1, but not so for Gly m 5 and Gly m 6. Previously, higher IgE sensitization rates to pollen during or following the pollen season compared to out of the pollen season have been reported [49-51]. The data of the current patient suggest that sIgE to birch pollen is 2.6-fold higher during the birch pollen season (*Table 1*), consistent with results described before [51]. However, studies evaluating sIgE seasonal variation are limited and focused mostly on symptomatic aeroallergens [51]. The clinical significance of this change in sIgE values is still uncertain, although it has been suggested that higher sIgE levels to birch are associated with higher symptom frequency [24,51,52]. Furthermore, studies that explore the sensitization pattern of birch pollen-related foods in relation to the birch pollen season are extremely limited and do so in patients with a well-established birch pollen-food-associated clinically relevant allergy [21,23,53,54]. As observed in the presented case, the sIgE values for Gly m 4 notably increase during the birch

pollen season, 3.3-fold for Gly m 4 compared to 1.5-fold for Gly m 5 and Gly m 6.

The current hypothesis suggests that patients sensitized to birch pollen (Bet v 1), which cross-reacts with a wide range of fruits and vegetables (e.g., Gly m 4), can experience an increase in food allergy episodes during and immediately following the birch pollen season due to seasonal boosting of pollen-IgE levels [14,15,23,30,31]. Rentzos et al reported that birch pollen allergic patients have clear signs of an ongoing intestinal mucosal inflammation (eosinophil infiltration and increased numbers of IgE+ cells mainly mast cells) which is aggravated during the pollen season [31]. This over-reactive immune system may be a reaction to the pollen itself rather than pollen-related food items [30,31,55]. Moreover, sensitized individuals produce more allergen-specific IgE antibodies; the binding of allergen to allergen-specific IgE antibodies on basophils leads to the cross-linking and activation of high-affinity IgE receptor (FcεRI) and low- affinity (CD23) responsible for allergic inflammation [56]. Carlsson et al reported a correlation between IgE bound on basophils with increasing IgE levels and increasing receptor expression [57]. The present BAT results show a different basophil activation dependent on the birch pollen season, with basophil activation starting to increase at the beginning of the birch pollen season and increase significantly during the peak of the season. These BAT results correlate with the clinical history which shows a difference in soy tolerance in and out of the birch pollen season. This BAT results suggest that the increase of pollen sensitization during the pollen season may increase the high (FcεRI) and low affinity (CD23) IgE receptors, thus increasing basophil activation.

The patient in the present case study shows a clinical history of different tolerance of processed soy off and during the birch pollen season, while chronically avoiding consumption of soymilk and soymilk products. To obtain the many commercially available soy products, soy must undergo various processing steps, conventional thermal methods being one of the most used processing techniques [58,59]. Higher heating temperatures lead to an altered protein structure, thereby increasing protein digestibility and quality [59]. If sugars are present during the heat treatment, a reaction known as glycation or Maillard reaction occurs [58]. Since thermal processing prompts conformational changes in food proteins affecting not only their digestion and absorption but also their recognition by immune cells and binding to IgE antibodies, and thus their allergenicity potential [58,60]. The results observed in the ImmunoCAP Inhibition test show high level of inhibition for Gly m 5 and Gly m 6, both allergens unlike Gly m 4, are thermo-stable and thus retain their allergenic potential [7]. Moreover, compared to raw SPE a decreased in allergenicity is observed for Gly m 6 for the two types of processed soy, which added to the low levels of sIgE (Gly m 4 > Gly m 6 > Gly m 5) and the medical history may suggest a tolerance development towards soy proteins.

Despite the main limitations of a case report, mainly generalization of the presented hypothesis to a broader population is not possible and the danger of result over-interpretation; the major advantage of a case study is the possibility of presenting novel and uncontrolled observations regarding clinical findings. To our knowledge this is the first time that a case is presented that shows a marked increase during the pollen season of basophil activation in a soy allergic patient whose food allergy a primary dietary form linked to

sensitization against soy storage proteins Gly m 5 and Gly m 6. Moreover, these results suggest that the change in soy tolerance during the birch pollen season were not only caused by Gly m 4 sIgE increase in the birch pollen season (as shown in *Figure 3*). Therefore, it is possible that the seasonal soy oral tolerance and worsening GI symptoms during the birch pollen season are due to an increase in IgE receptors, an over-reactive immune system and/or significant intestinal allergic inflammation. Moreover, it would be expected that this immune system reaction is reversible since it the patient experiences this change in diet restriction and symptomology every year. In future research, such patients should be followed for a long period of time (minimum of two years) to evaluate if this phenomenon is indeed reversible and to what degree.

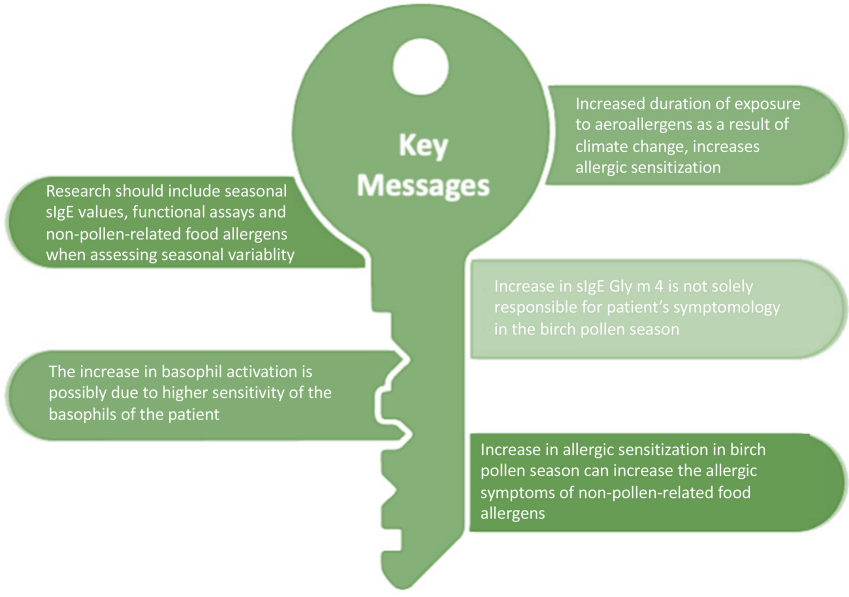

Figure 3. Key Messages.

Conclusion

The case presented illustrates that it is crucial that clinicians become aware that it may not be only birch pollen-associated soy allergy patients that can suffer an increase on gastrointestinal symptoms and changes in soy tolerance during the birch pollen season and, hence adjust dietary advice accordingly.

Moreover, the changes in the clinical reactivity to soy according to seasonality measured by the BAT and reflected in the clinical history of the patient, demonstrate the advantages of including a functional assay for monitoring allergic seasonal changes, which will be of particular importance since the effects of climate change influence allergic diseases [24,25,35,56]. Climate change not only increases the duration of exposure to aeroallergens; thus, increasing allergic sensitization but could increase the level of allergenic airborne pollen and allergic symptoms including those caused by non-pollen related food allergens, as demonstrated by this case report [25,35]. Thus, this single case study highlights the importance of future research to use seasonal sIgE measurements for birch plus food allergens and to include allergens that do not cross-react with birch pollen, in the case of soy allergic patients Gly m 5 and Gly m 6. Additionally, these studies should include not only seasonal measurements of allergic markers like sIgE and symptomatology but add a functional assay such as the BAT to evaluate clinical relevance.

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Section 2

The Maillard Reaction: the impact of food processing on soy allergenicity

Chapter 5

Receptor mediated effects of advanced glycation end products (AGEs) on innate and adaptative immunity: Relevance for food allergy

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Abstract

As of late, evidence is emerging that the Maillard reaction (MR, also referred to as glycation) affects the structure and function of food proteins. MR induces conformational and chemical modification of food proteins, not only on the level of IgG/IgE recognition but also by increasing interaction and recognition of these modified proteins by antigen presenting cells (APCs). This affects their biological properties including digestibility, bioavailability, immunogenicity, and ultimately their allergenicity. APCs possess various receptors that recognize glycation structures, which include receptor for advanced glycation end products (RAGE), scavenger receptors (SRs), Galectin-3 and CD36. Through these receptors, glycation structures may influence recognition, uptake and antigen-processing of food allergens by dendritic cells (DCs) and monocytes. This may lead to enhanced cytokine production and maturation of DCs and may also induce adaptive immune responses to the antigens/allergens as a result of antigen uptake, processing and presentation to T cells. Here, we aim to review the current literature on immunogenicity of AGEs originating from food (exogenous or dietary AGEs) in relation to AGEs that are formed within the body (endogenous AGEs), their interactions with receptors present on immune cells, and their effects on activation of the innate as well as the adaptive immune system. Finally, we will review the clinical relevance of AGEs in food allergy.

Introduction

In the past 30 years the consumption of ultra-processed foods has almost tripled from 11% to 32% of daily energy intake, with this increased consumption of ultra-processed food being associated with a higher hazard of increased mortality in numerous kinds of non-communicable diseases [1]. This diet pattern, mainly associated with Western countries, has a led to an increased exposure to advanced glycation end-products (AGEs). AGEs are a heterogenous group of compounds formed as products of the Maillard reaction (MR). The MR is a non-enzymatic browning reaction or glycation, that occurs between reducing sugars and a free amino acid group of proteins, peptides or free amino acids [2,3,4]. The MR happens when proteins are heated in the presence of reducing sugars e.g. during thermal processing of food plus it is often used in the food industry since it gives food a different taste, color, and aroma but additionally causes a number of modifications on the level of protein structure [5]. This alterations include aggregation, cross-linking, changes of hydrophobicity as well as protein charge and exposure of β-sheet structures which also occur upon heating and glycation of food proteins, which can also be affected by the MR [6,7].

Endogenous AGEs are produced and accumulated slowly within the body during the aging process plus under oxidative stress, inflammation and hyperglycemic conditions. Exogenous AGEs are also called dietary AGEs (dAGEs) and are obtained from processed food such as BBQ meat, bakery products, fast food but also soft drinks that contain high fructose corn syrup and cigarrete smoke [8,9]. In the case of endogenous AGEs, it has been observed that they can cause structural and functional protein alterations, as well as oxidative stress inflammation and therefore can have pathological

implications [9]. AGE accumulation can increase oxidative stress which in turn can induce cellular dysfunction and apoptosis ultimately leading to tissue or organ injury, therefore accelerating pathophysiological conditions [9]. Therefore, it has been proposed that both endogenous and exogenous AGEs can have a negative impact on health; nonetheless, it has to be noted that the evidence for the negative health effects of dAGEs is still lacking as many aspects such as bioavailability, metabolism as well as organ and tissue distribution and accumulation is not clear for the majority of them [10]. The binding of AGEs to the cell surface receptor for advanced glycation end products (RAGE) can lead to the production of pro-inflammatory cytokines and has been linked to the development and progression of a number of certain diseases such as diabetes type 2, cardiovascular diseases, some forms of cancer, neurodegenerative diseases such as Alzheimer's and Parkinson's, liver diseases and osteoporosis [9,11-13] and more recently to the development of food allergy [14-16].

Since the MR also promotes protein aggregation by inter or intra-molecular crosslinking between lysine and arginine residues, glycation can produce structural changes in proteins [17]. Therefore, hydrophobicity, charge, aggregation and exposure of β-sheet structures have been suggested to participate in the immunomodulatory function of heated proteins [16,18,19]. Heated and glycated proteins may act as signalling molecules in modulating innate and adaptative immunity, explaining their potential relevance in food allergy and in non-communicable diseases. However, due to the complexity of the MR the immunological and allergic properties of dAGEs are not only diverse but up to date not completely understood [17].

The aim of this paper is to review the current literature on exogenous and dAGEs regarding their interaction with a number of receptors present on immune cells, the effect of AGEs on activation of the innate as well as the adaptive immune system; and finally, to review the clinical relevance of AGEs in food allergy and in non-communicable diseases.

1. Maillard reaction products (MRPs)

1.1. Formation and structural changes in proteins

Maillard reaction products are components formed upon heat treatment applied during food processing [20]. High heat and prolonged cooking time of food that contains sugars increase the amount of AGEs in the food and is accompanied by the structural changes of the food proteins [21,22]. Therefore, MR and the heat-induced structural changes of proteins take place in parallel what makes difficult a clear distinction between an effect of heating and an effect of MR on the protein structure e.g., when considering the aggregation [21]. For MRP formation the initial step of heating is for the protein to start denaturing and unfolding which is partly reversible; next comes irreversible aggregation when temperatures further increase. The extent to which each individual protein will unfold and aggregate depends on the temperature plus time of heating, pH, protein concentration, presence of other proteins and stability of the native structure of the proteins [23]. This protein unfolding can also lead to the exposure of structural elements that have been located in the make-up interior such as the β-sheet structures and thus change the hydrophobicity [7]. Moreover, the neo β-sheet structures can be formed upon heating via amyloid fibril formation. In parallel to these processes, the MR can promote protein

aggregation and affect the type of aggregates that are formed (as shown in *Figure 1*, block A). Therefore, an effect of the heat treatment on the protein structure is a combination of an effect of the temperature itself and the interaction of amino acids with sugars due to the MR, as illustrated on *Figure 1*.

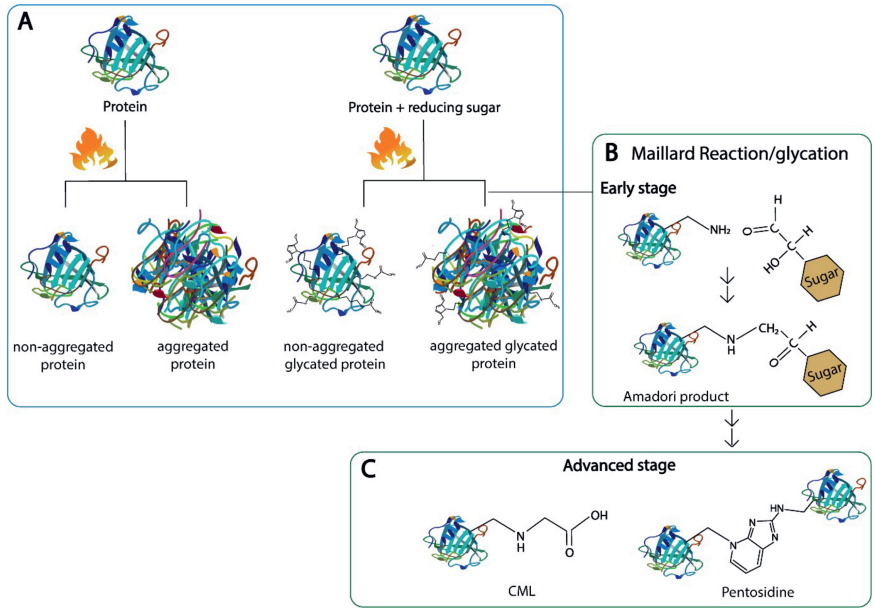

Figure 1. Structural changes of protein upon heating with and without reducing sugars. A: protein aggregation, B: Early Maillard reaction, C: Advanced stage of the Maillard reaction. N^{ε}-carboxymethyl lysine (CML) and pentosidine are shown as representatives for linear and cross-linking, heterocyclic advanced glycation end products, respectively.

The MR occurs in a series of stages which can be roughly divided in the early, intermediate and final stage; all mainly categorized by the products formed at each stage [24]. In brief, in the early stage of the MR the carbonyl compound reacts with the amino compounds to form an unstable Schiff base, this process is reversible, the so-called Schiff base subsequently rearranges to various amino-1-deoxy-ketose derivatives which are called Amadori

products (as shown in *Figure 1*, block B). Upon acidic hydrolysis, the Amadori product of lysine reacts to furosine, often used as a marker for the early stage of the MR [17]. The intermediate stage, highly reactive α-dicarbonyl compounds are formed from the Amadori products via enolization and dehydration. Lastly, in the final stage, the dicarbonyl compounds can either react with the amino groups of the amino acids, peptides and proteins or rearrange to form AGEs [5,17,24]. The type of AGEs highly depends on the type of sugar used in the reaction and on the processing conditions such as temperature, time of heating, pH, and water activity plus AGEs are bound to proteins with high contents of lysine and arginine units [25]. AGEs can be linear e.g.Nε-carboxymethyl-lysine (CML) or crosslinked e.g., pentosidine, where two side chains of the same proteins or different proteins are linked with each other (as shown in *Figure 1*, block C). Well characterized AGEs in food include: CML, Nε-carboxyethyl-lysine (CEL), pyralline, pentosidine and methylglyoxal-derived hydroimidazolones (MG-H1/H2) [5,17]. The MR has also been described to have an effect on digestibility, immunogenicity and allergenicity of food proteins; therefore, monitoring its impact on health and its use in the food industry is of great importance for the general public [16,26].

1.2 Endogenous AGEs: Formation and Structure

Endogenous AGE formation is part of the normal consequence of metabolism; nonetheless this can turn pathogenic if high levels start to accumulate in tissues and the circulation [2]. Endogenous AGEs can be formed in all tissues and body fluids within the human body when the carbonyl groups of reducing sugars react non-enzymatically with the free

amino groups on proteins [27]. Even though the structure of many AGEs that are formed *in vivo* have not been completely determined yet, the knowledge on AGEs produced in the human body has dramatically increased in the last decades [2]. Presently, four types of processes in the formation of AGEs under physiological conditions have been identified (i) monosaccharide autoxidation or auto-oxidative glycosylation, (ii) Schiff's base fragmentation, (iii) fructosamine degradation and (iv) direct reaction of α,β-dicarbonyl compounds [2]. Endogenous AGEs include structures such as pyrraline, CEL, pentosidine and CML [28]. All these structures were detected also in food [17].

Theoretically, as long as levels do not become too high the effects of endogenously produced AGEs are limited by detoxification pathways [20]. However, the rate for *in vivo* AGEs formation is determined by many factors such as the nature and concentration of the substrate group, the glycating agents, the availability of catalytic compounds, the redox balance, the half-life of the proteins and the presence of inhibitors (e.g. aminoguanidine and pyridoxamine) [2]. This relationship is observed in patients diagnosed with diabetes, where the elevated levels of glucose accelerate the formation of AGEs; moreover, the age specific receptor (RAGE) generates reactive oxygen species (ROS), activates inflammatory signalling cascades and therefore plays a key role in the pathogenesis of diabetic complications [29].

As stated previously, endogenous AGE formation is typically a slow process mainly affecting the function of long-lived proteins; however, their formation via reactive dicarbonyls such as methylglyoxal (MGO) can lead to quicker AGE formation [30,31]. Additionally, conditions of elevated stress and high circulating glucose levels occurring in diseases such as obesity and

diabetes, also increase endogenous AGE formation [32,33]. Presently, it is well documented that an imbalance in formation and accumulation of dAGEs contribute to a number of chronic inflammatory diseases such as diabetes, uremia, cardiovascular diseases plus neurogenerative diseases including Alzheimer's and Multiple Sclerosis [31-37]. Cigarette smoke has also been found to contain reactive glycation products and thus can increase AGE accumulation in tissues and blood of smoker [38].

1.3 Bioavailability of dietary AGES induced by food processing

With the growing awareness of endogenous AGEs and their potential harmful effect in the human body, it became important to determine whether dAGEs contribute to the human AGE pool and thus studies begun to describe the metabolic pathway of dAGEs. To date the available information regarding the absorption, distribution, metabolism and excretion of dAGEs is not entirely clear (as shown in *Figure 2*) [20], and it seems to be highly dependent on the AGEs structure and form (protein-bound vs free) [17]. Nonetheless animal and human studies concur that dAGEs are partially absorbed in the intestine (10% to 30%) [2,20,39]. The AGE molecular weight (MW) has an impact on the absorption rate, with low MW AGEs being relatively quickly absorbed, bio-transformed and excreted whilst high MW AGEs normally are absorbed more slowly probably due to insufficient degradation by gastrointestinal enzymes. Approximately 60% of the absorbed AGEs remain within the body for 72 hours on average [2,39,40]. In animal studies after 3 days, more than half of the absorbed AGEs were located in the kidney and liver, but they were also found in the heart lung and spleen [2,20]. Data currently show that approximately 30% of dAGEs that are taken up can

potentially be eliminated in the urine [22]. Nevertheless, studies of dAGEs in human are relatively scarce therefore the gastrointestinal absorption of AGEs has not been fully elucidated yet [41]. In the colon, there is increasing evidence that dAGEs may be able to modify local microbiota metabolism and hence modulate gut integrity; this local colonic action playing an important component of the pro-inflammatory role of the dAGEs in the body [33].

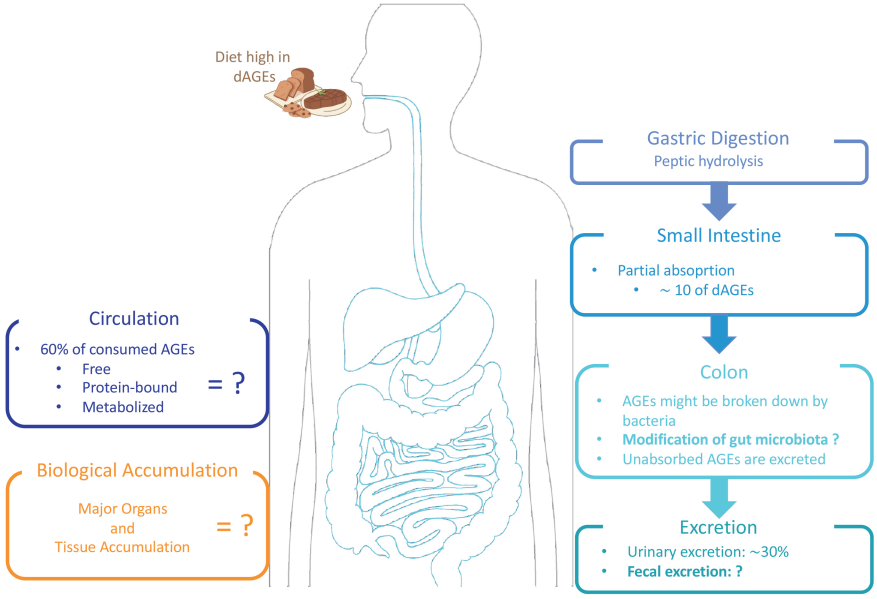

Figure 2. Suggested metabolic pathway of dAGEs: absorption, digestion, and excretion in the human body

However, the level of absorption as well as the reactivity in the body and metabolism of individual AGEs strongly depends on its chemical structure. MGO, an important marker for endogenous AGEs plus an AGE that is known for playing a role in diabetic complications, is quickly degraded during the digestion process in the intestine (80% to 95%). Hence, orally ingested MGO has no influence on the MGO level *in vivo* [42].

CML, which is highly abundant in food and because of the potential activity it has after ingestion, is normally used as a model of glycation products. Data shows that diets high in dietary CML (dCML) lead to an elevation of the same compounds in urine in proportion to the amount ingested; meaning that at least a percentage of dCML is absorbed and taken into circulation [40]. Therefore, preliminary data on the pharmacokinetics of dCML shows that it is characterized by a partial but rapid absorption and elimination [40]. However, the elimination of dCML seems to be incomplete since excreted CML in feces and urine does not exceed 47%, which would suggest possible retention in organs and tissues [43]. It is important to note that every glycation product differs chemically and metabolically, so not all results found on dCML can be extrapolated to every other glycation product [44].

Pyrraline, is an advanced MRP and usually it is identified in thermally treated food and its content increases with prolonged food storage time [45]. Moreover, it has been implicated in pathogenesis of ageing chronic renal failure (uremia) plus diabetes and its related complications such as inflammation, retinopathy, and nephropathy [46]. It has been shown that peptide-bound pyrraline is completely bioavailable, meaning it is proteolyzed plus reabsorbed during digestion and then rapidly eliminated [46]. Hence, it is unlikely that pyrraline will be metabolized in the body since it appears that 80% of the dietary pyrraline is absorbed plus rapid kidney elimination was observed for all absorbed pyrraline [46]. Even though, some dAGEs are efficiently cleared by the kidney, dietary intake has to be considered when evaluating the possible physiological effects of individual MRPs, since several studies show that individuals that consume a high AGE diet will have a

significant increase in serum and urinary AGE levels [47-49]. These are just some cited examples of the bioavailability and metabolic transit of individual dAGEs and dicarbonyl compounds, more extensive reviews have been described in detailed previously [44,50,51].

2. Interaction of AGEs with the immune system

The immune system not only provides protection to infection by pathogens but also tolerance to exposure against harmless antigens; therefore, an impaired immune function is associated with increased susceptibility to infections and increased disease severity. Regarding AGEs, it is known that MRPs can interact with different types of immune receptors, including the receptor for advanced glycation end-products (RAGE), and the MRPs/AGEs have been shown to contribute to chronic inflammatory states with negative health consequences [52]. Two separate mechanisms by which AGEs are known to produce effects in the human body have been described: (i) structural deformation or cross-linking of body proteins and (ii) interaction with AGE receptors [53]. AGE cross-linking with body proteins depends on sugar concentration and the turnover rate, it has been mainly related to the increased endogenous production of AGEs in diabetes and its comorbidities [20]. The other mechanism of action of AGEs is via AGE-sensitive receptors, circulating AGEs interact with AGE receptors and are capable of inducing a cellular signalling downstream [54]. Thus, AGEs not only have a direct impact on proteins and the extracellular matrix, but they can also interact with specific cell surface receptors activating the multiple mechanism including production of ROS and activation of the nuclear factor kappa B (NF-κB) pathway resulting in enhanced production of pro-inflammatory cytokines,

growth factors and adhesion molecules [2]. The AGE receptors are expressed in many cell types including monocytes, macrophages, endothelial cells and adipocytes [20]. Based on the reviewed information above, it may be concluded that the dAGEs load contributes to the AGEs in the circulation and therefore in the AGE pool *in vivo*. Thus, dAGEs might be able to affect the immune system as well as metabolism in the analogical way as endogenous AGEs.

2.1 Critical aspects of binding of AGEs to AGE receptors

To date, several AGE receptors have been identified, including: RAGE, oligosaccharyl transferase complex protein 48 (AGER1), 80 K-H protein (AGER2) and Galectin-3 (AGER3) [20,55-57]. Additionally, several AGE receptors have been identified that belong to the heterogenous scavenger receptor family which include: class A type I and II (SR-AI/II), class B type I (SR-BI), CD36 and lastly Toll-like receptors [58-61].

Each AGE-receptor has unique ligand binding domains that recognizes a particular structural element of the ligand. Ligands are recognized by the receptors via different mechanisms and the ligand-receptor interactions are dependent on the type of receptor and physicochemical characteristic of the ligand [62,63]. The structure of the different AGE receptors that have been studied in relation to dAGEs, their ligands, and the proposed sides on which each receptor recognizes the respective ligands are shown in *Figure 3*. Several interactions such as electrostatic, hydrophilic and hydrophobic interactions are involved in the binding of the receptors to their respective ligands [64,65]. Some AGE receptors show a common ligand motif e.g., polyanionic for SR-AI and therefore are more restricted in ligand recognition.

Other receptors, like Galectin-3, show no consistent ligand pattern and thus are less restricted for specific ligands; therefore, a higher variety of receptor-ligand interactions is possible (as summarized in *Table 1*) [64,66].

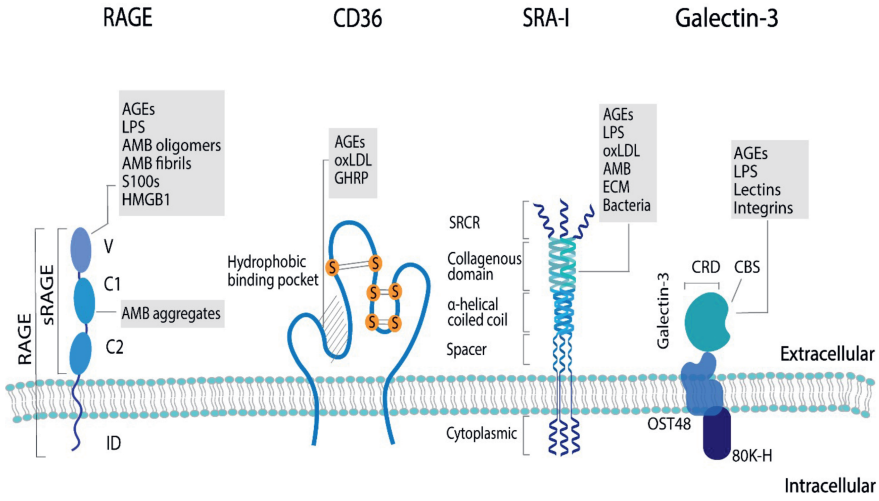

Figure 3. Structure and ligands of the AGE receptors mostly researched in relation to dAGEs: receptor for advanced glycation end products (RAGE), the soluble form of RAGE (sRAGE), CD36, scavenger receptor class A type I (SRA-I), and Galectin-3. Ligand binding domains of the different receptors are V-domain (V), C1- and C2-domain (C1 and C2), and intracellular-domain (ID), scavenger receptor cysteine-rich structure (SRCR), oligosaccharyltransferase 48 complex (OST48), carbohydrate-binding site (CBS), and carbohydrate recognition domain (CRD). Ligands for the different receptors are advanced glycation end products (AGEs), amyloid-β (AMB), (ECM), growth hormone-releasing peptide (GHRP), high mobility group protein 1 (HMGB1), lipopolysaccharide (LPS), and oxidised low density lipoprotien (oxLDL).

Table 1. Structural elements of AGE receptors that have been researched in relation to dAGEs: RAGE, Galectin-3, SR-A and CD36: their role in AGE binding and the forces that result in the interaction of AGEs with the respective AGE receptor.

Receptor	Structure	AGE binding sides	Forces for AGE interaction
RAGE	EC: one V-type domain, two C-types domains and a short transmembrane domain IC: cytoplasmic tail	V-type domain	Electrostatic
Galectin-3	Component of AGE-R complex has a carbohydrate recognition domain (CRD) and a carbohydrate binding side (CBS)	CBS	Hydrophilic interactions via hydrogen bonds, and hydrophobic interactions, specifically the CH-π interaction explains binding to lectins and lipopolysaccharides. For specific AGEs unknown.
SR-A	EC: scavenger receptor cysteine-rich structure (SRCR), collagenous domain, α-helical coiled coil, and spacer as well as an intracellular cytoplasmic	Collagenous domain	For specific AGEs unknown. All ligands are macromolecular and polyanionic. For apo-A and apo- E amphipathic α-helix suggested as a potential recognition motif. Dual cation-binding site proposed as main domain for ligand binding via SR-A, hence electrostatic interactions.
CD36	Two transmembrane domains, an EC loop with glycosylation sites and two short IC tails	Hydrophobic binding pocket located at the highly glycosylated sites	Electrostatic, via a positively charged moiety that binds to negatively charged ligands, based on studies with diacylglycerol and oxLDL as ligands.

By binding to AGE receptors, protein glycation may manipulate the uptake of dietary proteins by APCs as well as cellular signalling. For example, an enhanced uptake of glycated OVA by mature DCs as well as increase IL-6 production in the CD4(+) T cell co-cultures with AGE-OVA-loaded mature DC has been demonstrated [67]. Moreover, increased TNF-α production after the exposure of THP-1 macrophages with protein bound AGEs but not free

AGEs has been described [68]. Due to the large heterogenicity of AGEs and the diversity of potential receptor-ligand interactions described for the various AGE receptors, it has not yet been possible to define common motifs of AGEs that result in binding to AGE receptors. For example, for protein-bound CML it has been indicated that the negative charge of CML could be a potential interaction motif for the binding to RAGE, sRAGE, Galectin-3, and CD36 [69,70]. At the same time, other AGEs such as protein-bound pyrraline that has been shown to enhance T-cell activation via SRA-I does not carry a negative charge [45]. This shows that still little is known about structure-related binding of many AGEs to a certain receptor and its molecular consequences.

Several studies have investigated MRPs/AGEs binding to AGE receptors and glycation induced immune responses [45,56,60,65,67,69-73]. In general, two different approaches were chosen to induce the formation of AGEs in the investigated model proteins: (i) incubating either below the denaturation temperature or above the denaturation temperature of the protein in the presence of a reducing sugar. (ii) incubation with chemicals that result in a specific modification with an individual AGE. The latter method has repeatedly been shown not to affect the secondary structure of the protein [45,69] and also incubation below the denaturation temperature of the protein is considered to minimize 3D-structural changes. Nevertheless, Cardoso et al. showed that even heating at moderate temperatures can result in protein aggregation [7]. This was especially promoted by the MR. It is thus important to sufficiently monitor 3D-structural changes of the investigated proteins after glycation, which has so far only been done in a few studies [45,65,71,73]. This is crucial, as recent studies pointed out that not

only glycation but also other 3D-structural changes such as aggregation, increased surface hydrophobicity and β-sheet formation/exposure are determinants for binding to RAGE and its soluble form (sRAGE), Galectin-3, SRA-I and CD36 [65,69,71-73].

For example, it was shown that the binding of heated β-lactoglobulin to sRAGE differs depending on the heating conditions (dry vs. wet conditions, high vs. low temperature as well as presence or absence of reducing sugars) and that aggregation also plays an important role in the formation of sRAGE binding ligands. Notably, glycation was not an indispensable requirement for the formation of sRAGE ligands [73]. Likewise, the uptake of heated β-lactoglobulin by THP-1 macrophages can be explained rather by hydrophobicity, exposure of β-sheets and aggregation, than glycation itself [69]. Moreover, aggregates with a molecular weight above 100 kDa that are formed upon heating of β-lactoglobulin both in the absence and presence of reducing sugars were more potent ligands to Galectin-3, CD36 and SRA-I in both THP-1 cells and monocyte derived human dendritic cells than the fraction of lower molecular weight [71]. The fact that the presence of sugar was not required to induce binding to AGE receptors points out that it is also important that next to unheated controls, glycation controls (protein heated in the absence of reducing sugars) should be added to the sample sets when testing for AGE receptors responses. Not only is a better control of the 3D structural changes necessary, but the qualitative and quantitative assessment of the formed AGEs is another critical point when investigating the immunomodulatory properties of AGEs. Previously, a wide variety of methods to monitor glycation in the model food proteins were used to assess binding to AGE-receptors, which some resulting more informative than

others. Fluorescence intensity measurement as well as the OPA-assay were used, as they were easy and quick methods to determine the overall extent of glycation relative to the unheated protein; however, they do not allow for the quantification of specific AGEs [18,74-76]. To quantify specific AGEs, immunoassays such as ELISA have often been used; however, interference in the quantification by other substrates and optimization of the matrix is crucial for its optimal use. Additional analytical methods include reverse phase high performance liquid chromatography (RP-HPLC) ultraviolet (UV), gas chromatography mass spectrometry (GC-MS), and liquid chromatography mass spectrometry (LC-MS). Of which the latter two are not only targeted but also highly sensitive and are considered as the state-of-the-art methods for qualifying and quantifying individual AGEs [77]. In the search for specific AGE-receptor binding AGEs and their structural motifs that result in this binding this will be indispensable; nevertheless, until now these analytical methods are not comprehensively applied in this field of research. Nonetheless, a few individual protein-bound AGEs have been directly tested as ligands for specific AGE receptors such as pyrraline, CML, CEL, and MG-H1 [45,69,70,78,79]. In the most recent study, Zenker *et al.* attempted to discriminate the role of different proteins modifications in binding to AGE receptors by testing β-lactoglobulin which was selectively modified with CML vs. β-lactoglobulin that was glycated with lactose below its denaturation temperature. Their findings showed that protein-bound CML is a ligand for sRAGE, CD36, and Galectin-3 as measured by inhibition ELISA and that the negative charge of CML is a determinant for the binding. [69]. In summary, AGE receptors may be responsive to structural modifications other than AGEs and thus it is crucial to properly monitor 3D-structural changes, to add

glycation controls and to sufficiently characterize the AGEs that are formed during the applied glycation procedures.

2.2 Immunogenicity of AGEs

As previously stated, AGEs have shown to contribute to chronic states of inflammation within the human body. Inflammation is part of the body's immune defences, both innate and adaptative; therefore, compromising a series of cellular and chemical barriers that aim to control endogenous and exogenous harmful stimuli [80]. One of the potential immunological modulatory effects of AGEs is the capacity of AGEs to be recognized by receptors on the surface of the immune cells [14,15,81].This not only has the capacity of contributing to low grade inflammation and local inflammation in tissues expressing RAGE but also to food allergy, since induction of oxidative stress and enhanced cytokine expression are the consequences of this binding (as shown in *Figure 4*) [82].

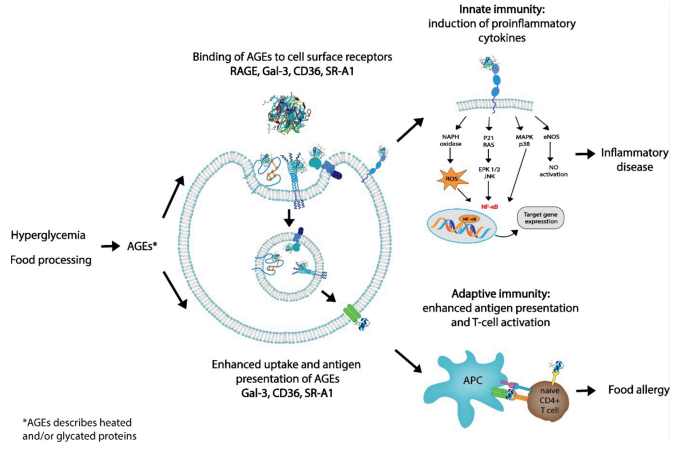

Figure 4. Role of dietary advanced glycation end products (AGEs) in innate and adaptive immunity. By targeting AGE receptors dAGEs may influence innate immunity via interaction with RAGE or by binding to AGE receptors which internalize the ligands: Galectin-3 (Gal-3), CD36, and scavenger receptor class A type I (SR-A1). Presentation of antigen to T-cells may

facilitate the T-cell activation and skewing possibly leading to the allergic responses. In that way dAGEs may contribute to both innate and adaptive immunity.

By modulating immunogenicity of food proteins by pathogen recognition receptors (PRR) which includes macrophage scavenger receptors and the RAGE additional downstream immunological effects can be that they promote T cell activation and differentiation leading to Th2 responses in food allergy [44,83]. Further potential immunological modulatory effects of AGEs are the ability to create novel epitopes that are recognized by IgE, the capacity of enhancing inflammatory conditions and oxidative stress and causing a reduction in the diversity of the intestinal flora which is associated with an enhanced susceptibility to allergies [17,45,52,63,65,71,81-84].

Regarding the immunogenicity of AGEs by receptor recognition, it is crucial to note that some receptors like RAGE only bind the AGEs and produce cytokine as a result, but other receptors like Galectin-3 also internalize the proteins, leading to enhanced antigen presentation of the protein to which the AGEs are bound, which may lead to activation of food protein-specific T-cells [71,83,85,86]. Therefore, initiation of adaptative immunity by AGEs will occur indirectly via antigens presentation, ultimately leading to T-cell activation.

2.2.1 Influence of AGEs on the Innate and Adaptative Immune System

The innate immune system recognizes microbes or foreign objects directly through pattern recognition receptors (PRRs), including RAGE [74]. RAGE is the most researched AGEs receptor since in response to the AGEs load is the main up-regulator of cell activation [9,28,30,55,62,63]. It is known that interaction of AGE-RAGE triggers various intracellular signalling cascades

which are followed by the transcription of a range of genes which perpetuate the pro-inflammatory signals [87]. More specifically, AGE-RAGE binding leads to an activation of NF-κB, a key player in activation of pro-inflammatory pathways including increase in the cytokine expression, growth factors and adhesion molecules [2,3,67,88] but also generation of ROS [54,87]. There is a body of evidence that interaction of RAGE with endogenous AGEs induces oxidative stress and inflammation [20] and increasing data on similar effect of dAGEs; however these studies are mostly based on *in vitro* work or experiments in rodents [71-73,86,88].

Current evidence suggests that RAGE shares ligands and intracellular signalling pathways like NF-kB activation with TLRs and can therefore cooperate in strengthening inflammatory response [61,89,90]. Moreover, studies show crosstalk between TRLs and RAGE, acting together through the recruitment of homo and hetero oligomers that strengthen inflammatory responses [91]. Liu et al reported that TLR4 expression increased in AGEs exposed macrophages which was then followed by the activation of RAGE/ROS signalling; thus, in macrophages, over-expression of RAGE elevated both ROS and TRL4 expression [92]. Therefore, it is possible that RAGE/ROS/TLR4 signalling is responsible for AGEs induced macrophage polarization [92]. Macrophages have a high degree of plasticity, meaning they can alter their function rapidly and polarize either to a more pro-inflammatory (M1) state, or an anti-inflammatory (M2) state [93,94]. Currently, evidence shows that macrophages treated with endogenous AGEs lead to polarization of macrophages into M1 state via MAPK signalling [95,96]. However, as most studies are conducted with endogenous AGEs and

not dAGEs, it remains unknown whether dAGEs are capable of inducing the same response in macrophages.

In order to induce an adaptive immune response, dAGEs first need to be internalized by APCs and subsequently presented to T-cells (as shown in *Figure 5*). This is strongly influenced by the efficiency of antigen binding, uptake and processing; as well as the activation status and production of cytokines by myeloid APC [97]. Receptor-mediated endocytosis is an efficient way of antigen uptake that makes possible an activation of adaptative immune responses at low antigen exposure and may be facilitated by Galectin-3, Fc receptors, dectin-1, 2 and 3, DC-SIGN and mannose receptors [16]. Recently, more evidence has been found that dAGEs are recognized and internalized by a number of the forementioned receptors expressed also by DCs [3,18,20,22,71,85]. Teodorowicz at al showed the binding and internalization of heat treated and glycated β-lactoglobulin by human monocyte-derived DCs [71]. Ge et al reported that AGEs obtained from bovine serum albumin induced maturation of DCs and increased their capacity to stimulate T cell proliferation and cytokine secretion [98]. Nevertheless, Price et al. showed that AGEs derived from adrenocorticotrophic hormone failed to show maturation markers of DCs and their capacity to stimulate primary T-cell proliferation [99].

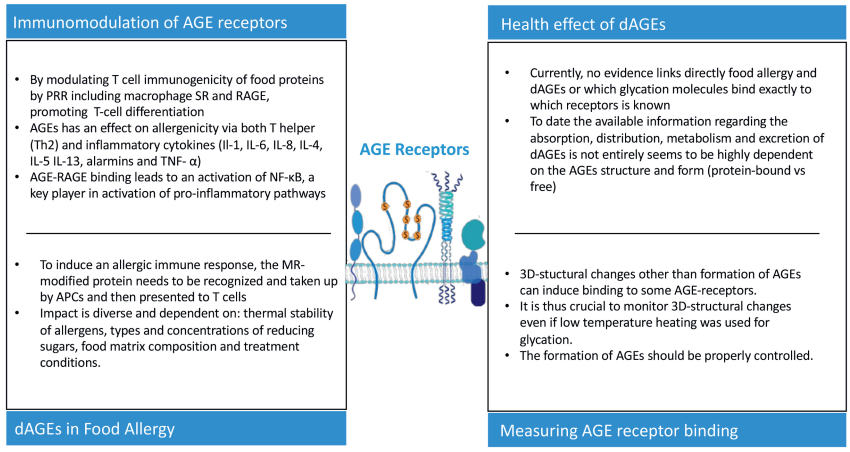

Figure 5. Key messages regarding AGE receptors and their role in the innate and adaptative immune system plus the health effects of dAGEs

By using ovalbumin (OVA), an egg white allergen as a model, Heilmann et al attempted to identify specific glycation structure(s) that had a food allergenic potential by influencing T-cell immunogenicity in the murine study [45]. Their research is one of a few attempting an identification of AGEs structures responsible for activation of T-cell immunity using OVA modified with CEL, CML, and pyralline (Pyr). T-cell immunogenicity of different glycated-OVA was assessed by co-culturing murine OVA specific CD4+ T-cells with bone marrow derived DCs. OVA modified by Pyr (Pyr-OVA) showed an enhanced production of IL-2, IL-17A and IFN-y compared to native OVA, demonstrating an increased CD4+ T-cell immunogenicity. Furthermore, the scavenger receptor (SR) was involved in the uptake of Pyr-OVA by bone marrow dendritic cells (BMDCs). Therefore, this study showed that pyralline was capable of inducing enhanced allergen uptake by DCs via association with SR class A (SR-A), and thus enhance CD4+ T cell activation and IgE production, aiding in the understanding into how the MR enhance the potential

allergenicity of food allergens [45]. Similarly, Ilchmann et al. demonstrated the uptake of glycated OVA by myeloid DC via receptor mediated endocytosis involving SR class-A type I & type II; plus showing that the production of IL-4 was enhanced by OVA-specific CD4+ T-cells [85].

There are also studies showing that glycation may not always lead to an activation of T-cell immunity. For example, Perusko et al described the immunological effect of glycated ß-lactoglobulin (BLG), demonstrating that glycation significantly increased the uptake by BMDCs via receptor mediated endocytosis via SRs [100]. Nevertheless, despite higher degradation by lysosomal enzymes, glycated BLG demonstrated lower ability to induce production of Th1 and Th2 type cytokines in co-culture of BMDC with BLG specific CD4+ T-cells [100]. In contrast, different study investigating the immunogenicity of bovine β-lactoglobulin showed that heat-induced formation of amyloid-like structures, aggregates and increased hydrophobicity are the features determining the binding to APCs [18]. Both studies did not include the effect of enzymatic digestion of the processed/glycated protein on the binding to the specific receptors. The importance of glycation *in vivo* was suggested by a study which showed that glycated aggregates of β-lactoglobulin are less sensitive to digestion and therefore maintain their binding capacity to RAGE and Gal-3 [71]. Finally, β-lactoglobulin modified with CML was shown to be recognized by sRAGE, galectin-3, and CD36 [69]. Recently it has been shown that the RAGE expressed on T cells is involved in activation of T cell signalling cascade and may be an important mechanism in response of T-cells to inflammatory mediators. It suggest that RAGE may also play a role in direct activation of T-

cells via AGEs present in the circulation contributing to inflammation and enhanced T cell reactivity [83].

Hence, during the processing of antigen various steps such as antigen uptake, activation of DCs, generation of peptides, stability and density of MHC peptide complex can affect the immunogenicity of the antigen [101]. Since antigen fate is determined by intracellular degradation of antigen in APC, antigens having higher susceptibility to endolysosomal enzymes have a weaker capacity to prime T cells [101]. Therefore, the ability of dAGEs to activate the T-cell immunity may be determined by a number of factors including heterogeneity of AGEs formed under different conditions as well as the unique amino acid composition of protein determining formation of amyloid-like structures, hydrophobic motives and aggregates which are the features determining the binding to APCs. In conclusion, heterogeneity of AGEs and the diversity of their receptors makes difficult to formulate the unequivocal conclusions on structure-function relation in activation of adaptive immunity. Therefore, more well defined, and unified studies are needed in order to define the AGEs structures responsible for binding to certain receptors and the consequences of this binding on the level of both innate and adaptive immunity.

3. Association between dAGEs and Food Allergy

IgE mediated food allergy prevalence is rapidly increasing, particularly in Western countries and evidence suggests an immune system dysfunction in the development and persistency of food allergy [102]. Since human genetics are not capable of radical modification in the past decades, it is more plausible that the way genes function has been altered by

environmental factors influencing epigenetic processes like methylation, ubiquitination and histone acetylation [103,104].

It has been suggested that a Western diet, typically high on AGEs has an effect on allergenicity via both T helper (Th2) including IL-4, IL-5, IL-13, the pro-inflammatory cytokines Il-1, IL-6, IL-8, and TNF-α, and alarmins [89]. Type I allergic immune responses are primarily characterized as T-helper cell (Th2) driven, which results in the formation of allergen-specific IgE antibodies, leading to mast cell activation upon secondary contact with the responsible allergen [44,82].

By modulating T cell immunogenicity and antigen presentation of food proteins by pathogen recognition receptors (PRR) which includes macrophage SR and RAGE; they contribute to T cell differentiation and Th2 responses [44,82,83]. Further potential immunological modulatory effects of AGEs are the ability to induce novel IgE binding epitopes, the capacity of enhancing inflammatory conditions and oxidative stress and causing a reduction in the diversity of the intestinal flora which leads to an enhanced susceptibility to allergies [16,17,41,82].

The possible influence of the MR on the potential allergenicity of certain food allergens has been a topic of recent interest [14-17]. So far, studies have shown the effects of the MR in food allergenicity are diverse, mostly dependent on the thermal stability of allergens but also on the types and concentrations of reducing sugars, food matrix composition and treatment conditions (e.g. temperature, pH, duration and moisture) (as shown in *Figure 5*) [17]. The affinity of allergens for specific IgE antibodies may be influenced by glycation, which in turn has an impact in the electric charge and hydrophobicity of proteins [18,19]. Moreover, glycation with AGEs

induces either masking of epitopes or generation of neo-allergens [15]. Extracts from roasted peanuts have been shown to induce higher levels of IgE than raw peanut, and Mueller et al described that binding of peanut allergens that had been specifically modified by AGEs to RAGE, establishing that RAGE does interact with AGE-modified recombinant Ara h 1 but not with unmodified recombinant Ara h 1 [104]. Teodorowicz et al described that MR-type neo-allergens in processed soy caused a strong allergic reaction in soy-sensitized individuals [105]. Evidence shows that to induce an allergic immune response, the MR-modified protein needs to be recognized and taken up by APCs and subsequently by presented to T cells (as shown in *Figure 5*) [85]. Moreover, several studies have identified RAGE and several other receptors as described above from the scavenger family as the main receptors recognizing glycated food proteins [45,56,57,59,60,71].

The false alarm hypothesis, proposed by Smith et al., suggests that the signalling of immune cells by RAGE-activated APCs is important for the role of AGEs in food allergy [106]. Moreover, it has been proposed that dAGEs might induce alarmin signalling, thus have the potential to mimic tissue damage through glycation [106,107]. In other words, dAGEs could mimic innate alarms and skew towards allergic responses in certain subjects that have a genetic and environmental predisposition [106]. Currently, there is no direct evidence that AGEs trigger food allergy through interaction with RAGE, though RAGE is highly expressed on DCs, macrophages, T lymphocytes and B cells [2,3,22]. Thus, evidence that directly links food allergy to the AGE-RAGE axis is lacking plus it is still not known which glycation molecules bind exactly to which receptors *in vivo*. We propose here that activation of APC via not only RAGE, but also other receptors induces a proinflammatory environment,

but that uptake and antigen presentation via AGE receptors that can internalize the food allergen with AGEs is a key mechanism linking activation of the innate immune system by AGEs with the activation and differentiation of adaptive, food allergen specific Th2 responses (*Figure 5*). So far, there is no precise evidence to predict the consequence of food-derived AGEs on the allergenicity of food proteins and further studies are needed to understand the biological and the immunological characteristics and consequences of MPRs (as shown in *Figure 6*) [3,22].

4. Conclusions

The evidence reviewed above show that dAGEs may act as signalling molecules in modulating innate and adaptative immunity, potentially contributing to the low-grade inflammation, food allergy and in non-communicable diseases. Thus, dAGEs may influence activation of the immune system in two different manners. Firstly, via interaction with RAGE which does not lead to internalization of the ligand [71] but does activate pro-inflammatory pathways as described for endogenous AGEs [108]. Further research is necessary to determine which RAGE ligands are involved in promoting RAGE-dependent responses. In vitro studies suggest that protein bound CML might play a role in RAGE activation [69] while heat induced protein aggregation may also play an important role [71]. Since RAGE is capable of recognizing various ligands characterized by ß-sheets and fibril formation. This could place RAGE in the group of PRRs that are important for activation of innate immune response via food-derived ligands and contributing to non-communicable diseases [96,97].

Secondly, binding of dAGEs to the receptors RAGE, Galectin-3, CD36 and SR-A, which internalize the ligands and mediate the interaction of APCs with the adaptive immune system, and which may facilitate T-cell activation and skewing, thus leading to the allergic responses. Although the number of studies showing the T-cell skewing by dAGEs is limited the enhanced uptake and presentation of food allergens with dAGEs by APCs is by now well documented.

Therefore, it can be implied that the interaction of dAGEs and specific receptors on APC play an important part in the immunogenic effects of dAGEs. However, not all studies use the same types of proteins and methods of glycation and characterization of AGEs and structural changes of proteins. Further, information of the effects of digestion on glycated proteins and their effects on APC is limited at present. As a result, no definitive conclusions on interaction of AGEs with the immune system *in vivo* can be drawn at present. Future human studies are needed to elucidate the relevance of these mechanisms in health and disease.

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Chapter 6

Soy derived Maillard reaction products (MRPs) are recognized by sRAGE and promote pro-inflammatory response in human peripheral blood mononuclear cells (PMBCs)

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Abstract

The Maillard reaction (MR) occurs frequently in food processing techniques which produce soy-based products that are consumed more frequently in Western diets like meat replacers. Due to the MR, changes incur in protein structure and functionality as the consequences of formation of Maillard reaction products (MRPs). Previously it has been suggested that MRPs may play a role in increasing the allergenicity of these highly processed products. Moreover, the interaction between advanced glycation end products (AGEs) and the receptor for advanced glycation end products (RAGE) receptor may be a mechanism by which AGEs can modulate the immune response. However, there is a knowledge gap between the structure-function dependency of MRPs formed during soy processing and their interaction with RAGE. The aim of the present study is to characterize the MRPs formed during different heating times of soy proteins with glucose by analyzing the biochemical changes and to relate them to the functional changes including antioxidant capacity, binding to soluble RAGE (sRAGE) and stimulating immune cells to secrete pro-inflammatory cytokines.

The results observed in the present study show that the type of MRPs/AGEs obtained in SPE – glucose system strongly depends on heating time under conditions. Moreover, these results suggest that structural protein changes during heating without glucose had less binding potency to the sRAGE when compared to glycated proteins. This hypothesis is strengthened because the binding capacity of glycated proteins to sRAGE positively correlated with time of glycation; therefore, formation of advanced glycation end products (AGEs). Thus, the results in the present study

demonstrated time dependent differences in the biochemical characteristics of glycated soy when compared to heated soy, which could be attributed to the different stages of MR and the diversity in the obtained MRPs.

Additionally, incubation of glycated soy sample for 90 minutes (G90) resulted in increased expression of pro-inflammatory cytokines (IL-1ß, IL-8 and TNF- α), suggesting that formed AGEs interact with immune cells activating possibly via various AGE receptors, including RAGE.

1. Introduction

In recent decades, soy proteins are more frequently used in food products not only for its reported beneficial health effects but because they represent inexpensive and excellent sources of quality proteins [1,2]. However, since soy is rarely consumed raw, the majority of soy-based products contain highly processed soy proteins [2]. The production of these soy-based products usually involves thermal processing such as cooking, roasting, irradiation and autoclaving, during which the Maillard reaction (MR) may occur [3]. The MR occurs between free amino groups of amino acids, peptides and proteins with the carbonyl group of a reducing sugar, known as non-enzymatic browning reaction or glycation as well [4-6]. The MR occurs always during food processing or cooking, when proteins and a reducing sugars are present together [7]. The speed of reaction and the type of formed products are highly dependent on several variables; physical ones such as temperature and heating time; as well as chemical ones such as pH, water activity and presence of substrates [8]. The chemistry of the MR is very complex encompassing a whole network of various reactions, with the formation of Amadori products seen in the early phase, the rearrangements of these Amadori products in the advanced phase and finally formation of advanced glycation end-products (AGEs) [9]. Therefore, the stages of the MR can be summarized as: (i) slow formation of Schiff bases, (ii) early formed unstable AGEs precursors that might be able to cause rearrangement of Amadori products and (iii) formation of late and irreversible AGE products [10]. Thus, the MR leads to chemical modifications of food proteins and hence the formation of new flavors, colors, aroma, and other neo-formed compounds known as the Maillard reaction products (MRPs) [11]. The term

MRPs covers both early, intermediate and advanced products, thus it encompasses a variety of heterogenous and complex group of compounds commonly found in processed foods [12].

Recently, MRPs have been linked to the occurrence of many diseases, including allergies; although, there is little consensus on this topic and only a few MRPs have been reported as potentially harmful [12]. For instance, dietary supplementation in mice with N^{ε}-(carboxymethyl)lysine (CML) caused elevated serum levels of CML as well as insulin resistance, which altered cardiac function by altering myocardial glucose metabolism and promoting myocardial remodeling [13]. Additionally, it has been reported that CML promotes macrophage lipid uptake via CD36 and RAGE receptors, which may lead to the formation of foam cells [14]. Therefore, in certain pathologies like common non-communicable diseases: cardiovascular diseases, diabetes and allergy the dietary MRPs may possibly add to the pool of endogenous AGEs and promote pathological complications [13-18]. Furthermore, AGEs are reported to affect the elasticity of the extracellular matrix (ECM), the binding of cells, and the turnover of ECM proteins; additionally, they can interact with a number of receptors that induce oxidative stress and activate inflammatory cascades [1,3,9,10,19]. For example, the receptor for advanced glycation end-products (RAGE) is expressed on the surface of various cells including endothelial, intestinal and immune cells interacting with a diverse class of ligands such as AGEs, S100, amyloid-ß and AGEs [9]. The ligation of AGEs by RAGE can induce the generation of reactive oxygen species (ROS) by a NADPH oxidase which eventually leads to the activation and translocation of NF-κB [9,20-23]. The induction of NF-κB leads to the activation of several, primarily pro-inflammatory, genes with a variety of roles in inflammation [9,19].

Moreover, the AGEs-RAGE mediated activation of macrophages and their associated cytokines (IL-1, IL-6 and TNF-a) can also contribute to the induction of a persistent state of inflammation [24,25].

In the present study we analyzed the level of the MR of soy proteins glycated at different heating time points by the means of biochemical characteristic and formation of MRPs / AGEs and we related these structural changes with functional properties measured by their antioxidant capacity, binding potential to sRAGE and their ability to stimulate the immune cells to secrete pro-inflammatory cytokines.

2. Materials and Methods

2.1. Soy protein extracts (SPEs)

The protein extraction from soy flour (SPEs) was performed according to L'Hoccine et al with some minor modifications, precipitation pH was adjusted to 9.0, the washing of precipitate was done with deionized water (Milli Q, MQ, Millipore, St Louis, MO, USA) and performed once instead of twice to improve protein recovery [26]. The starting material was 5 grams of soy flour (Sigma Aldrich, St Louis, MO, USA, S9633) which contains 2.2 grams of protein, according to the manufacturer. The flour was dissolved in water at 55°C at the ratio of 1:10 (w/v). The supernatant was separated from the insoluble part by centrifugation (30 minutes, 9000g at 4°C) and the pellet was collected to perform the re-extraction. It was stirred for 2h at room temperature (RT) and centrifuged at 9000g for 20 minutes at 4°C, the obtained precipitate was washed twice and spun down. The pH of the protein extract was measured and adjusted to 7.5 with 2M NaOH and the protein concentration was determined by absorbance at 280 nm using NanoDrop.

The SPEs obtained consisted of 44.2% dispersible protein and was estimated to be 14.2 mg/ml.

After extraction, the SPE was dissolved in PBS and distributed in Eppendorf tubes, glucose was added in a ratio 1:2 (w/w, protein/glucose), to the control the same volume of PBS was added. All samples were heated at 100°C for 5, 10, 20, 30, 45, 60, 90 and 120 minutes in the presence as well as absence of glucose in a heating block. Non-heated control was included as the reference point. After heating, the samples were cooled down on ice and stored at-20°C.

2.2. Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Allergen extracts were separated by SDS-PAGE under reducing conditions using Biorad equipment. Proteins were loaded in an amount of 20 µg per well on 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.

2.3 Structural properties of soy proteins

2.3.1 O-phthaldialdehyde (OPA) assay

The assay was performed as described previously by Nielsen et al [27]. The proteins were diluted to the concentration of 2 mg/ml, appropriate blanks were prepared. Samples were distributed in the volume of 30 µl per well into 96-wells plate, as was a standard curve of L-leucine (2.5-0.078 mM). To each well 200 µl freshly prepared reagent of sodium tetraborate (0.10 M),

SDS (3.5 mM), OPA (6.0 mM) and dithiothreitol (5.7 mM) was added. The plate was incubated at RT for 20 minutes after which the absorbance was measured at 340 nm. The amount of free amino groups was calculated from the standard curve obtained for L-leucine (normally linear for these concentrations).

2.3.2 UV-Vis

Intermediate MRPs were detected by recording the UV-absorbance at 294 nm and the advanced MRPs, at 420nm, the results of the protein samples were corrected with the blank. The proteins were diluted to 2 mg/ml and pipetted onto a clear 96-wells plate, a blank of PBS was added.

2.3.3 Fluorescence and Fructosamine Assays

The maximum excitation and emission were determined previously (excitation 340 nm, emission 435 nm), the fluorescence was recorded to detect the presence of advanced MRPs. The samples in concentration of 2 mg/ml were pipetted into a white, non-transparent plate. The maximum excitation of the samples was tested from 320 to 380 nm with the emission wavelength at 425 nm. The maximum emission was tested from 380 to 480 nm in steps of 10 nm. Finally, the fluorescence was measured at the optimal excitation and emission wavelength.

A fructosamine commercial kit (Kamiya Biomedical Company, Seattle, WA, US) was used. The methods were adapted to 96-well plate since this kit was not designed for small scale; thus, the volumes used were 4 times lower than in the original protocol. The samples were diluted to 2 mg/ml and pipetted into 96 well plate in a volume of 12.5 µl. Then 250 µl reagent was

added and the plate was incubated for 10 minutes at 37°C. The absorbance was measured at 535 nm. The plate was incubated for another 5 minutes at 37°C and the absorbance was measured again at 535 nm. The concentration of fructosamine was then calculated according to the following formula, where A_1 is the absorbance from the first measurement and A_2 the absorbance from the second:

$$\frac{A_2\ Sample - A_1\ Sample}{A_2\ Calibrator - A_1\ Calibrator} \times Conc.of\ Calibrator = Conc.Fructosamine\ in\ Sample$$

2.3.4 Quantification of N^{ε}-carboxymethyllysine (CML)

CML was quantified using uHPLC-ESI-MS/MS according to a method previously described by Zenker et al [28]. Samples were diluted to 2.5 mg/mL in ultrapure water and mixed with hydrochloric acid to a final ratio of 0.63 mg protein/1 mL 6 M hydrochloric acid. Solutions were saturated with nitrogen and heated for 22 h at 110 °C. Hydrolysates were centrifuged (4500 ×*g*, 10 min, 20 °C) using a Heraeus multifuge X3R (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and filtered through a 0.2 µm Polytetrafluoroethylene syringe filter (Phenomenex, Torrance, California, USA). An aliquot was dried under nitrogen and dissolved to the same volume in ultrapure water. Samples were centrifuge (10,000 ×*g*, 20 min, 20 °C) using an Eppendorf multifuge 5430R (Eppendorf, Hamburg, Germany). Subsequently, they were diluted with acetonitrile to reach 50% acetonitrile and spiked with internal standard CML-d2. Standard solutions were prepared in a concentration range between 25 ng/mL and 750 ng/mL and spiked with CML-d2. Final concentration of CML-d2 in all sample and standard solutions was 250 ng/mL. CML was separated on a Kinetex 2.6 µm HILIC 100A, 100 ×

2.1 mm (Phenomenex, Torrance, California, USA) at 35 °C column temperature. Eluent A was ultrapure water with 0.1% formic acid, eluent B was acetonitrile with 0.1%, and eluent C was 50 mM ammonium formate. Flow rate was set to 0.4 mL/min using the following gradient (time [min]/eluent B [%]/eluent C [%]): (0/80/10), (0.8/80/10), (3.5/40/10), (6.5/80/10), (8.0/80/10), (11/80/10). Electron ionisation was conducted in positive mode. Spray voltage was set to 3500 °C, vaporising temperature was 250 °C, and sheath gas pressure was 60 psig. Capillary temperature was set to 290 °C.

2.3.5 Browning

The proteins were diluted to 2 mg/ml and pipetted in triplo onto a clear 96-wells plate. The absorbance was measured at 625, 495, 445 and 550 nm. This value was then corrected and converted to the transmission (10^{-A} = T). The transmission values were then used to calculate X ($X = T_{625} * 0.42 + T_{550} * 0.35 + T_{445} * 0.21$). The browning index (Br) was then calculated as follows:

$$Br = x - \frac{0.31}{0.172} . 100$$

2.4 Functional properties of soy proteins

2.4.1. Antioxidant Assay (DPPH)

The method was adapted from Gu et al [30]. Samples were diluted to 2 mg/ml, a blank of PBS and the positive control of Trolox were prepared. The samples were plated in six-fold (125 µl per well), methanol was added to half of the samples and DPPH dissolved in methanol (0.2 mM) was added to the other half. The contents of each well were mixed and the plate was

allowed to stand in the dark at RT for 30 minutes. After this the absorption was measured at 535 nm and the radical scavenging activity (RSA) was calculated using the formula:

$$RSA = \left[1 - \frac{A_{sample} - A_{control}}{A_{blank}}\right] \times 100$$

To confirm that the concentrations used were no cytotoxic to the cells, a cell cytotoxicity assay was performed using Caco-2 cells as model for the enterocytes in the small intestine. The protocol used listed as Supplemental Section 1. No cytotoxicity of SPEs was observed after 24 hours of incubation of Caco-2 cells with SPEs with any of the tested concentrations 5, 50 and 500 µg/ (as shown in Supplemental *Figure 1*)

2.4.2. sRAGE inhibition ELISA binding assay

This assay was performed as previously described by Zenker et al [28] to determine sRAGE binding affinity. The coating material was glycated SPEs with glucose (90 min, 100°C in wet conditions). Transparent high binding ELISA plate (Greiner Bio-One, Kremsmuenster, Austria) were coated with the SPE glycated for 90 minutes (G90) for 12 h at 4°C. Sample protein concentration was adjusted to 25 µg/mL with 1.5% BSA (*v*/*w*) in 0.025% tween in 10 mM PBS (PBST) with the optimal protein concentration chosen based ona pre-experiment. Before addition to the ELISA plate, the samples were pre-incubated with 1 µg/mL sRAGE in a ratio 1:1 (*v*/*v*) for 45 min at 37 °C on a NuncTM polystyrene plate (Thermo Fisher Scientific, MA, USA), then the coated ELISA plate was blocked with PBS with 3% BSA (*v*/*w*) for 1 h at RT and washed with PBST (washing was repeated after each step of ELISA). After blocking, the pre-incubated sRAGE/sample mixture was transferred into the

ELISA plate and incubated for 1 h at 37 °C. After washing, anti-sRAGE antibody was added at a concentration of 0.25 µg/mL and the plate was incubated under shaking for 30 min at room temperature. After washing, anti-mouse polyclonal goat HRP conjugated antibody at a concentration 0.25 µg/mL was added and the incubation was continued for 30 min at room temperature. The signal was detected with TMB. The color reaction was measured at 450 nm vs. 620–650 nm reference using a Filter Max F5 multi-mode microplate reader (Molecular Devices, San Jose, CA, USA). Each sample was measured in triplicate. Amyloid-β was used as a positive control, while ovalbumin was used as a negative control. Inhibition was calculated with the formula below, where Abs_{Max} is the absorbance obtained from sRAGE without competition agent and Abs_{Min} is the absorbance obtained from blank sample (PBS) without sRAGE, Abs_{sample} is the absorbance obtained from the mixture of sRAGE and each sample. High inhibition indicates high sRAGE binding affinity.

$$Inhibition\ [\%] = \left[\frac{Abs_{max} - (Abs_{sample} - Abs_{Min})}{Abs_{Max} \times 100}\right]$$

2.4.3. PBMCs stimulation and Cytokine measurement

After informed consent, blood samples were collected from 5 healthy adult donors in EDTA tubes. Peripheral blood mononuclear cells (PBMCs) were isolated essentially as described previously [29].

After the second wash, the PBMCs were resuspended in 1ml of RPMI 1640-Glutamax medium supplemented with 10% FBS, 1% MEM non-essential amino acids, 1% Na-Pyruvate and 1% Pen/Strep and counted before further use for monocyte isolation. Monocytes were isolated by using EasySep Human Monocyte Isolation kit (Stem cell technologies, #19359), then seeded

at 1x10^6 cells per 2ml per well on a 24-well plate and rested for 2 hours prior to stimulation, after which the cells were adhesive PBMC-derived monocytes.

The adhesive PBMC-derived monocytes were stimulated with glycated soy at 25 ug/ml for 3 hours. RNA isolation was done by using the RNeasy mini kit (Qiagen). For harvest, cells were washed with PBS, then RLT buffer containing 1% β-mercaptoethanol was added directly on the well, followed by passing the sample through a needle fitted to a 1 mL syringe. To the homogenized lysate, 350ul of 70% ethanol was added, and the total volume of 700 ul was added to the RNeasy mini column followed by centrifugation for 30 seconds at 10 000 rpm. After washing, 80ul of DNase solution was added to the column and incubated for 15 min at RT. The column was washed twice with buffer and eluted with 30ul of RNase free water. RNA concentration was measured on the nanodrop. cDNA was synthesized using the SuperScript III kit (Invitrogen) according to manufacturer's instructions with a T-professional PCR (Westburg, Leusden, Netherlands), using a total of 250-500ng RNA per sample. For qPCR analysis, 1x SYBR Green master mix, 3uM of forward and reverse primers, and 1ug of cDNA were mixed together per tube to be analyzed. Samples were run in a Qiagen rotor gene q 5plex HRM device (Qiagen, Hilden, Germany). The primers are listed in *Table 1*. Gene expression was calculated using the data retrieved from the Rotor Gene Q software (Qiagen) and transformed to fold change using the Pfaffle method. Expression is normalized to the PUM1 gene. Graph Pad Prism 9.5.1. (Boston MA, USA) and Student T-test were used for the statistical analysis

Table 1. Primers used in the PBMCs stimulation and cytokine measurement

Genes	Forward	Reverse
PUM1	TGAGGTGTGCACCATGAAC	CAGAATGTGCTTGCCATAGG
IL-1B	TTCGACACATGGGATAACGAGG	TTTTTGCTGTGAGTCCCGGAG
IL-8	CTGATTTCTGCAGCTCTGTG	GGGTGGAAAGGTTTGGAGTATG
TNFa	CTT-CTG-CCT-GCT-GCA-CTT-TG	GTC-ACT-CGG-GGT-TCG-AGA-AG
IL-10	TCAAGGCGCATGTGAACTCC	GATGTCAAACTCACTCATGGCT
RAGE	GCT-TGG-AAG-GTC-CTG-TCT-CC	CAC-GGA-CTC-GGT-AGT-TGG-AC
LGALS3	GTGAAGCCCAATGCAAACAGA	AGCGTGGGTTAAAGTGGAAGG
CD209	TCAAGCAGTATTGGAACAGAGGA	CAGGAGGCTGCGGACTTTTT
CD86	CTGCTCATCTATACACGGTTACC	GGAAACGTCGTACAGTTCTGTG

3. Results and Discussion

The extent of the MR under 8 different heating times at 100°C was assessed by various methods, which taken together provide a comprehensive view of the stages of the MR and the type of MRPs obtained at each stage.

3.1. SDS-PAGE

The SDS-PAGE pattern on the heated controls and glycated soy proteins, H-SP and G-SP respectively (as shown in *Figure 1A* and *1B*, respectively), and the molecular weight distribution observed from 10 to 250 kDa. The SDS-PAGE spectrum of the raw soy protein showed the characteristic bands of *β*-conglycinin (*α'*, *α*, and *β*) and glycinin (As and Bs) subunits (as shown in *Figure 1C*). The discussed results will focus on the soluble components; however, protein concentration was diminished partly because a pellet was formed.

As the heating time increased, the intensity of the characteristic protein bands weakened, and more intensive bands with high molecular weight (upper part of the gel) were observed. The G-SP showed slightly more

reduction in bands when compared to the H-SP (as show in Figure 1A and *1B*). Additionally, the smeared zones at the top became darker, particularly for the G-SP; suggesting that glycation promotes the formation of aggregates with higher molecular weight. This concurs with previous studies that evaluated the effects of glycosylation on different proteins [31-33]. Bu et al suggested that the formation of new polymers could lead to the changes in spatial structure of proteins which causes the changes in the antigenicity and allergenicity of glycated proteins [31].The results of the SDS-PAGE show that heating and glycation affect the structure of SPEs; moreover, formation of high molecular aggregates was more intense in the G-SP when compared to heated and appeared at the 10 minutes of glycation.

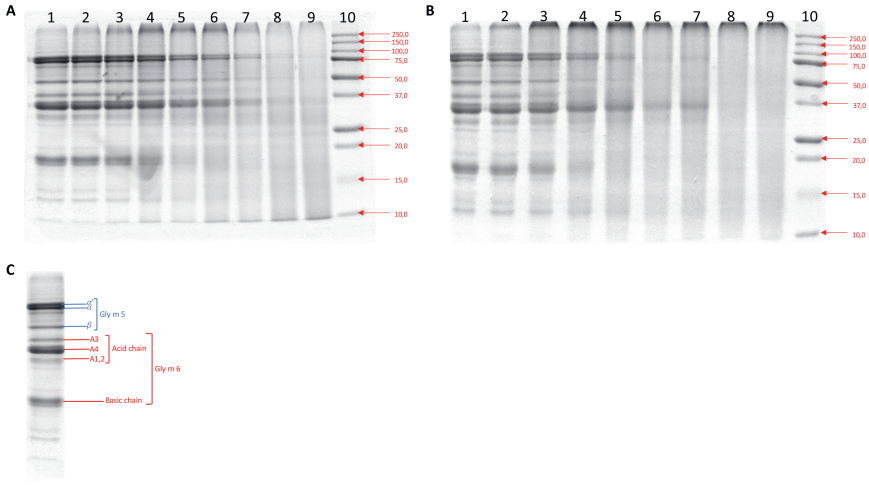

Figure 1. SDS-PAGE results: (A) SDS-PAGE of Heated Soy Proteins, (B) SDS-PAGE of Glycated Soy Proteins, where lane 1 = raw soy, lane 2 = glycated soy for 5 minutes, lane 3 = glycated soy for 10 minutes, lane 4 = glycated soy for 20 minutes, lane 5 = glycated soy for 30 minutes, lane 6 = glycated soy for 45 minutes, lane 7 = glycated soy for 60 minutes, lane 8 = glycated soy for 90 minutes, lane 9 = glycated soy for 120 minutes , lane 10 = marker, (C) Separation of the denatured soy proteins by molecular weight

3.2. Structural properties of soy proteins

3.2.1 OPA assay

The OPA assay was used to measure the amount of free amino groups in H-SP and G-SP. The results showed a decrease in the ratio between G-SP and H-SP, the decrease reached the higher value after 60 minutes of glycation being at the level of 0.77(as shown in *Figure 2A*). Thus, significant reduction in free amino groups, which are major reactants of the MR, were observed in all the glycated samples except 5 and 110 minutes of glycation, concurring with previous research which found that the MR decreases the amount of free amino acids in a condition dependent manner [34-36]. Moreover, the present study shows that the reduction of free amino groups stabilizes after the first hour, possibly because the first stage of the MR has slowed down and so has the attachment of sugars to free amino groups. This would suggest that after one hour that MR has moved on to intermediate stages characterized by the degradation of Amadori products and formation of the stable MR products.

3.2.2 UV-vis

The UV-Vis results showed the absorbance at 294 nm for the G-SP increased linearly with time, reaching a plateau after 90 minutes, with an absorption 5 times higher when compared to the H-SP (as shown in *Figure 2B*). A similar linear increase was observed for the G-SP at 420 nm, reaching a maximum after 120 minutes, 7 times higher when compared to the H-SP (as shown in *Figure 2C*). The increase in absorption at 294 nm correlates with the formation of intermediate MRPs, while the absorption at 420 nm correlates with the formation of advanced MRPs.

Previous studies reported a strong, linear increase in the amount of absorption of glycated solutions over time, concurring with the results in the present study [37-40]. Thus, it appears that intermediate MRPs start to be produced immediately, with absorption found after 5 minutes of heating, while the advanced MRPs appear approximately after 10 minutes of heating. Additionally, the net production of intermediate MRPs reaches the plateau after 90 minutes, possible attributed to the production of these intermediates going down or the production of advanced MRPs going up; however, most likely, it is caused by a combination of both factors.

3.2.3 Fluorescence and Fructosamine Assays

To continue characterization of a type of MRPs formed at different time points of heating of SPE, fructosamine an early MRP and important AGE precursor was measured [41]. The results showed that the concentration of fructosamine in the H-SP was constant and did not exceed 0.19 mM; while in G-SP, a linear increase in fructosamine concentration was observed immediately after heating, reaching a plateau after 45 minutes (1.9mM) (as shown in *Figure 2D*). This results, together with OPA outcomes, indicate that the early stage of the MR starts after 5 minutes of heating with most of it occurring in the first hour of heating when most likely the MR reaction reaches the advanced stage. It was previously reported that during the digestive process of meal-resembling systems, high fructosamine levels correlated with further formation of fluorescent adducts which suggest pathways going mostly into the formation of cross-linking fluorescent products (e.g., pentosidine) [41]. Our finding on formation of MRPs, both early and advanced, during conditions mimicking the digestive process,

including concentration of reactants and time of reaction, supports the concept of intraluminal generation of AGEs as another source of exogenous AGEs.

To assess the formation of fluorescent AGEs in SPE -glucose system, the fluorescence was measured. An increase in fluorescence in G-SP was observed immediately with a significant increase after 5 minutes, the maximum was reached after 30 minutes which was 3-times higher when compared to the H-SP (as shown in *Figure 2E*). Previously, fluorescence has been described to detect advanced MRPs; additionally, fluorescent compounds have been reported as precursors of browning pigments during the MR [35,42]. Moreover, modification of proteins with fluorescent compound pentosidine has been reported to be linked with inflammation, chronic kidney disease and cardiovascular diseases [43]. Additionally, the level of fluorescence in the G-SP samples started to decrease after around 40 minutes of heating (as shown in *Figure 2E*), which suggests that other non-fluorescent advanced reaction products are being formed, since not all advanced MRPs possess fluorescence properties [44]. This finding concurs with previous studies, that report that fluorescence intensity reached a maximum during heating after which a gradual decrease in intensity was observed [45,46].

3.2.4 Quantification of N^{ε}-carboxymethyllysine (CML)

The results above show that soy glycation is capable of generating both early stage and advanced MRPs. CML is a common marker of non-fluorescent AGEs both endogenous and these formed in food during processing [47]. CML was quantified in all G-SP for the different selected

heating times to determine the level of the advanced stage of the MR (as shown in *Table 2*). The results in the present study showed small amounts of CML were found in raw soy (0.06 mg/1g protein) and G-SP at 15 minutes (0.07 mg/1g protein). CML quantities positively correlated with the increase in heating time, with the highest CML quantity observed for the G-SP at 120 minutes (0.39 mg/1g protein).

Previous studies have reported that AGEs (e.g., CML) may function as epitopes that activate immune cells with CML possibly functioning as a potent epitope capable of exacerbating allergenicity [28,48-50]. The results in the present study show that formation of CML time dependent in the G-SP samples. Moreover, Mueller et al reported previously that CML-modified peanut allergens (Ara h 1 and Ara h 2) where preferably recognized by RAGE and contributed to the activation of the RAGE receptor, hence the allergenic response [48].

Table 2. CML quantities determined by uHPLC-ESI-MS/MS for raw and glycated soy at different heating times.

Sample	Mg per 1 gram protein
N^{ε}-carboxymethyllysine (CML)	
Raw Soy	0.06
Glycated Soy for 15 minutes	0.07
Glycated Soy for 30 minutes	0.12
Glycated Soy for 45 minutes	0.16
Glycated Soy for 60 minutes	0.23
Glycated Soy for 90 minutes	0.35
Glycated Soy for 120 minutes	0.39

3.2.5 Browning

The browning of the H-SP was stable and did not exceed the value of 1.5; while the browning of the G-SP showed a linear increase with time and

after 120 minutes their value was 13.6 times higher compared to the H-SP at the same time (as shown in *Figure 2F*). The browning index is often used as a predictor of melanoidin formation, the final brown products of the MR [39,50]. The antioxidant activity of melanoidins has been repeatedly studied, with the ability of melanoidins of radical scavenging and Fe^{2+} chelation reported as the most important antioxidant mechanisms [51-55]. However, the inability to access pure melanoidins makes it challenge to accurately determine their true contribution to the antioxidant activity in the presence of other antioxidants such as polyphenols and isoflavones [39].

In summary, the results presented in this section show that the formation of intermediate MRPs starts after the first minutes of heating as shown by the UV-vis assay, with the fructosamine results showing the main activity of intermediate MRPs in the first 30 minutes. Moreover, formation of advanced MRPs started after ten minutes of heating reaching their plateau after the first hour of heating as shown by the UV-vis and Fluorescence results. Additionally, CML accumulation was also in a time dependent manner, with the lowest amount of CML for raw soy and the highest for G-SP at 120 minutes. Lastly, browning products were reported to be present on G-SP after ten minutes of heating indicating the advanced stage of the MR and formation of melanoidins. All of these results show that the type of MRPs / AGEs obtained in SPE – glucose system strongly depends on heating time under conditions.

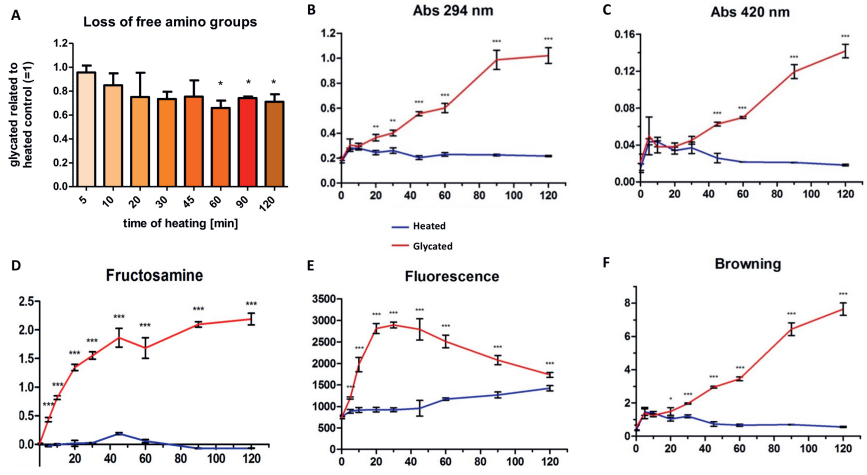

Figure 2. Results of the Structural Properties of heated and glycated soy proteins assays: (A) OPA assay, (B) UV-vis at 294 nm, (C) UV-vis at 420 nm, (D) Fructosamine assay, (E) Fluorescence assay and (F) Browning assay

3.3 Functional properties of soy proteins

3.3.1 Antioxidant Assay (DPPH)

The DPPH was used to screen the antioxidant activity of soy extract by determining the radical scavenging activity (RSA). The RSA of H-SP was variable for the first 30 minutes and did not exceed 18%, the RSA for the control remained stable between 45 to 120 minutes and did not exceed 14%. For the G-SP the RSA was somewhat variable for the first 10 minute after which it showed a linear increase (R^2 of 0.96) up to 47% until 120 minutes of heating, 4-times higher when compared to the H-SP at the same time (as shown in *Figure 3A*). These results suggest that the antioxidants form in the advanced stages of the MR, results that concur with the browning assay, which could indicate that the MRPs that have an antioxidant function are in part melanoidins which contribute to the browning index. The results from the present study are in line with previous studies [35,37,46,56]. Moreover,

Kim et al described that the RSA for MRPs made of glucose increased as a function of heating time [37].

3.3.2 sRAGE inhibition ELISA binding assay

The sRAGE inhibition ELISA can be used as a screening tools to indicate whether a specifically treated soy protein is a potential ligand for RAGE and thus can initiate an immune response. The specificity of this assay is shown in *Figure 3B*, were the G-SP at 90 minutes (G90) was set as the competition agent and ovalbumin (OVA) as the negative control. The results showed no inhibition for OVA while the G90 was able to bind to the receptor and inhibit the binding of sRAGE to the plate for all the tested concentrations (as shown in *Figure 3B*). This effect was dose-dependent with 100% of inhibition observed for the highest concentration of 500 µg/ml, 95% for the 50 µg/ml concentration, 63% and 21% of inhibition were observed for 5 and 0.5 µg/ml, respectively, and no inhibition was observed for the lowest concentration of 0.05 mg/ml (as shown in *Figure 3B*). Furthermore, this dose-dependent binding to sRAGE was observed for all the G-SPs obtained at different timepoints of heating (G20, G30, G45, G60, G90 and G120) (as shown in *Figure 3C*). The highest sRAGE inhibition curve was observed for G90 and G120, both reached an inhibition level very similar to the one observed for amyloid-β, the positive control (as shown in Figure 3C) Amyloid-β was able to inhibit sRAGE up to 97% at the highest concentration tested while OVA (negative control) showed no inhibition (as shown in *Figure 3C*).

The concentration of 25 µg/ml was chosen in order to compare the binding capacity observed for different G-SPs and corresponding at this concentration the sRAGE binding of G-SPs heated 45 minutes and longer was

significantly higher compared to their H-SPs while the observed sRAGE inhibition of G-SPs heated 30 minutes and shorter was similar to their heated controls (Figure 3D). An increase in inhibition capacity was observed, the longer the heating time, increasing from 22% to 86% for G20 and G120, respectively (as shown in *Figure 3D*). For the G-SPs, after 45 minutes of heating there was a linear increase in inhibition capacity with the maximum showed by G120 (as shown in Figure 3D). Moreover, a significant correlation between heating time and % of inhibition was observed for the glycated samples (r = 0.97), while no correlation was found for the heated samples and heating time (r =-0.72) (as shown in *Table 3* and Figure 3F).

Table 3. Correlation time of heating and capability to inhibit the sRAGE of glycated and heated soy samples in the ELISA based RAGE inhibitory binding assay for the concentration 25 µg/ml. In Figure 2G linear regression observed for the glycated SPEs is depicted with R= 0.9456

	Glycated SPEs	Heated SPEs
Number of XY Pairs	6	6
Pearson r	0.9724	-0.7166
95% confidence interval	0.7630 to 0.9971	-0.9662 to 0.2272
P value (two-tailed)	0.0011	0.1091
P value summary	**	Ns
Is the correlation significant? (alpha = 0.05)	Yes	No
R square	0.9456	0.5135

In summary, these results suggest that structural protein changes during heating without glucose had less binding potency to the sRAGE when compared to glycated proteins. This hypothesis is strengthened because, additionally, the results show that the binding capacity of glycated proteins to RAGE is positively correlated with time of glycation; therefore, also formation

of advanced glycation end products (AGEs). Moreover, the described results in the present study indicate that the binding capacity of the formed MRPs increases by heating soy protein for longer periods of time in the presence of glucose, suggesting that RAGE binds to soy proteins that have been modified by MRPs/ AGEs and it doesn't interact with raw soy. These results concur with previous findings peanut allergens [48,,57]. Previously, it has been suggested that the interaction between sRAGE and endogenous AGEs could be a mechanism by which AGEs could modulate the immune response [48-50,56-60]. Nonetheless, previous studies did not aim to characterize the stage of MR during different time points of heating and linking it to sRAGE binding capacity [58,59]. The results of the present study suggest that the types of MRPs are important factors that can influence the binding capacity of sRAGE, concurring with reports regarding AGEs formed by ovalbumin (OVA) and ß-lactoglobulin [29,61]. AGE-OVA and OVA modified with pyrroline are taken up by dendritic cells via scavenger receptor class A; while ß-lactoglobulin modified with CML was recognized by sRAGE in an inhibition ELISA assay [29,61]. Moreover, longer heating time periods in the presence of glucose can lead to the formation of more advanced MRPs (as shown in *Figure 2C*). The ELISA based sRAGE inhibitory binding assay results suggest that the amount of MRPs formed can be an important contributor to enhanced sRAGE binding capacity of G-SPs. This theory is strengthened by the established link between formation of MRPs and their influence on the binding capacity since biochemical analysis showed a rapid increase in MRPs formation between 90 and 120 minutes confirmed by UV absorbance at 420 nm, formation of CML as well as increased browning index. Interestingly the level of fluorescent AGEs among the others pentosidine tended to decrease in G-SPs heated

longer than 45 minutes which suggest that rather non-fluorescent AGEs are responsible for binding to sRAGE.

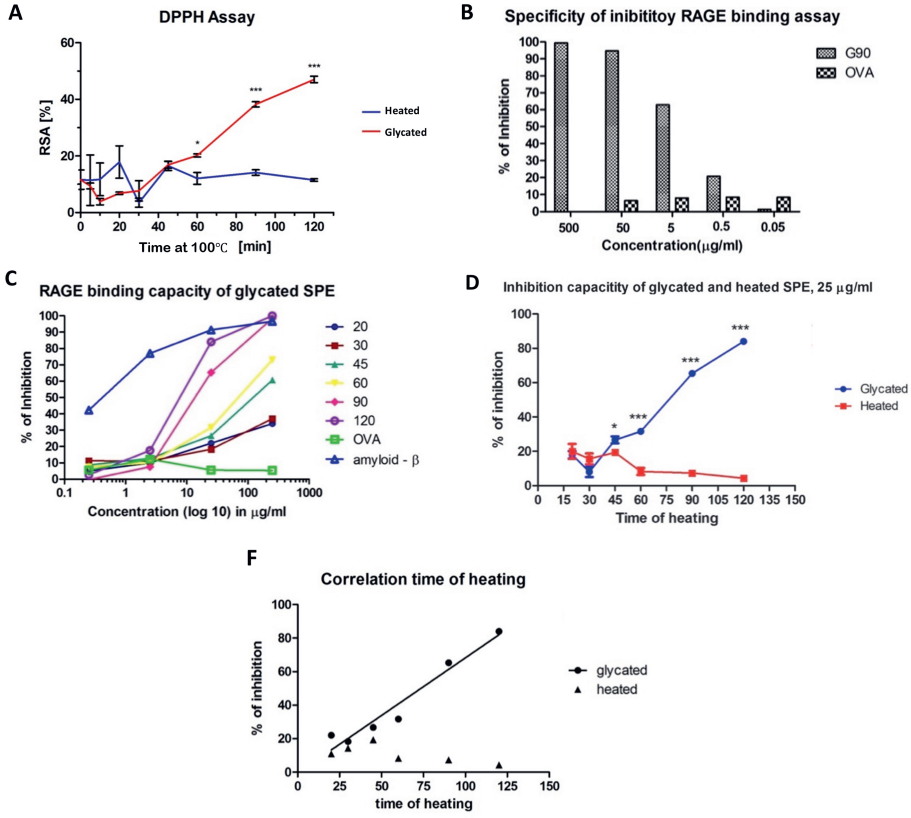

Figure 3. Results of the Functional Properties of heated and glycated soy proteins assays: (A) Radical Scavenging Activity (RSA) of the samples, (B) Specificity of the ELISA based inhibitory sRAGE binding assay, an extendedly glycated SPE (20 µg/ml) was used as coating protein; (C) sRAGE binding capacity of G-SPs; (D) Inhibition capacity for G-SPs at a concentration of 25 µg/ml; (E) Comparison between the inhibitory capacity of G-SPs and H-SPs at a concentration of 25 µg/ml, tested with a two-tailed unpaired t-test with a $p<0.05$ (n=3); (F) Inhibition capacity of G-SP compared to the H-SPs at a concentration of 25 µg/ml, G-SPs were separated into three groups G20 + G30, G45 + G60 and G90 + G120, significant differences were determined using a two-tailed unpaired t-test with $P < 0.05$; (G) Correlation between time of heating and capability to inhibit sRAGE at a concentration of 1 µg/m. OVA as the negative control and

amyloid–β as the positive control, anti-RAGE and detection antibody dilutions were 1:1000, incubation with TMB was 12 minutes and sRAGE concentration at 1μg/ml.

Recently, it has been hypothesized that the MR creates novel IgE epitopes in food allergens [22,64,65]; however, at the moment there is no solid evidence of the presence of IgE antibodies specific to glycated food allergens [64,65]. Nonetheless, it has been reported that IgE reactivity of food allergens could be enhanced or reduced by the MR via induction of conformational change and protein aggregation [64-67]. Moreover, some glycated proteins show an increase in immunoreactivity when compared to non-heated raw samples *in vitro* studies using e.g., macrophages and dendritic cells [22]. Mueller et al reported that RAGE binding to peanut allergens modified by AGEs and showed no interaction with RAGE and unmodified peanut allergens [48]. At present, although it has been suggested that certain structural and functional changes of MRPs may have an impact on their potential allergenic activity, MRPs have been the subject of infrequent research when compared to other non-digestible dietary elements [12]. Moreover, the effects of the MR on allergenicity have not yet been clearly elucidated since it seems they not only depend on the type of protein but also on the conditions of the MR itself such as temperature and time of treatment [67]. Moreover, information of the effects of digestion on glycated proteins *in vivo*; therefore, no definitive conclusions of the effects of dietary AGEs in allergenicity *in vivo* can be drawn at [23].

3.3.3 PBMCs stimulation and cytokine measurement

RAGE ligands, such as the high motility group box 1 protein (HMGB-1) have been reported to play an important role in the development of allergic

sensitization [68]. Furthermore, RAGE ligands, e.g. HMGB-1, chemical blocker of RAGE (FPS-ZM1), S-100 proteins, amyloid-β, among others bind to the extra-cellular domain, initiating intra-cellular signalling which leads to ROS generation and the activation of the NF-κB [58,60]. The activation of the transcription factor NF-κB induces gene expression of pro-inflammatory cytokines [48,58,62]. The results described above confirm that soy- derived AGEs can bind to the sRAGE receptor. Previously it has been reported that this binding might have an effect on the cellular level; thus, the capacity to stimulate PBMCs and to produce cytokines (IL-B, IL-8, TNF- α and IL-10) of the H-SP and the G-SP heated for 90 minutes (G90) was analyzed (*Figure 4*).

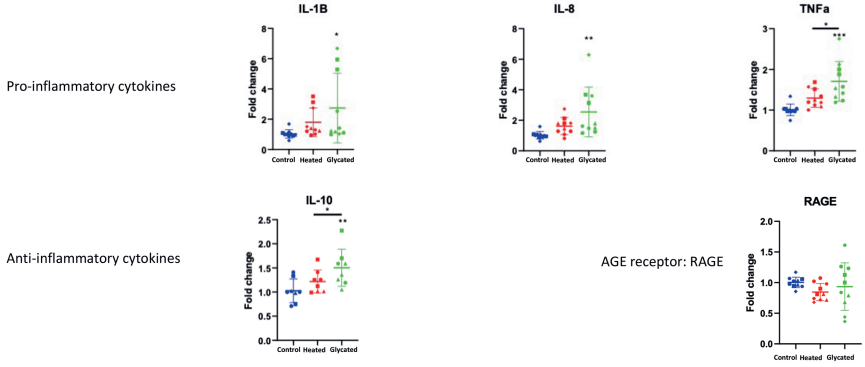

Figure 4. *Cytokine secretion* by peripheral blood mononuclear cells (PMBCs) derived from blood of 5 healthy donors after stimulation with heated soy and glycated soy for 90 minutes.

Comparison of cytokine expression induced among the different donors in response to H-SP and G90 showed consistently higher transcription levels of all investigated cytokines for the G90 samples when compared to the H-SP, although variation between the blood donors was observed (as shown in Figure 4). The expression of RAGE did not change significantly although for

some donors a tendency to an increased expression of RAGE was observed (as shown in *Figure 4*).

Previously, different studies have reported that AGE formation affects macrophage function by promoting the release of various growth factors and cytokines [63,69]; thus, concurring with the present results. Moreover, the difference in cytokine transcription observed between the H-SP and G90 may suggest that glycation could induce an increase in pro-inflammatory mediators hence possibly increasing the inflammatory state in the donor. Additionally, the increased expression of RAGE in glycated samples (as show in the *Figure 3B*, *3D* and *3E* and in *Figure 4* for several donors) may amplify this inflammatory process. Smith et al described recently that RAGE can activate nuclear factor kappa-light-chain-enhancer of activated cells (NF-κB), inducing intracellular pro-inflammatory processes which lead to cytokine response [70]. These cytokines can influence the innate and adaptative immune system which includes activation of NADPH oxidase causing reactive oxidative and nitrogen intermediates [70-72]. Furthermore, it has been previously reported that the RAGE pathway is capable of promoting pro-inflammatory cytokine production (IL-1β, IL-6 and TNF-α) [73]. This findings concur with the results from the present study where incubation of G90 with PBMCs isolated from healthy donors resulted in increased expression of pro-inflammatory cytokines (IL-1ß, IL-8 and TNF-α). Moreover, it has been suggested that the RAGE/ NF-κB pathway induces macrophages to secrete inflammatory cytokines [74]. The results observed in the present study suggest that RAGE binding might result in activating different intra-cellular signalling transduction cascades resulting in the observed cytokine profile. However, further research is needed focusing which focuses on the

characterization of the structural changes that are responsible for the increase binding capacity of RAGE in the on glycated and heated SPEs.

4 Conclusions

In this study we evaluated eight different time/temperature combinations of the MR on the structural and functional properties of soy proteins. We demonstrated time dependent differences in the biochemical characteristics of glycated soy when compared to heated soy, which can be attributed to the different stages of MR and the diversity in the obtained MRPs. The present study showed that for soy proteins, the MR starts immediately and that intermediate MRPs are already formed at 5 minutes of heating and continue to be formed in the linear manner until 120 minutes of heating (as shown in *Figure 3B* and *3C*). Moreover, AGE formation (CML levels) may be more important regarding sRAGE mediated immunogenicity than aggregate formation (observed on SDS-PAGE) since time-dependent increase of AGEs formation was positively correlated with sRAGE binding (G-SPs heated longer than 30 minutes), while aggregate formation was present in all G-SP samples. Furthermore, our results show that highly modified soy proteins are capable of better interaction with sRAGE since the binding capacity of the G-SP was increasing with the prolonged heating time in the presence of glucose. Additionally, H-SP were also capable of interacting with the receptor however on the very low level. Incubation of G-SP at 90 minutes (G90) with PBMCs isolated from healthy donors resulted in increased expression of pro-inflammatory cytokines (IL-1ß, IL-8 and TNF- α) but also anti-inflammatory cytokines (IL-10), suggesting that formed AGEs interact with immune cells activating possibly via various AGE receptors, including RAGE.

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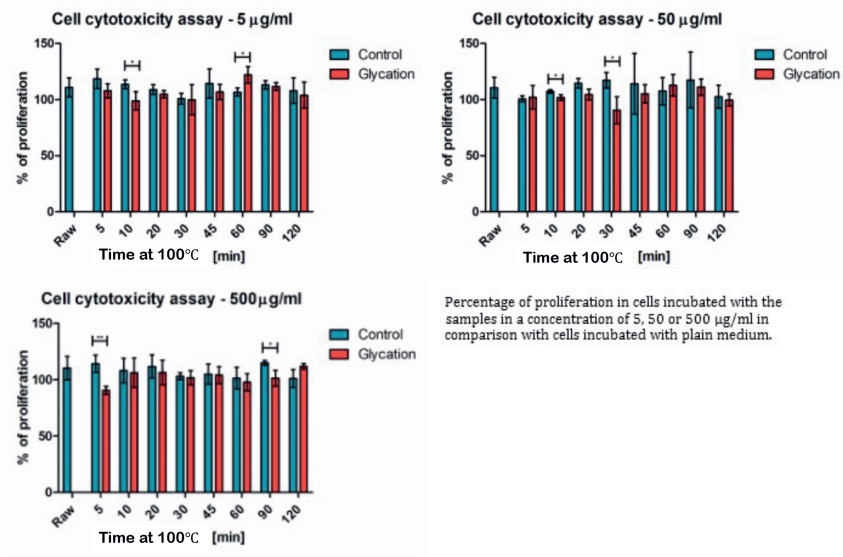
Supplementary Assay: Cell Cytotoxicity Assay Protocol and Results

1.1. Protocol

The Caco-2 cells were seeded in 96-wells plates at a density of $5 \cdot 10^4$ cells/ml and incubated to obtain a full confluency, with the medium changed every second day. The cell cytotoxicity assay was performed first to eliminate a direct cytotoxic effect of SPE on Caco-2 cells. To measure the cytotoxicity of the samples the CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (Promega, WI, US) was used. The cells were incubated with proteins in 3 different concentrations: 500, 50 and 5 µg/ml. Medium and medium with glucose were used as negative controls, while SDS in medium was used as a positive control. At the start of the test, the old medium was removed, and the cells were incubated in triplo with 100 µl of either protein, one of the negative controls or the positive control during 24 hours at 37°C. The next day the protocol of the commercial assay was followed, and the absorbance was measured at 490 nm.

1.2. Results

No cytotoxicity of SPEs was observed after 24 hours of incubation of Caco-2 cells with SPEs with any of the tested concentrations 5, 50 and 500 µg/ml (shown in supplemental *Figure 1*). For all three concentrations, the amount of proliferation in the control and G-SP was stable, with the control samples showing a higher mean and median when compared to the G-SP. However, the mean and median of the G-SP was always above 100%.



Percentage of proliferation in cells incubated with the samples in a concentration of 5, 50 or 500 µg/ml in comparison with cells incubated with plain medium.

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Chapter 7

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 minutes (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 [1]. Moreover, soy is an inexpensive and an excellent source of quality proteins which contains all the essential amino acids [2]. 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 [1]. Nonetheless, soy has been classified as one of the eight most common food allergens [3,4]. At least 28 allergenic proteins in soy have been suggested to bind to IgE [5,6], of which 8 have been recognized by the WHO/IUIS Allergen Nomenclature Subcommittee [7]. 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) [6,8]. 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 [9,10]. 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 [5]. 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 [11-15]. One of the reactions which occurs commonly during thermal processing of food is the Maillard Reaction (MR, glycation), a non-enzymatic reaction

between reducing sugars and a free amino acid group of proteins or peptides [12,14]. 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 [16,17]; as well as specific IgE and IgG binding [18,19]. 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 [20]. For instance, the MR can reduce the IgG/IgE binding capacity for the major cherry allergen, Pru av 1 [20], silver carp [21], buckwheat allergen Fag t 3 [17], soybean allergen ß-conglycinin [23], squid allergen [24], milk allergen [25,26], hazelnut allergen, Cor a 11 [12] and shrimp tropomyosin [27]. On the other hand, MR increased the immunoreactivity of peanut allergens, Ara h 2 [28,29] plus Ara h 1 [30,31] and scallop tropomyosin [32]. 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 [14,19]. Moreover, the methods used to assess the changes in allergenicity varies per study. Several studies use IgE binding methods like ELISA or Western Blot [20-22,24,26-31]; 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 [12,15,30,33]. Lastly, scarcely studies address the sensitization profile of glycated proteins in *in vitro* or *in vivo* studies [34,35].

Even though the analysis of IgE binding to epitopes is a good indicator of IgE-allergen complex formation and the activation of effector cells [36,37], an increase in IgE binding capacity does not equal an increase in allergenicity

or vice versa [36]. 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) [38]. The BAT is that assay is capable of distinguishing between sensitization but tolerant patients and those patients that are clinically allergic [39,40]; 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 [41].

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 ≥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), H10, 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 minutes: short term heated (SH SPE)
2. Raw SPE heated at 121°C with glucose for 10 minutes: short term heated with glucose (SH SPE + Glu)
3. Raw SPE heated at 121°C without glucose for 30 minutes: long term heated (LH SPE)

4. Raw SPE heated at 121°C with glucose for 30 minutes: 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 [44].

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 2h. The free binding sites were blocked with 2% BSA blocking buffer for 1.5 hours. The patients' serum was pre-incubated for 30 minutes 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 minutes. The percentage of inhibition was calculated according to the formula:

$$\% \text{ of Inhibition} = (IgE0\% - IgEX\%)/IgE0\% \times 100$$

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 [44]. 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 hours 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:

$$\% \text{ of inhibition} = (IgE0\% - IgEx\%) / IgE0\% \times 100$$

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 *Figure 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 15V for 35 minutes. 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 1h 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 minutes 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 minutes; the blot was placed in the cassette and the film was exposed for 60 minutes. 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) [43]. 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% [43]. 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 [38,40], but since it uses several measuring points at multiple allergen concentrations, it lowers the risk of false outcomes [40].

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 minutes, heated without glucose for 10 and 30 minutes, 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.

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/F	19.5	<0.35	9.75	19.7
2	23/F	9.59	<0.35	3.58	12.3
3	32/M	2.69	<0.35	2.75	1.78
4	34/F	3.35	<0.35	2.39	2.88
5	25/F	19.8	2.51	4.86	25.5
6	26/F	2.66	4.84	1.67	2.74

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 *Figure 1A*). Five bands (160, 130, 98, 25 and 16 kDa) were 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 *Table 2*). 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 *Figure 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 >250kDa; 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.

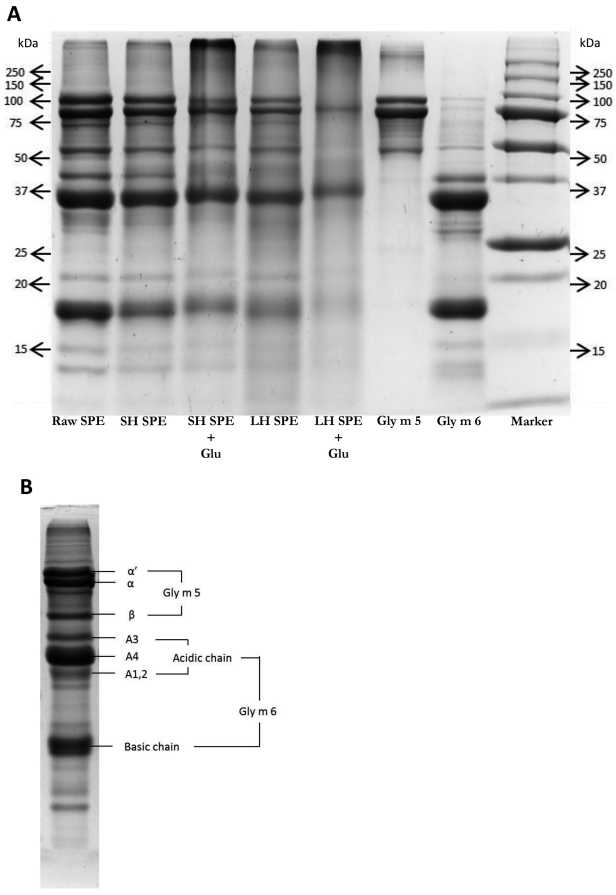


Figure 1. A: Changes in SDS-PAGE pattern of the soy proteins upon progressive Maillard reaction and heat treatment; B: separation of the denatured soy proteins by molecular weight (in kDa). Nomenclature of the soy specific bands according to WHO/IUIS Allergen Nomenclature Sub-committee.

Table 2. Overview of the protein fractions present on the SDS-PAGE

Molecular Weight (kDa)	Raw SPE	SH SPE	SH SPE + Glu	LH SPE	LH SPE + Glu	Gly m 5	Gly m 6
200	200						
160	160	160					
150							
130	130	130					
100							
98	98	98					
90						90	90
82	82	82	82	82		82	82
75						75	
73	73	73	73	73	73	73	73
65	65	65	65	65		65	
55	55	55		55			
50	50	50	50	50		50	50
45							45
40	40	40	40	40		40	40
37							
34	34	34	34	34	34	34	34
33	33	33	33	33	33		33
29	29	29	29	29		29	29
27	27	27	27	27		27	27
25	25	25				25	
23	23	23		23			23
20	20	20	20	20			
18	18	18	18	18	18		18
16	16	16					
15							15
14	14	14	14				
13	13	13	13	13	13		13
12							12

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 *Figure 2*) with extract were comparable with ImmunoCAP

results (as shown in *Figure 3*) with purified proteins (Gly m 5 and Gly m 5 in the present study).

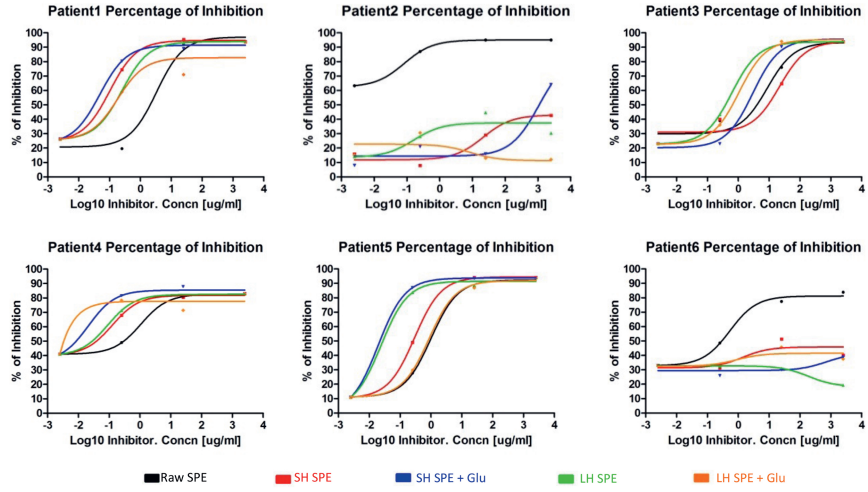


Figure 2. Competitive ELISA results with ^{10}log inhibitor concentration (in µg/ml) and the observed % of Inhibition without competitor (100%) for the different sample preparations as indicated by the colors.

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 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 *Figure 4*). 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 *Figure 3*)

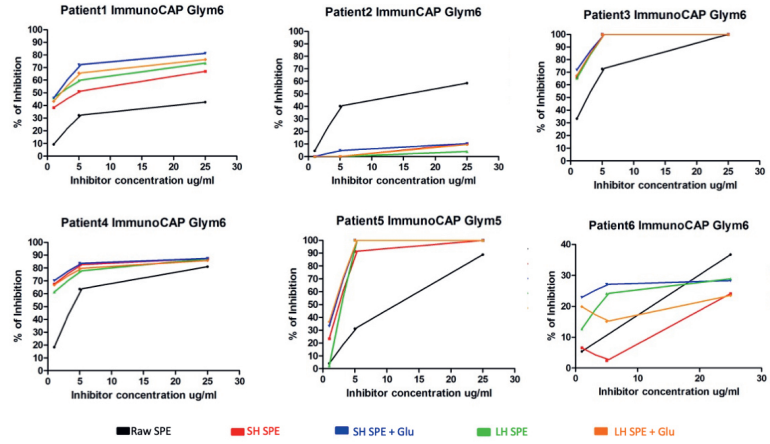


Figure 3. ImmunoCAP Inhibition Results % of Inhibition Against Gly m 6. For both major allergens (Gly m 5 and Gly m 6) the outcomes per patient are in-line; thus, only the results for Gly m 6 are presented.

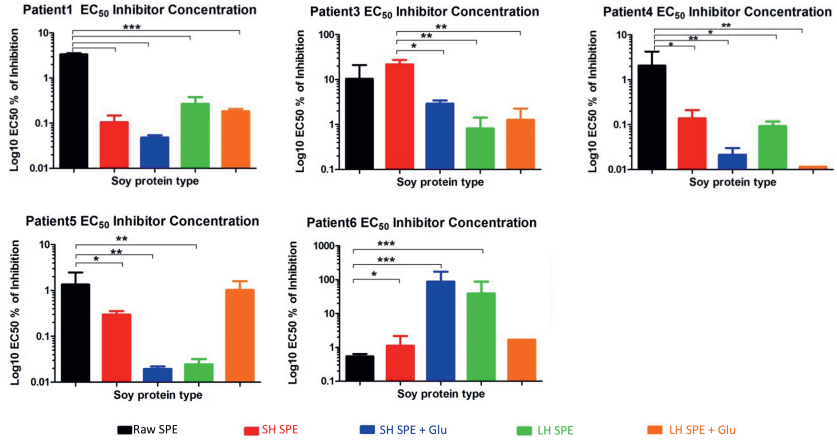
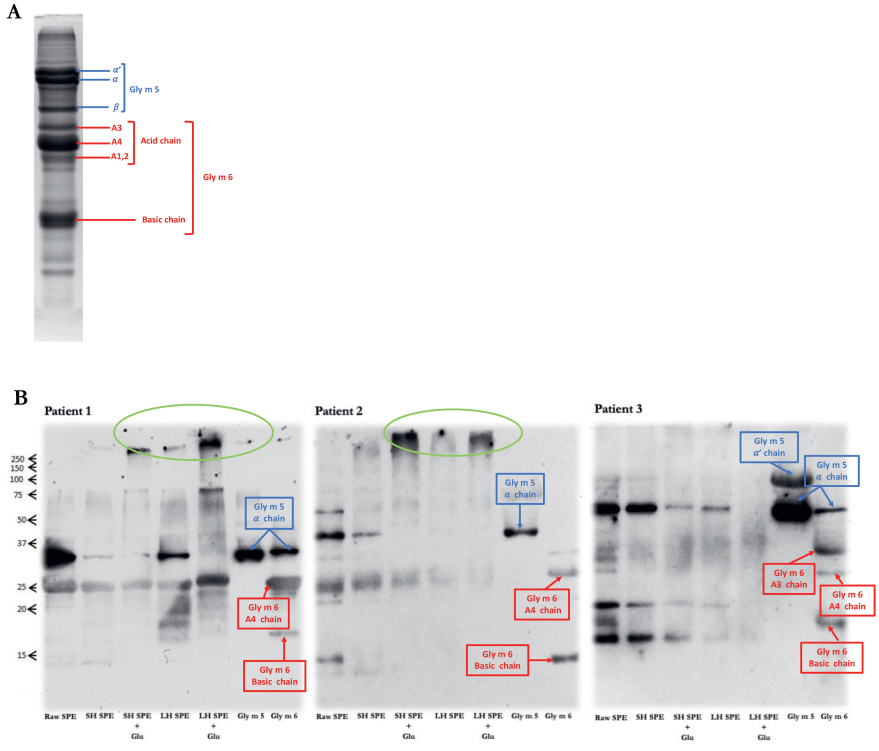


Figure 4. ELISA EC_{50} of Inhibition Concentration for the different sample preparations as indicated by the colors. The results for patient #2 are not represented because no inhibition was observed with the treated SPEs (EC50 could not be calculated)

3.3. Qualitative assay: Western Blot (WB)

For most patients, the WB analysis showed IgE binding to glycated proteins (as shown in *Figure 5B*). 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 *Figure 5A* and *Figure 5B*). 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.



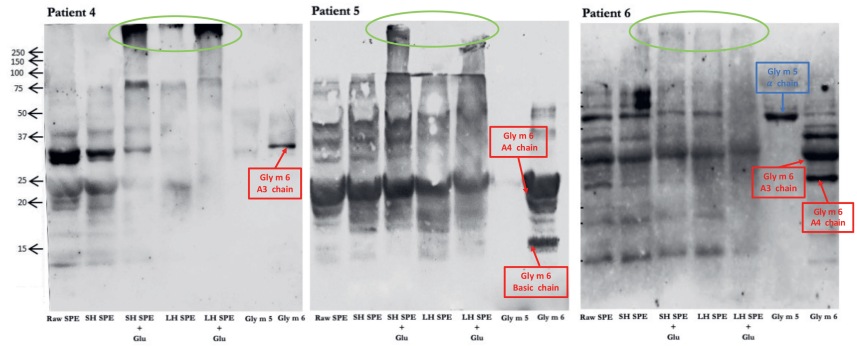


Figure 5. A: Separation of the denatured soy proteins by molecular weight; B: Individual Western Blot results for the different sample preparations per patient. Green circle = Large Aggregates containing advanced glycation end products (AGEs), Blue color = Gly m 5, Red color = Gly m 6.

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 (*Figure 6*). 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 *Figure 6*).

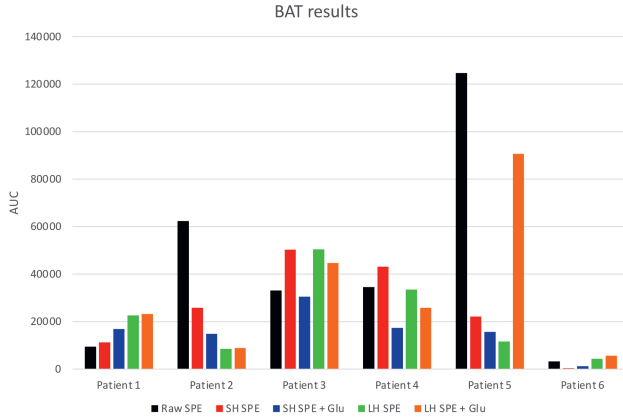


Figure 6. Results for the ratio of the area under curve (AUC) for the basophil activation test (BAT) from the Gly m 5/Gly m 6. Panel A shows different sample binding profile per patient where the colors indicate the different sample preparations tested. Panel B shows the difference in binding by comparing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

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 3*). 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 *Table 4*). 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.

Table 3. 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	↓	↓	↓	↓	↓	↑	↓	↑

Table 4. Comparison between results of the IgE binding tests and the BAT assay.

	# of Patients	Patients	Percentage of Patients (%)
Increase of allergenicity for all treatments for all two types of assays	1	1	17%
Decrease of allergenicity for all treatments for all two types of assays	1	2	17%
Same results for SH SPE for all two types of assays	5	1,2,3,4,6	83%
Same results for SH SPE + Glu for all two types of assays	3	1,2,6	50%
Same results for LH SPE for all two types of assays	4	1,2,3,4	67%
Same results for LH SPE + Glu for all two types of assays	3	1,2,3	50%

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 [12,30,33]. 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 [44]. 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 [12,19,33,40]. 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* [38]. 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 [40].

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 [14,19,26,45]. 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 [37]. 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 [24,37,48]. 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 [48]. 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 [19,36]. 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 [25], 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 minutes 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 minutes 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 [30]. Therefore, it is likely that aggregated structures are more effective in enhancing the degranulation capacity [30,46]. Similar findings were reported by Lehmann et al regarding Ara h 2 and Ara h 6, where the functional assay (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 [49]. 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 [12]. 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 [50]. 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 [51]. This discrepancy between IgE binding and effector cell activation has previously been reported for other allergens [12,30,49,52].

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 [53]. 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 [33]. 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.

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Chapter 8

General Discussion

General Discussion

The aim of this thesis was to evaluate the food-specific immunoglobulin E levels and the contribution of the basophil activation test (BAT) in the diagnostic accuracy of clinical soy allergy. Moreover, the sensitization patterns to different soy allergens in accordance with their cross-reactive homologous allergens was taken into consideration when evaluating soy allergy diagnostic markers. Finally, since soy is rarely consumed raw, the effects of food processing techniques, exemplified by the Maillard Reaction (MR), in soy allergenicity is evaluated as well.

The discussion will focus on the possible clinical implications of the reported findings as well as the immunological implications of our results in clinical soy allergy diagnosis. Furthermore, the interpretation and implications of the diagnostic studies and current drawbacks of clinical soy allergy diagnosis will be discussed. Additionally, recommendations for improvement in future clinical soy allergy research, which could be applied generally to other food allergens, will be considered. The following points will be further explored in this section:

1. Factors that can influence the diagnostic accuracy of Clinical Soy Allergy
2. The Role of Cross-Reactivity in Clinical Soy Allergy Diagnosis
3. The Role of the Basophil Activation Test in Clinical Soy Allergy Diagnosis
4. Recommendations for the Improvement in Clinical Soy Allergy Diagnosis

5. Implications of Processing Techniques on Immunogenicity and Allergenicity of Soy Proteins
6. Conclusions

1. Factors that can influence the diagnostic accuracy of Clinical Soy Allergy

When a case of food allergy is suspected, identification of the possible culprit allergens as well as the sensitization pattern is needed, usually done with a serologic test for specific IgE (sIgE) [1]. Presently, sIgE levels evaluate the relationship between allergic disease and sensitization from a dichotomous perspective, either positive or negative [2]. Thus, the presence of an allergen-specific sIgE antibody in the blood defines allergic sensitization, not the presence of a clinically relevant allergy, as stated in the review presented in Chapter 2 [3]. Therefore, a positive sIgE level is not enough to make a food allergy diagnosis without a direct link to the patient's clinical history of allergic symptoms [1,3]. During the review presented in Chapter 2 as well as the diagnostic studies conducted for the present thesis it was noted that certain factors can impact the diagnostic accuracy of sIgE. Thus, when interpreting the diagnostic accuracy of sIgE to soy components, it is important to understand and consider factors such as the study population selection, allergens used, and the specific IgE patterns of the selected patient population which can influence the diagnostic marker.

1.1 Selection of Study Population

The study population in the present diagnostic studies consisted of randomly selected adult subjects previously diagnosed with soy allergy based

on a positive SPT and/or sIgE level with a clear clinical history of soy allergy who visited the Allergic Clinic in Rijnstate Hospital (Arnhem, The Netherlands). Once patients agreed to participate in the studies, a blood sample was donated and a detailed questionnaire was completed to obtain more information regarding their allergic clinical history and dietary information.

Presently, soy allergy prevalence in Europe when diagnosed with an oral food challenge (OFC) is reported to be less than 1%, when sensitization is assessed by sIgE it climbs to <2.9% [4]. Determining the sample size in a clinical trial is a very common issue; generally, it is mathematically calculated based on the sample size necessary to identify a statistically significant outcome with P set for statistical significance [5]. However, due to the low prevalence of soy allergy in the general population, recruiting soy allergic patients normally requires a long recruitment period and even then, the result is usually a small study size [6]. Nevertheless, a small sample size is usually a challenge to the current analytic approach and design standards in a clinical research setting [7]. Therefore, researchers must be prepared to appropriately argue for the strength of their research even when a small sample size is presented. It is important to note that in certain settings, such as rare diseases or other small populations, it may not be a feasible approach to obtain a large sample size [5,8]. Hence, in settings such as a low prevalence food allergy like soy, a small sample size reflects the size of the selected population [5,7]. Moreover, it has been suggested that research in small samples commonly represent health concerns in underrepresented populations [7,8]. To increase the recruitment patient pool, including multiple clinical centers is strongly recommended, this setting will hopefully

lower the required recruitment period as well. Importantly, as well, since soy allergy rarely presents as an exclusive food allergy is that when recruiting patients, patients are selected based on their soy allergy diagnosis and avoid, for example, selecting a sub-group of soy allergic patients from an already established study on peanut allergy.

An additional barrier that occurs during the recruitment of soy allergic patients is that the current reference standard in diagnosing FA is a double-blind, placebo-controlled food challenge (DBPCFC) [9]. Generally, performing a DBPCFC is not only-time consuming and must be observed by a trained nurse for a full challenge, but it also carries inherent risks including acute allergic reactions which can be potentially life-threatening and can cause emotional distress, as discussed in Chapter 2 [10,11]. In Chapter 3, performing an DBPCFC or OFC was excluded as part of the diagnostic panel due to a high prevalence of anaphylaxis (64%). An OFC was also not possible in the case presented in Chapter 4 since it has been reported that conditions such as uncontrolled eczema and severe allergic rhinitis are further reasons to defer an OFC [11]. Moreover, it can be argued that by not including OFC as part of the diagnostic panel, the recruitment process was less limited because patients with a history of anaphylactic shock would probably be reluctant to participate in a study were an OFC was mandatory. An OFC can be omitted if there is a high probability that the patient will react to the selected food as predicted by the food reaction history such as a history of a severe reaction, including to trace amounts of the food, or when the food is implicated in fatal or near-fatal food-induced anaphylaxis [11].

Nonetheless, at present due to scientific rigor, the majority of food allergy research studies include either DBPCFC or OFC in their protocols [11-

15]. In food allergy research, an OFCs is performed for mainly 3 purposes: (i) to establish the diagnosis, (ii) establishing a baseline threshold for eliciting symptoms and/or (iii) evaluating oral immunotherapy [16]. However, at present there is no established methodology for systemically assessing food allergy in research settings and OFC are not standardized to allow valid comparisons between studies [17]. Moreover, it has yet to be unequivocally established that an OFC is necessary for IgE-mediated food allergy when the patient has an clear and convincing clinical reactivity to a known food allergen plus associated positive sIgE results (SPT and/or sIgE values) [15-18].

Therefore, by establishing a clear definition of a convincing history of IgE-mediated food allergy and a classification of specific sIgE values, it can be possible to deferred from using an OFC to diagnose a food allergy in research due to the high probability of a reaction as indicated in *Table 1* [12,13].

Table 1. Schema for considering deferring using and oral food challenge (OFC) to diagnose a food allergy strictly limited to research setting, not applicable for clinical use, allowing for the sIgE result to be interpreted in the clinical context as defined by the clinical history. Based on Stiefel et al (2012) [12]

		Likelihood of allergy from specific IgE (kU/L)		
		Low	**Intermediate**	**High**
Likelihood of allergy from clinical history	**High** Convincing history (e.g., urticaria and wheezing after 2 exposures)	Probable allergy, OFC is necessary	Very probable allergy, possible to avoid an OFC	Allergy, possible to avoid an OFC
	Intermediate Suggestive history (e.g., urticaria after 1 exposure)	Possible allergy, OFC is necessary	Possible allergy, OFC is necessary	Allergy, it is possible to avoid an OFC
	Low Little suggestive history (e.g., non-IgE symptoms)	Low probability of allergy, evaluate differential diagnosis	Possible allergy, re-evaluate clinical history, OFC may be necessary	Possible allergy, re-evaluate clinical history, OFC may be necessary

Stiefel et al described a tool that combines the clinical history and sIgE levels to categorize patients, where patients with a high likelihood of allergy can be diagnosed in the absence of an OFC [12]. Thus, it has been proposed that specific tests, in this case sIgE, may be linked with a specific probability of allergy; however, this probability is strongly influenced by the clinical context [12-14]. However, at the moment, cut-offs that predict 50% and 95% likelihood of a clinical reaction based in food sIgE values have only been calculated for egg, cow's milk and peanut based on several studies of referral populations [9,18]. Therefore, at the moment no cut-off levels have been clearly established for soy; thus, before this proposed methodology for OFC deferment can be implemental, these sIgE values need to be established based on studies with sufficiently large adult soy allergic populations. Nonetheless, once these threshold values have been established, integrating the clinical history and sIgE results may prove a useful tool useful to defer from implementing an OFC for food allergy diagnosis in a research setting, as proposed in *Table 1* [12,19]. It is important to note that the linking of specific tests to the probability of an allergy considering the clinical history may be applicable if restricted to a research setting and should not be applicable in a purely clinical setting in food allergy diagnosis. Moreover, the diagnostic studies presented in the present thesis show that it is possible to design an adequate clinical research protocol without necessarily performing an OFC or a DBPCPC to diagnose a food allergy [20]. Hence, the information provided in *Table 1* could prove useful in future clinical research protocols that want to evaluate food allergies but due to a variety of reasons are not able to perform either an OFC or a DBPCFC as part of their diagnostic protocol.

1.2 Allergens

Allergens are mainly proteins in biological materials such as plant pollen, animal's epithelia and food. Allergen extracts are used to determine sIgE levels which presently, along with the clinical history, is an important part of the screenings performed to diagnose an allergy [21]. However, the use of allergen extracts is not without difficulties [21,22]. Mainly, the lack of standardization of the allergens used as substrates since these allergens can differ in terms of their allergenic content due to the natural variability of the allergen source [21-23]. A previous study reported that commercial extracts prepared from natural sources showed a more than 10-fold variation regarding the total protein content and the amount of the major birch pollen allergen Bet v 1; thus, deliver variable *in vivo test* results [24]. Due to the advantages of recombinant allergen-based preparations, this variability might be overcome by using them instead of natural allergens, as seen in *Table 2* [21,22]. Purified allergen proteins from plants consisting of different allergens present in multiple closely related isoforms, also known as isoallergens such as Bet v 1, which contains more than 30 different isoforms of which some of these involved in allergic sensitization [25]. Moreover, isoforms are highly similar in their primary amino acid sequences but show differences in IgE binding and activation capacity of allergen-specific T-cells [26]. However, by definition, recombinantly expressed allergens contain only one isoform (as shown in *Figure 1*).

Table 2. Natural vs Recombinant Allergens Extracts. *Potency of allergens might be influenced by post-translation modifications and glycosylation. Adapted from Valenta and Niederberger [21]

Natural Allergen Extracts	Recombinant Allergens
May contain low amounts or lack very important allergens	Amounts can be controlled on the basis of mass units
Contain allergens with varying potencies* and ratios	Potencies and ratios can be adjusted for each molecule
May be contaminated with allergens from other sources	Represent pure molecules
May induce new sensitizations	Hypoallergenic forms of recombinant allergens which do not induce degranulation of IgE+ mast cells or basophils but maintain their capacity to elicit IgGs and stimulate T-lymphocytes can be used for desensitization purposes ('vaccines') and be tailored to the patient's sensitization profile
Comparison between different products and batches is not possible	Consistent and reproducible products and batches are possible
Precise monitoring of immunotherapy is not possible	Precise monitoring of immunotherapy is possible and it can be modified to suit different treatment strategies

Advanced proteomic tools, including mass spectrometry, permit the simultaneous identification and quantification of different allergens and their isoforms in complex allergen preparations [27]. The relative abundance of different isoforms in different allergen preparations could hinder the diagnosis or treatment of the specific allergy. Recombinant isoforms exhibit a similar antigenicity as a native allergen, including cross-reactivity for IgE antibodies. An allergic patient displays an individual response with respect to allergen-specific B and T cells, showing a unique reactivity toward individual allergens, isoallergens, and variants. Typically, allergens are defined by their capacity to bind IgE antibodies and induce immediate hypersensitivity reactions, despite the fact that T cell reactivity was shown to be crucial in the

regulation and maintenance of an allergic disease [28]. This T cell reactivity needs more intense study in order to define a protein as a potential allergen.

Currently, the allergen extracts used for SPT and sIgE analysis have many components from which the majority are not significant for provoking an allergic reaction; thus, many allergens have been purified and are available as recombinant allergens extracts for sIgE antibody assays (as shown in *Figure 1*) [29]. In the present thesis allergen extracts are defined as heterogenous mixtures of allergenic and non-allergenic components. Presently, recombinant allergens can be produced as molecules that mimic the properties of the natural allergen, as modified variants with either reduced or increased allergenic the relevant epitopes of complex allergen source [21].

In Chapter 3, the use of a recombinant Gly m 8 (rGly m 8) in the indirect BAT (iBAT) vs native (nGly m 8) in the ImmunoCAP generated contention because the biological activity of a protein depends on the specific assay conditions and thus, theoretically a guarantee on the use of a recombinant protein product in specific applications cannot be provided. Moreover, it was suggested that in the case of rGly m 8 it was possible that the protein did not fold properly and hence there was a discrepancy between positive sIgE values to nGly m 8 (ImmunoCAP) and the capacity of rGly m 8 (BAT) to induce basophil degranulation (see *Table 2*).

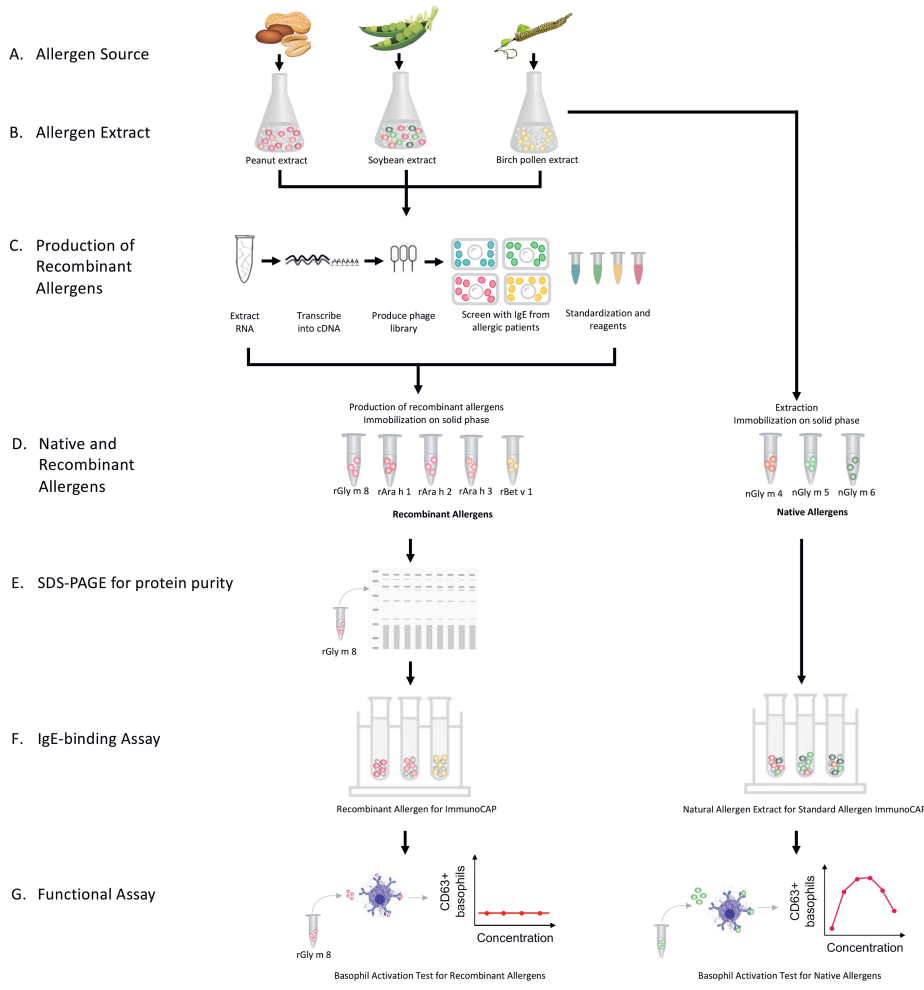

Figure 1. Steps required for recombinant allergen production; both recombinant allergens and natural allergens can be used in IgE-binding based assays.

In Chapter 3 a Dot Blot was performed, which even though does not guarantee proper protein conformation, it did show that there were IgE binding epitopes to rGly m 8 in the patient's sera. Moreover, Ueberham et al previously reported that immunization of mice with rGly m 8 protein led to the recovery of antibodies that bound with high affinity to both native and

recombinant Gly m 8 [30]. Nonetheless, further research regarding the immunoreactivity of recombinant allergens is necessary to guarantee that these allergens work in different applications, until then doubts regarding the immunoreactivity of recombinant allergens will remain. Moreover, as done in Chapter 3 to demonstrate the immunoreactivity of the selected recombinant allergen an SDS-PAGE can show the purity of the allergen while a Western Blot can establish the immunoreactivity by detecting IgE-binding. Nonetheless, it is possible that the different isoforms of Gly m 8 analyzed in Chapter 3 may have had an impact on the IgE measurements and thus, the difference in observed results compared to previous studies [11,15]. This discrepancy in study results due to isoform level difference has been reported previously as a factor that could hinder food allergy diagnosis [27].

Presently, the conventional (direct) BAT has been proven useful in the diagnosis of food allergies since basophils surface markers (CD63 or CD203c) are upregulated following cross-linking of surface IgE [33]. Additionally, the CD63-based BAT has been validated for recombinant allergens in the detection of food allergies [33,34]. However, one of the major pitfalls of the direct BAT is that the analysis needs to be performed rapidly after blood collection (within 24 hours) [35]. A way to circumvent this issue is to use donor basophils sensitized with the patient's IgE, known as the passive BAT [35]. Notably, the study presented in Chapter 3 is the first time that the iBAT has been successfully used for rGly m 8, however, validation of the iBAT for rGly m 8 is still pending.

In addition to the variability issue, some natural allergen extracts contain very low amounts or even lack important allergens, an issue that can be resolved by using a mixture of recombinant allergens (as shown in *Table 2*)

[21]. In Chapter 4, we encountered this issue when attempting to diagnose the clinical reactivity of Gly m 4 in a soy allergic patient with birch pollinosis. The currently available soy allergen extracts generally contain very low amounts of Gly m 4 (0.01–0.1%; or 0.1-1 mg per g of total protein); thus, tests for allergen-specific IgE tend to be not sensitive enough in cases of suspected Gly m 4-induced soy allergy [47-48]. Moreover, to our knowledge, the BAT with rGly m 4 (Indoor Biotechnologies, USA) has only been reported in two studies, both in patients with Gly m 4-exclusive soy allergy [38,39]. The patient reported in Chapter 4 did not qualify as a Gly m 4-exclusive soy allergy because positive sIgE and BAT values for both Gly m 5 and Gly m 6. Even though the patient presented a higher Gly m 4 sIgE seasonal variability during the birch pollen season when compared Gly m 5 and Gly m 6, a BAT for Gly m 4 was not to performed. Mainly because a negative result could either be due to the lack of patient reactivity or the low content of the Gly m 4 in the extract [36]. More importantly, the patient did not consume non-processed soy; thus, the clinical data to confirm the BAT results in this case was not available. Moreover, at present there is a lack of validation of the BAT for Gly m 4 in soy allergic patients. Furthermore, because this patient did not consume soymilk products and the patient's tolerance seasonality hence subsequently symptomology was limited to processed soy products, on which Gly m 4 is normally not detected since it degrades during heating, [38-40], the patient's clinical reactivity to Gly m 4 wasn't of major clinical importance for the case presentation in Chapter 4.

Presently, the final amount of an allergen in an extract will depend on both the raw material and the extraction methodology used [21,38,41]. Moreover, different plant strains may contain different amounts of allergens,

each allergen family consists of a mixture of isoforms, and allergens are susceptible to degradation by enzymatic activity released during the mechanical crushing of the food [41]. Thus, standardization of food extract according to total protein content or single allergen content is frequently not possible [21]. Recombinant allergen preparations have been evaluated and tested; showing generally highly specific results plus they have greatly contributed to the understanding of sensitization profiles and cross-reactivity. The use of single allergenic molecules, such as recombinant allergens, instead of extracts to detect sIgE antibodies is called component-resolved diagnosis (CRD) which gives a higher diagnostic precision. However, at present, the specificity and sensitivity levels are not accurate enough to become a new standard and thus replace the OFC [29]. Although CRD should be a milestone in food allergy diagnosis, the fact that only some of the most relevant allergens are available for commercial diagnostic assays is a major hurdle that needs to be resolved. For soy allergens in particular, studies with recombinant allergens are few and far between [29,42,43]. In Chapter 3 for example, the only soy recombinant allergen used was Gly m 8, whilst all of the allergens used for peanut and birch were recombinant allergens (as shown in *Figure 1*). This demonstrates a great need for a food-by-food approach where the sensitivity and specificity of these molecular diagnostic test are compared to the conventional diagnostic tests such as DBPCFC, particularly in low prevalence food allergies such as clinical soy allergy. Thus, there is a need for more clinical research studies that asses the relevance of molecular diagnosis as well as validate recombinant allergens for each food allergen.

1.3 IgE Sensitization Pattern in Soy Allergy as it regards to Birch and Peanut Allergy

Exposure to different allergen sources depends on climate, socioeconomic factors, nutritional habits and other factors and all have an influence on the allergic sensitization pattern of the individual allergic patient [22]. For the diagnostic studies presented in Chapter 3 and Chapter 7, one clinical center was involved for patient recruitment, the Outpatient Allergic Clinic in Rijnstate Hospital (Arnhem, The Netherlands), in total 30 patients were recruited for the diagnostic studies presented.

In an international multicenter study in Europe (n = 30), the IgE binding pattern to soy proteins was reported as very diverse with storage proteins expressed at high levels, 67% of the studied population had a concomitant peanut allergy and more than two-thirds were sensitized to Bet v 1, a major birch pollen allergen [6]. As shown in *Table 3*, among the study population in Chapter 3 (n = 30), Gly m 4 is a prevalent allergen (87%) and the rate of Bet v 1 co-sensitization is high (87%). The rate of peanut co-sensitization is much lower (27%) (as shown in *Table 3*).

As it regards to birch co-sensitization, in the total study population of Chapter 3, the highest sIgE results were observed for Bet v 1 with a median value of 15.15 kU/l (as shown in *Table 3*).

Table 3. Demographic and sIgE sensitization characteristics of the study population presented in Chapter 2

	Total (n= 30)	sIgE Gly m 4 positive (n = 26)	sIgE Gly m 8 positive (n = 11)	sIgE Gly m 5/ Gly m 6 positive (n = 7)	sIgE Gly m 4/Gly m 6/ Gly m 5/Gly m 8 positive (n = 2)
Male	6 (20%)	5 (19%)	1 (9%)	2 (29%)	0 (0%)
Age (years)	46 (20-69)	50.5 (20-69)	32 (20-68)	26 (21-55)	25.5 (25-26)
ImmunoCAP sIgE values (kU/l)					
IgE Gly m 4	3.81 (0.35 – 37.8)	4.91 (0.63-37.8)	3.72 (0.35-37.8)	0.35 (0.35-4.84)	3.67 (2.51-4.84)
IgE Gly m 5	0.35 (0.35-9.75)	0.35 (0.35-4.86)	0.35 (0.35-9.75)	2.75 (0.35-9.75)	3.27 (1.67-4.86)
IgE Gly m 6	0.35 (0.35-25.5)	0.35 (0.35-25.5)	1.32 (0.35-25.5)	2.88 (0.86-25.5)	14.12 (2.74-25.5)
IgE Gly m 8	0.35 (0.35-5.59)	0.35 (0.35-5.59)	0.6 (0.38-5.59)	0.47 (0.35-5.59)	2.96 (0.38-5.59)
IgE Bet v 1	15.15 (0.35-100)	17.85 (2.84-100)	26.1 (0.35-100)	0.35 (0.35-100)	56.75 (13.5-100)
IgE Ara h 2	0.35 (0.35-100)	0.35 (0.35-100)	5.71 (0.35-100)	92.9 (1.64-100)	94.45 (92.9-100)
Co-sensitization to Soy homologous allergens Bet v 1 and Ara h 2					
Birch pollen	26 (87%)	26 (100%)	6 (55%)	3 (43%)	2 (100%)
Peanut	8 (27%)	4 (15%)	6 (55%)	7 (100%)	2 (100%)

There is a strong association between soy allergy and birch pollen allergy, since soy is a clinically relevant birch pollen-related allergenic food with cross-reaction mediated by Bet v 1 and Gly m 4, as substantiated by the fact that 100% of the patient sensitized to Gly m 4 were also co-sensitized to Bet v 1, as shown in *Table 3* [45]. At present in the Netherlands, there are three pollen monitoring stations: Leiden, Helmond and Drachten, operating since 2018 [44]. The station closest to the area where the recruitment center was located (Arnhem) is Helmond, which historically has relatively high levels of airborne birch pollen, which could explain the high prevalence of birch co-sensitization and the high sIgE Bet v 1 values among the selected population in Chapter 3 [44,44]. In the future, having multiple recruitment centers

distributed along the Netherlands would confirm if the birch co-sensitization prevalence identified in the Dutch adult soy allergic patients corresponds to the patterns identified in selected population of Chapter 3.

As it regards to peanut co-sensitization, in Europe, a relevant proportion of soy allergy patients is also sensitized to peanut although peanut-independent soy allergy is also reported [45]. The study population presented in Chapter 2 showed a rate of peanut co-sensitization was much lower (27%) than in previously reported in studies of soy allergic patients, where Klemans et al reported 60% of concomitant peanut allergy and Kattan et reported 89% of peanut co-sensitization [11,15]. Thus, possibly due to cross-reactivity between the homologous proteins Gly m 8 and Ara h 2, the higher rate of peanut co-sensitization in previous studies, may have overestimated the value of Gly m 8 as a diagnostic marker as stated in Chapter 3. However, no obvious reason for this peanut co-sensitization difference among study populations has been identified, although with Kattan et al there is an age discrepancy [15]. Therefore, when the peanut co-sensitization pattern among the soy allergic populations is not considered, over- or underestimating the diagnostic value of a particular sIgE sensitization may occur as evidenced in Chapter 3.

Savage et al suggested that there are two soy allergy phenotypes: early-onset and late-onset [48], with the second phenotype (late-onset) possibly related to either birch pollen cross-reactivity or peanut allergy [45-50]. In Chapter 3, two sensitization profiles were identified: Peanut-Associated (26%) and Peanut-independent/PR-10-Associated (74%) SA groups (as shown in *Figure 2*). These sensitization patterns in soy allergic patients may be in-line with these two soy-allergy phenotypes; however, establishing this

phenotype pattern is limited due to the retrospective data collection method used [48]. Additionally, Bet v 1 has been reported to induce cross-reactive IgE that reacts with related allergens in peanut (Ara h 8) and soy (Gly m 4) [49]. Mittag et al reported cross-reactivities between rBet v 1, rAra h 8 and rGly m 4 via IgE inhibition studies in the sera of birch pollen allergic patients who also had a history of peanut and soy allergy [50]. Furthermore, one IgE-binding surface area present on all three molecules was identified [50]. Recently, Tedner et al presented a 24-year follow-up on the longitudinal development of peanut sensitization profile among 4089 participants, reporting that the co-existing birch sensitization was more common in the participants that developed a peanut sensitization (33/1565 participants, between 8 to 24 years of age) [51]. This finding suggests that this *de novo* peanut sensitization was due to cross-reactivity caused by an initial birch pollen sensitization rather than the onset of a genuine peanut allergy [51].

As previously stated, soy allergic patients with pollen-food syndrome can be sensitized to other legumes, in particular peanuts by cross-reactivity among the Bet v 1-like protein Ara h 8 (as shown in *Figure 2* and *Figure 3*) [52]. In Chapter 3, a soy-peanut-birch sensitization pattern was observed in four patients (#1, #2, #4 and #5), as shown in *Table 3*; however, Ara h 8 was not part of the analysis in Chapter 3. Due to the described cross-reactivity between aero-allergens (Bet v 1) and food allergens (Gly m 4 and Ara h 8), further research in this sub-group of patients is advisable since this cross-reactivity has been reported to be not associated with severe symptoms and our study population has a high prevalence of severe symptoms such as anaphylactic shock (AS) [51-33]. Moreover, the majority of Bet v 1 related food allergens are linked to mild local allergic reactions in birch pollen allergic

patients, which can be also evaluated in this subgroup of patients [6,33,36]. Furthermore, it has been reported that Gly m 4 sensitization in birch pollen allergic patients is related to severe and generalized symptoms in the absence of Gly m 5 and Gly m 6 sensitization, which does not occur in the evaluated subgroup of patients since they are also sensitized to Gly m 5 and Gly m 6 [55].

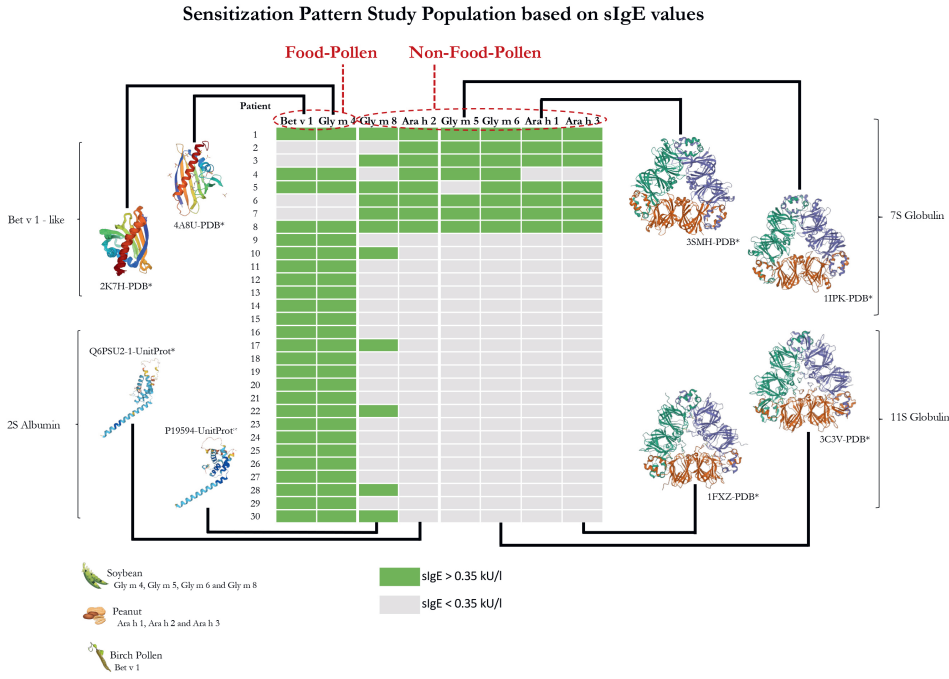

Figure 2. Sensitization Pattern of the Study Population in Chapter 2 based on the serum specific IgE (sIgE, kU/L) as well as the structural view of the evaluated allergens according to the Allergen Nomenclature, WHO/IUIS Allergen Nomenclature Subcommittee Database [53]

2. The Role of Cross-reactivity in Clinical Soy Allergy Diagnosis

Allergens are proteins with antigenic determinant, or epitopes, that are unique plus capable of eliciting an immune response [56]. Moreover, cross-reactivity and co-sensitization have distinct frequencies of occurrence and

different molecular bases (as shown in *Figure 3*) [52,56]. Allergen co-sensitization can occur when multiple IgE-mediated sensitizations are present against structurally unrelated allergen groups at the same time [56,57]. Allergens that contain homologous T-cell epitopes can lead to cross-sensitization by activating primary allergen-specific memory T cells, and subsequently induce the generation of secondary allergen-specific IgE antibodies [58]. Cross-reactivity is often used to describe the relation between two allergens (as shown in *Figure 3*) [52,59]. The more structurally similar the two allergens are, the more likely a cross-reactive IgE antibody can be found (as shown in *Figure 2*); thus cross-reactivity is more appropriately described as the relation between two allergens and an antibody [58,59]. A subset of cross-sensitization and/or cross-reactivity reactions can occur in which the initial sensitizer is not known [56]. In soy allergy since the seed storage proteins, Gly m 5, Gly m 6 and Gly m 8 are major contributors of protein content (80%), these proteins are recognized as potential diagnostic markers for severe allergic reactions to soy (as shown in *Figure 3*) [49,21,58]. Additionally, in birch-endemic areas, soy allergy is usually based on the cross-reactivity between the birch pollen allergen Bet v 1 and its soy related allergen Gly m 4 [46,50,58]. In the diagnostic studies presented in this thesis, the clinical relevance of cross-reactive allergens Gly m 4 and Bet v 1 plus Gly m 8 and Ara h 2 (as shown in *Figure 2*) and their effect in soy allergy diagnosis were discussed [46,52,58-61].

Cross-reactivity, can be defined as the ability of a secondary allergen or allergens to recognize IgE antibodies and invoke a cellular response (T-cell, mast cell or basophils) upon exposure when the host has already been sensitized to a primary allergens that shares one or more epitope with the

secondary allergen, known as the cross-reactive epitopes (as shown in *Figure 3*) [57]. Additionally, allergens that contain homologous T-cell epitopes can lead to cross-sensitization by activating primary allergen-specific memory T-cells; subsequently inducing the generation of secondary allergen-specific IgE antibodies. In other words, when a sensitized individual is exposed to a source containing homologous proteins/allergens via ingestion, inhalation or contact, the allergen specific IgE antibodies preformed due to the primary allergen can recognize these secondary allergens via cross-reactive epitopes which may lead to cross-linking on basophils and mast cells, resulting in mediator release [62]. Presently, the structural information obtained from recombinant allergens can now be combined with IgE cross-reactivity data to predict conformational epitopes; in turn these predicted epitopes could be a starting point for introducing structural changes into a known allergen that will create an allergen derivative which can be used for allergen-specific immunotherapy [63].

The route of exposure of the primary and secondary allergen may differ (i.e., Bet v 1 – inhalation and Gly m 4 – ingestion) [21,57,64]. Moreover, depending on the number of cross-reactive epitopes and binding affinity, the IgE antibody's binding affinity to the primary allergen may be stronger when compared to the secondary allergen [57]. Due to cross-reactivity, allergy to one allergen can also result in allergic complaints to a structurally related allergen [64]. In IgE-mediated allergies, there are two distinct IgE receptors: high-affinity IgE receptor (FcεRI) and the low-affinity receptor (CD23) [65]. Human intestinal epithelial cells can directly take up allergen-IgE complexes via the low-affinity IgE receptor CD23 thereby protecting the allergen from lysosomal degradation enabling the capture by DC and activation of mast cells

[66]. Thus, such food antigen-IgE complexes with CD23 are crucial in the induction of pathophysiology in the gastrointestinal tract. Chang et al reported that the capacity of the low-affinity receptor to activate mast cells *in vitro* and drive allergic reactions *in vivo* could explain unexpected cross-reactivities [67]. Moreover, it was reported that affinities as low as 10^{-7} M were sufficient to trigger type I allergic reactions by binding to allergens bivalently on the surface of mast cells leading to high-avidity interactions [67]. Therefore, allergen cross-reactivity can be described as low-affinity but high-avidity binding between IgE antibodies and cross-reactive allergen [67]. Additionally, for mast cell degranulation the affinity threshold is an important qualitative modifier that depends on the read-out system and the concentrations used for testing; thus, clinically, low affinity may translate into a high threshold for the cross-reactive allergen and/or milder symptoms [59,67]. Importantly, neither sensitization nor the detection of IgE antibodies to a protein signifies the diagnosis of a clinical allergy because many factors are needed to determine clinical outcomes, as explained in Chapter 2 [61]. Moreover, in cross-reactivity, not only the affinity and avidity between the IgE antibodies and cross-reactive allergens determines clinical relevance but other factors such as the physicochemical stability and amino acid sequence or structural homology of the secondary allergen play a role in the clinical relevance of IgE cross-reactivity [57].

Since allergens for soy and peanut share IgE epitopes (as shown in *Figure 3*), it appears that the rate of peanut sensitization is high amongst soy allergic patients; as well as the high co-sensitization rate reported between soy and birch (as shown in *Figure 2* and *Figure 3*) [11,36,50,51]. A study reported that in a group of 39 peanut sensitized patients, 87% were sensitized

to soy; however clinically relevant allergy to soy occurred in about 35% determined by a DBPCFC [68]. These findings concur with a report from Smits et al, who concluded that although co-sensitization between legumes is frequent, particularly between soy and peanut (as shown in *Figure 3*), the majority are not clinically relevant [64]. Moreover, the authors reported that the co-sensitizations in this patient group are mainly due to 7S and 11S globulins with a reported amino acid sequence identity ranging from 45% to 55% [64].

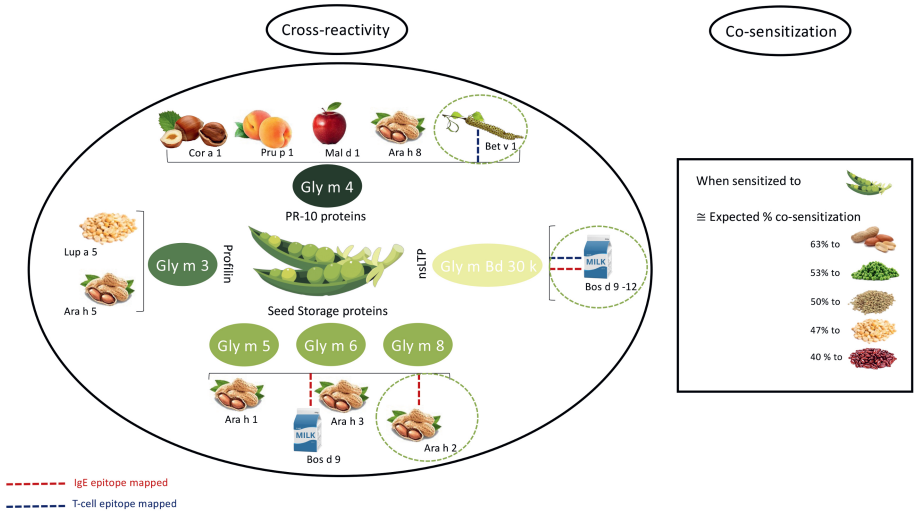

Figure 3. Overview of different reported cross-reactivities of several soy allergens and the secondary allergen sources against which cross-reactivity is documented, where the clinically relevant cross-reactive allergens are encircled (dotted light green) [57]; the expected co-sensitization to legumes when soy allergy is diagnosed is also represented in the following order: peanut (65%), green pea (53%), lentil (50%), white lupine (47%), and bean (40%)[64].

In Chapter 3, we hypothesized that the higher prevalence of peanut co-allergy in previous study populations [11,15] could have influenced the diagnostic value of Gly m 8, possibly due to the cross-reactivity between Gly

m 8 and Ara h 2 (2S albumins). The cross-reactivity between these two δ-conglutin proteins has been reported previously [52,61,60]; nonetheless, data regarding the clinical implications of their cross-reactivity is lacking [69,70]. Presently, clinically relevant cross-reactivity between 2S albumins of different species has been reported as uncommon [64,37]. Sequence homology between peanut and soy 2S albumins is approximately 40% and in order for cross-reactivity to be clinically relevant a sequence homology of greater than 70% has been described as necessary [21,60,23]. Therefore, proteins that share <50% sequence identity are rarely reported to be cross-reactive [73]. However, an exception to the homology sequence percentage is IgE cross-reactivity that is based on conserved structures of cross-reactive carbohydrate determinants [73]. For example, in peanut allergy, even though there is a sequence identity of 59%, the similar tertiary structures means that Ara h 6 is reported to cross-react extensively with Ara h 2 [74]. Moreover, Hazelbrouck et al described that mainly IgE antibodies specific to peanut 2S albumins are mainly non-cross-reactive; however, low-affinity cross-reactivity may still impact diagnostic accuracy [74]. Presently, data regarding the implications of 2S albumins structural features on the sensitization patterns displayed by allergic patients or their potential cross-reactivity is lacking and serological cross-reactivity has been reported as broader than clinical cross-reactivity [62].

As shown in Chapter 3, six patients from the Peanut-Associated SA group were clinically allergic to both soy and peanut demonstrated with positive BAT results to Ara h 2 and positive BAT results for soy allergens Gly m 5 and Gly m 6 but negative results for Gly m 8. These results are presented in *Table 4* and suggest a lack of correspondence between positivity of sIgE

against Gly m 8 and iBAT positivity; with five patients presenting a positive Gly m 8 sIgE value (results > 0.35 kU/l) but all of these patients showing negative results in the rGly m 8 iBAT Gly m 8 (basophil activation <15% CD63+). The exception is patient #2 which shows negative Gly m 8 sIgE and hence the expected negative iBAT for Gly m 8. The iBAT was used to evaluate the clinical relevance of Gly m 8 sensitizations in Chapter 3; thus, the fact that Gly m 8 did not induce basophil activation suggests that sIgE sensitization against Gly m 8 was not clinically relevant. Moreover, there was total correspondence for sIgE and iBAT results for Ara h 2 for all six patients (as shown in *Table 4*), which corresponds with the medical history of these patients who describe a history of peanut allergy that started at an early age with recorded medical episodes of anaphylactic shock after peanut consumption. Therefore, the lack of correspondence for Gly m 8 (1 out of 6 patients) compared to Ara h 2 (6 out of 6 patients) and Gly m 6 (5 out of 6 patients), reinforces the suggestion made in Chapter 3 that Gly m 8 sIgE positivity was most likely due to cross-reactivity to Ara h 2 and not clinically relevant in these group of patients. Additionally, the lack of correspondence between sIgE and iBAT results for Gly m 8, and to a lesser extent Gly m 5 (3 out of 6 patients), shows the need to include functional assays such as the BAT to evaluate the clinical relevance of sIgE sensitization as suggested in Chapter 2 and 3 in future research.

Presently, cross-reactivity often creates a dilemma in determining the clinical relevance of sIgE sensitization profiles in occasions yielding a false positive result and in turn, increasing the possibility of allergic patients following unnecessary treatments or elimination diets [52,60]. Therefore, it is vital that clinicians and researchers understand the difference between sensitization and clinical allergy when interpreting tests results [60,61].

Therefore, special attention is needed when analyzing the clinical relevance of cross-reactive IgE antibodies and although CRD may be helpful is not universally available and still does not replace the OFC [29,52,60]. Van Erp et al proposed that the BAT could be useful in assessing the clinical relevance of IgE sensitization due to cross-reactivity [75]. As shown in *Table 4*, the use of the BAT aided in the diagnosis of clinical soy allergy; however, further research is necessary to investigate the conformational IgE epitope profile of the major soybean allergens such as Gly m 4, Gly m 5, Gly m 6 and Gly m 8 and analyze how those epitopes are involved in cross-reactivity with their homologous proteins (as shown in *Figure 3*).

Table 4. Correspondence between sIgE and BAT/iBAT results for soy allergens Gly m 5, Gly m 6 and Gly m 8 as well as peanut allergens Ara h 2; sIgE positivity >0.35 kU/l and BAT/iBAT positivity >15% CD63+cells; for patients 1,2 and 6 the selected BAT allergen concentration was 300 ng/ml; for patients 3 and 5, 1200 ng/ml and for patient 4, 1000 ng/ml; selected concentration for the BAT Gly m 8 was 1000 ng/ml; selected concentration for the iBAT Ara h 2 was 10 ng/ml.

	Soy Allergens						Peanut Allergen	
	sIgE	BAT	sIgE	BAT	sIgE	iBAT	sIgE	iBAT
Patient	Gly m 5		Gly m 6		Gly m 8		Ara h 2	
1	+	+	+	+	+	-	+	+
2	+	-	+	+	-	-	+	+
3	+	-	+	+	+	-	+	+
4	+	+	+	+	+	-	+	+
5	+	-	+	-	+	-	+	+
6	+	+	+	+	+	-	+	+

Moreover, to confirm the clinical relevance of these cross-reactivities not only functional assays but inhibition assays (with their superior binding specificity and ability to detect low amounts of allergens) and histamine

release assay, a functional assay variant of the BAT, should be performed which would confirm the ability of these epitopes to bind IgE and activate effector cells.

3. The Role of the Basophil Activation Test in Clinical Soy Allergy Diagnosis

In Chapter 2, a review summarizes the current data regarding the clinical application of the BAT in the field of FA diagnosis and highlighting the increasing interest in the diagnostic application of this technique. A BAT is a flow cytometry-based assay that detects the expression of activation markers (mainly CD63 or CD203c) on the basophil cell membrane as a consequence of IgE cross-linking [76]. At present, the BAT has been validated for several IgE-mediated food allergies showing a high sensitivity and specificity; thus, there is expectation that in the future the BAT could decrease the use of OFC [76-80]. Moreover, the BAT has been shown to be able to differentiate between allergic patients and patients who are only sensitized to an allergen [76,78]. Additionally, as a functional assay the BAT uses multiple allergen concentrations thus providing dose response curves. Since there is a large degree of variability in the individual basophil response to an allergen, these dose response curves allow for a detailed comparison between different patients [80].

As mentioned previously the direct BAT, which measures the activation of the patient's own basophils imposes the time constraint since the assay must be performed within 24 hours after blood collection; moreover, approximately 10% of patients present non-responding basophils to IgE-receptor-mediated signaling, known as anergy [76,81]. To circumvent these

two issues an iBAT can be performed for which isolated basophils from pooled healthy blood donors are stripped from receptor bound IgE and passively sensitized with sIgE from allergic patient's serum [76,81]. However, the iBAT is a more time-consuming procedure compared to the BAT [81]. Nonetheless, universal clinical application of the BAT or iBAT requires data validation regarding degranulation metrics and allergen standardization, as well as implementation guidelines and standardization procedures to ensure the reproducibility and reliability of the results [76-80]. Presently, the cut-offs for BAT positivity are not clearly established and the ones defined in one population are not necessarily directly transferable to another one [82,29]. The results of the diagnostic studies presented in this thesis show the versatility of the addition of the BAT in the diagnostic panel of clinical soy allergy and how this addition can benefit the diagnosis algorithm (as shown in *Figure 4*).

In Chapter 3, the iBAT was performed to evaluate the clinical relevance of Gly m 8 sensitization in Peanut-Associated SA group; to further validate the assay an iBAT for Ara h 2 was performed in the same group of patients. The results strengthen the validity of the iBAT by showing a strong correspondence between the positive basophil activation by Ara h 2 and the recorded medical history of the patients. Moreover, the negative iBAT for Gly m 8 results despite sIgE sensitization highlight why it is crucial to verify the clinical relevance of sensitization with the use of functional assay as the iBAT; thus, avoiding under or overestimating the diagnostic value of sIgE sensitizations.

In Chapter 4, the case of a patient whose clinical symptoms are exclusively to processed soy and increase during the birch pollen season is

presented. Outside and during the birch pollen season, the patients Gly m 5 and Gly m 6 sIgE levels did not increase remarkably (1.5-fold). However, the BAT results show different basophil activation results according to seasonality. The basophil activation is negative outside the birch pollen season (December), increases at the start of the season (March) and significantly rises during the peak of the season (May). Moreover, these BAT results correlate with the clinical history which shows a difference in soy tolerance in and out of the birch pollen season. In this study the BAT results showed the changes in clinical reactivity to soy during the birch pollen season which is reflected in the clinical history of the patient. The results in Chapter 4, demonstrate that for measuring allergic seasonal changes it can be advantageous to include a functional assay. Since the effects of climate change can also increase the level of allergenic airborne pollen and allergic symptoms, monitoring the effects of these changes in food allergens and to include non-pollen related food allergens is relevant. Moreover, as the data in Chapter 4 showed, the BAT can aid in monitoring clinical relevance of sIgE seasonal variation [84].

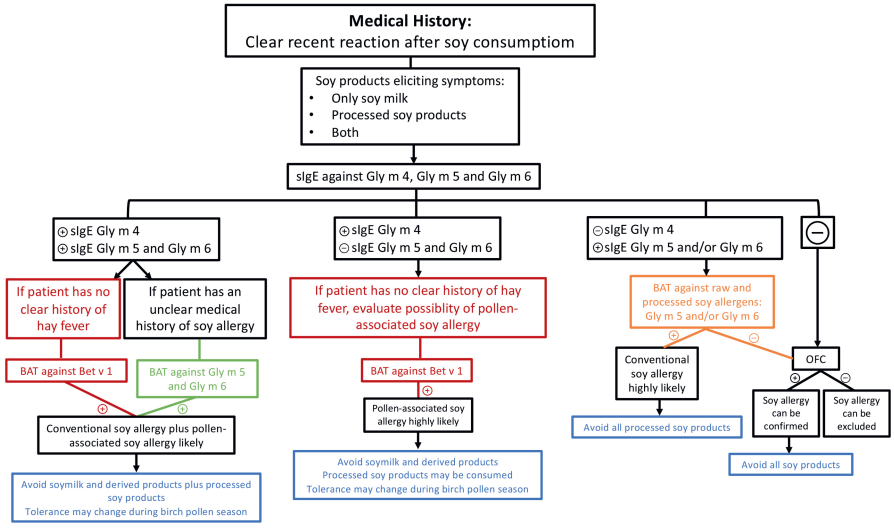

Figure 4. Proposed future algorithm for soy allergy diagnosis which includes three components evaluated in the present thesis: (1) the use of the basophil activation test (BAT), as indicated in green; (2) consideration of co-sensitization pattern as it relates to Bet v 1, as indicated in red; and (3) the evaluation of raw as well as processed soy allergens with the BAT, since soy is rarely consumed raw, indicated in orange. This algorithm illustrates how the dietary recommendation can be more targeted per patient, as indicated in blue. Inquiry about the type of soy products consumed before symptoms occurred will aid in confirming the test results.

In Chapter 7, the effect of heating and glycation on soy allergenicity was measured, and the BAT was used to analyze if processed soy proteins were more or less potent to degranulate basophils. In this study, the area under the curve was calculated (AUC) which has been described as a reliable BAT marker of sensitivity and reactivity [76,78,82]. The analyzed soy protein conditions were raw, heated with glucose for 10 and 30 minutes (glycated), heated without glucose for 10 and 30 minutes (heated); the allergen concentrations used were 10, 300 and 1200 ng/ml. The BAT results showed that in five out of six patients the AUC decreased in glycated 10-minute

samples when compared to the heated 10-minute samples; thus, suggesting that glycation was responsible for masking epitopes. Furthermore, this study shows the value of adding a functional assay to the assessment of the effect of food processing on soy allergenicity because the correspondence between the IgE binding and the basophil stimulation tests was low; only 2 out of 6 patients. This lack of correspondence could be due mainly because IgE binding tests do not reveal information regarding the functional properties of the allergenic components upon interaction with effector cells like mast cells and basophils [40,85]. Even though IgE binding test indicates sensitization and not the manifestation of clinical symptoms, most studies assessing the effects of food processing on allergenicity do so via IgE immunoblotting and IgE binding tests. The value of adding a functional assay, such as the BAT, is that the results of the BAT are not only dependent on the amount of IgE but also on IgE affinity and avidity to relevant epitopes and the possibility of allergen-induced IgE crosslinking [76]. This study indicates that clinicians and researchers assessing allergenicity with only IgE binding tests, should view conclusions from individual assays with care and further research on this topic would greatly benefit from the inclusion of a functional assay such as the BAT.

The three studies (Chapter 3, Chapter 4 and Chapter 7) presented in this thesis show that the BAT can have different applications in the daily clinical setting such as differentiating between sensitization and a true food allergy [76,82]. Hence, the BAT can decrease the need for OFC when diagnosing FA, preventing potential anaphylactic reactions (as shown *in Figure 4*) [76,78]. Additionally, the BAT can be useful in measuring the effects of food processing in soy allergenicity and thus, other food allergens. Therefore,

further research is needed to correlate BAT reactivity with clinical outcomes in FA to validate the BAT for different food allergens and thus, reduce the need of OFC.

4. Recommendations for the Improvement in Clinical Soy Allergy Diagnosis

In diagnosing FA, the most important aspect will always be the patient's clinical history, which must be reviewed in the context of clinical manifestations but also the epidemiology of FA to provide an adequate differential diagnosis. For example, patient A presents with complaints of generalized urticaria which started 15 minutes after soymilk ingestion, this patient has routinely tolerated soymilk in large amounts, is not atopic, and at the time had a viral infection; additionally, the urticaria resolved after one week. Patient B has a history of peanut allergy since childhood and atopic dermatitis; after ingestion of soymilk, the patient developed urticaria within half an hour which resolved with antihistamines. By understanding the clinical history and epidemiological risks, the most likely diagnosis in the case of patient A is that the urticaria appeared because of the viral infection and in the case of patient B the most likely diagnosis is soy allergy. Therefore, for patient A allergy testing will be unnecessary, and for patient B further testing will likely be confirmatory. Thus, it is important to understand the medical history will remain the most important criteria when diagnosing a clinical food allergy.

One of the main pitfalls in misdiagnosing food allergy is that most regular tests (sIgE/SPT) do not reveal when a clinical food allergy is present, as explained in Chapters 2 and 3 [10,76]. Many patients present adverse

reactions to foods that are not due to an allergy, particularly IgE-mediated food allergy, but most likely due to intolerances or toxic reactions to foods that do not induce an immune-mediated reaction (as shown in *Figure 5*) [86]. The presence of allergen-specific IgE against a tested allergen implies allergic sensitization and by-itself is not indicative of a clinical allergy [76,86].

The most common target for immediate reactions to food is the gastrointestinal (GI) system, aside from the symptoms of immediate GI hypersensitivity described in *Figure 5*, the main clinical presentation is the oral allergic syndrome (OAS) [77]. OAS is a local reaction thar occurs primarily in patients with respiratory allergies who have specific IgE directed against panallergens. Panallergens are related proteins that share homologous epitopes and thus are responsible for many IgE cross-reactions [79]. Moreover, panallergens share highly conserved sequence regions, structure and functions. OAS has been reported to be caused by cross-reactivity among PR-10 proteins [87]. In Chapter 3, OAS was reported as a symptom by37% of the study population. It has been reported that the majority of patients with OAS are capable of tolerating the triggering food when it is consumed cooked, since the epitopes are destroyed by the heating process [77].

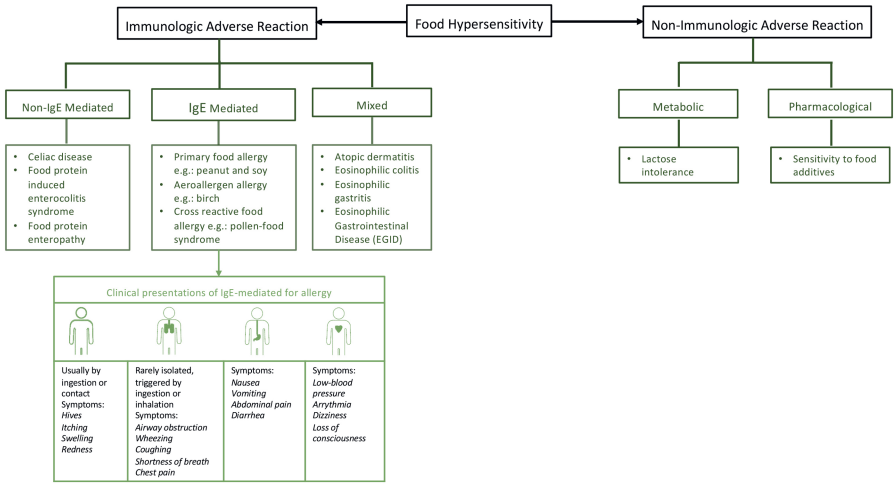

Figure 5. Overview of the classification of Food Hypersensitivity and the description of the most common clinical presentations of IgE-mediated food allergies according to systems: cutaneous, respiratory, gastrointestinal and cardiovascular/neurological [77]. Immunologic adverse reactions are frequently known as allergies; while the non-immunologic adverse reactions are known as intolerances.

Allergies to both pollen and food sources have been suggested to occur by sensitization to these type of allergens, due to cross-reactivity between panallergens from different sources [79]. For example, patients with birch pollen may have OAS after ingesting some vegetables like carrots, celery and soy, or fruits like apple, kiwi, apricot, cherry, apple, peach and pear [77,79]. Among the currently described panallergens, the named Bet v 1 cluster includes a number of vegetables and fruits [79]; in Chapter 3, 26 out of the 30 patients were sensitized to Bet v 1 and Gly m 4. Moreover, in their clinical history and the questionnaire, many of these patients self-reported other food allergies aside from soy and peanut, such as to celery, apple, kiwi and peach (data unpublished). In future research, the clinical significance of cross-reactivity in this specific group of patients among the panallergens Bet v 1

cluster, could aid in clarifying which ones of these allergens are important players in the clinical manifestation of allergic sensitization of birch pollen allergic patients. Moreover, the possibility that patient's tolerance regarding pollen-food related allergies may be affected by the birch pollen season; thus, enquiring about oral seasonality tolerance of food allergens may provide interesting data in future research.

Presently, the first line of allergy diagnostic test are usually SPT and/or sIgE, the proposed cut-off value are 3 mm and 0.35 kU/L, respectively for SPT and sIgE [88]. Both have been described as, generally, highly sensitive but with a low specificity [76,89-91]. Nonetheless, both are useful to exclude the diagnosis of food allergy since they have a high negative predictive value (NPV) [88]. Generally, SPT shows a high sensitivity for food allergens that contain stable allergenic proteins such as peanut, fish, milk or eggs [92]. For soy extracts, the sensitivity and specificity have been reported both at 55% (as shown in *Table 5*) [69]. Nonetheless, the reported specificity and sensitivity of food allergens can vary per study [69,93]. Moreover, for most allergens including soy, generally there is a poor correlation between the clinical history or the OFC and SPT results [92,93]. Generally, sIgE values need to be interpreted with caution and correlated with the clinical history of an IgE-mediated allergy since there is a high rate of false-positive [69]. Usually, the sensitivity of sIgE of food allergens is greater than 90% whilst the specificity can be less than 50% possibly due to cross-reactivity between related proteins [69]. Thus, sIgE can't differentiate between sensitization and a true IgE-mediated allergy, as reported in Chapter 3 with regards to Gly m 8 [69]. For soy sIgE, the reported sensitivity is 83% whilst the specificity lowers to 33.3% [69]. Thus, for clinicians and clinical researchers the medical history

is vital and serves as guide for the interpretation of the results of these tests in case an OFC is not performed.

Presently, the most promising test for the improvement of FA diagnosis that are slowly making the transition from research into clinical practice are cellular tests like the BAT, which uses whole blood, and the mast cell activation test (MAT), which uses serum or plasma [37,90,91]. As stated previously, in these tests it is the quality of sIgE more than the quantity that will determine the degree of mast cells and basophil activation, both responsible for the manifestation of allergic symptoms [76]. Recently, Hemmings et al reported several elements contributing to the activation of mast cells and basophils such as: the diversity of the IgE response in terms of the profile of allergens recognized by sIgE, the binding avidity to the allergens, the sIgE levels and the proportion of IgE that is allergen-specific [94]. Currently, due to the lack of standardization and validation, it is not feasible to employ the BAT and MAT as screening test; however, soon they could be used in clinical practice as a confirmatory test (as shown in *Table 5*) [95].

Table 5. Strengths, drawbacks and applications in of current and future tests for Clinical Soy Allergy Diagnosis, widely used tests are marked with an (*) [67]

	Medical History*	SPT* sIgE*	CRD	BAT	OFC*
Strengths	Specific questions that will aid in the proper interpretation of the auxiliary test and in obtaining necessary information to identify the likely pathophysiologic basis of adverse reaction.	SPT/sIgE is a rapid way of detecting sensitization; both can be done in patients of almost any age, lower reactivity is possible in infants and the elderly. Most allergen preparations are standardized.	Adds valuable information about a patient's sensitization profile, including sensitization to a genuine or a cross-reacting allergen, can predict the risk of severity of a clinical reaction.	Higher specificity and negative predictive value compared to SPT and sIgE. Could replace an OFC in high-risk individuals. Differentiates sensitization from clinical allergy	Current gold standard for FA diagnosis, valuable for screening clinical reactions to food allergens.
Drawbacks	Lack of time, trust and/or resources in the health care specialist can present a barrier in retrieving information from patients. In certain situations, language and culture-specific barriers may also be present.	High sensitivity but low specificity for both tests and their positivity can only detect sensitization not a clinical allergy.	Most components are not commercially available and very few are coming into the market, it is expensive and the threshold value is only known for Ara h 2 [29]	Experienced personal and specific laboratory equipment are required. For direct BAT: needs to be performed 24h after blood collection and there is 10% of non-responders, expensive. For indirect BAT: more time consuming	Time-consuming, requires trained personal, may contraindicated in high-risk patients. Very expensive as patients need to hospitalize for several time periods.

Soy Allergy	SA is one of the most common allergies in children, so the clinical history will be given by a parent/guardian - Previous reaction(s) to soy known or recorded? - Soy products consumed (highly vs low processed) - Reaction(s) to other allergen(s)	SPT [69] Sensitivity – 55% Specificity – 55% sIgE [69] Sensitivity – 83% Specificity – 38%	Presently, soy CDR studies have given mixed results; the threshold values for a clinically relevant allergy are unknown [45,53,70,95]	No current studies report on the sensitivity or specificity of the BAT for soy allergens, while such data are necessary for BAT standardization and validation for universal clinical use [76]	Oral provocation with appropriate soy-containing product (soy powder, tofu soy drink), as well as mild processed that contains soy allergens of low abundance (e.g.: Gly m 4)

Component resolved diagnostics (CRD) test against specified allergens utilizing purified or recombinant allergens and identifying the specific molecules to which patients are sensitized [96]. Therefore, this type of test can represent the next generation of sIgE testing since sensitization to different food proteins may carry different prognostic implications [96]. For example, CDR allows for the identification of patients sensitized to Gly m 4, Gly m 5 and Gly m 6 plus Ara h 2 who do not suffer from either a clinical peanut or a soy allergy [23]. Additionally, as discussed in Chapter 7, Gly m 5 and Gly m 6 are major soy allergens stable to heat and gastric digestion plus they have been reported to be associated to anaphylactic reactions; thus, patients who present a SA mainly to Gly m 5 and Gly m 6 need to avoid soy processed products since food processing techniques will not heavily alter the content of Gly m 5 and Gly m 6 [38]. Therefore, CRD can help identifying what patients are sensitized to allergens that are easily degraded by heat or

digestion, offering additional specificity on food allergy diagnosis [97]. Additionally, CRD is particularly useful in the identification of cross-reactive allergens and differentiation sensitization from clinical allergy [96,97]. However, at the moment CRD testing requires purified native and/or recombinant allergens, thus it is rather expensive; furthermore, since not all food allergens are available, the most cutoff values for different allergens are currently unknown [29]. Moreover, presently, the diagnostic accuracy value and clinical utility of CRD has not been settled; moreover, it is an expensive technique and thus not very cost effective as yet [29,97]. Thus, despite its limitations, the OFC remains the current reference standard for clinical food allergy diagnosis, severity assessment and assessment of treatment response [95]. However, upcoming tests (e.g.: the BAT) have the potential of providing a more precise diagnosis as well as reducing the future need for OFC. To reach that goal it is crucial to gather enough evidence for these types of diagnostic tests to achieve regulatory approval and thus their incorporation into clinical practice. Diagnostic studies such as the ones presented in Chapter 3 and Chapter 7 are a step in achieving this goal.

5. Implications of Processing Techniques on Immunogenicity and Allergenicity of Soy Proteins

Most foods are subject to thermal processing prior to consumption; thus, soy is rarely consumed raw and there are relatively few products that contain unprocessed or low processed soy (e.g.: soy sprouts, soy milk and edamame) [98]. Therefore, most soy-based products contain highly processed soy proteins [98]. Currently, the majority of food processing techniques are high temperature treatments that induce a Maillard reaction (MR), a non-

enzymatic reaction between sugars and amino acids, peptides or proteins, which alter protein structure leading to protein denaturation, degradation and several conformational changes [99,100]. The MR produces a complex array of compounds known as Maillard reaction products (MRPs), identified as early, intermediate advanced products [99,100]. Thus, the term MRPs encompasses a variety of heterogenous complex group of compounds, which include Amadori products, early glycation products, advanced glycation end products (AGEs) and melanoidins [101]. AGEs formed during the MR are also known as dietary (dAGEs) or exogenous AGEs; endogenously formed AGEs are formed when endogenous sugars react with endogenous protein at 37 °C [99,101].

It has been reported that the MR occurring during food processing may destroy, mask, or modify sequential epitopes of an allergen; additionally, conformational epitopes may also change, thereby the MR may affect food allergenicity [102-105]. It has been postulated that heating at higher temperatures in the presence of sugar leads to more pronounced structural changes, for example, roasting peanuts was suggested to affect their immunogenicity and enhanced peanut-induced sensitization in a mice model [106]. The knowledge that MR factors such as temperature and time are key in order to control the type of products obtained [107-110]; thus, when the MR is developed under controlled conditions, the generation of dAGEs may be limited. At present, developing processing techniques which would lead to the limited generation of allergenic/immunogenic structures in soy food product is highly relevant particularly because of the rise of food allergies in the general population and other non-communicable diseases that have been linked to AGEs [109-112]. Nonetheless, at present the information regarding

the influence of specific heat treatments on the antigenicity and allergenicity of soy allergenic proteins is mostly lacking. At present, it has been widely reviewed that the MR can increase the interaction and recognition of modified food proteins by antigen presenting cells (APCs) [99-106,109-112]. APCs possess various AGE receptors that recognize glycation structures [112-115]. Therefore, by targeting these AGE receptors, dAGEs may influence innate immunity via interaction with RAGE or by binding to AGE receptors and presentation of antigen to T-cells that may facilitate T-cell activation and Th2 differentiation, possibly leading to production of specific IgE [99]. To provide a better understanding on how dAGEs may contribute to both innate and adaptative immunity plus their effects on human health, a comprehensive and up to date review on this highly relevant topic for public health in relation to allergic disease was presented in Chapter 5. This review explained the mechanisms by which AGEs (dietary and endogenous) have been reported to be capable of inducing immune effects, focusing on their interaction with AGE receptors [99]. However, as highlighted in Chapter 5, currently, information regarding the absorption, distribution, metabolism and excretion of dAGEs has not been entirely elucidated [99]. Currently, the consensus from animal and human studies is that dAGEs are partially (10% to 30%) absorbed in the intestine with the AGE molecular weight playing a role on the absorption rate [115-117]. In animal studies, after 72 hours, more than 50% of the absorbed dietary AGEs were mostly located in the kidney and liver, with some found in the heart, lung and spleen [115,116]. At present it has been reported that 30% of dAGEs can potentially be eliminated in the urine [118]. Moreover, it has been suggested that dAGEs can modify colonic local microbiota metabolism hence modulating gut integrity which may be a mechanism by

which dAGEs play a pro-inflammatory role in the body [119]. Crucially, the effect of the dAGEs on activation of AGE receptors *in vivo* has not been well researched yet [99,101].

As described in Chapter 5, various AGE receptors have been identified, each with a unique ligand binding domain that recognizes a particular structural element of the ligand [99,111]. Ligand-receptor interactions have been described to be dependent on the receptor type and the physicochemical characteristic of the ligand [120,121]. Nonetheless, a clear classification of AGE receptors based on physiological effect has yet not been established which creates communications issues regarding scientific findings on the field since it is not uncommon that multiple names have been assigned to the same receptor. To address this lack of system classification a committee of multiple expert organizations recently published a consensus to define scavenger receptors and proposed a systemic nomenclature and classification where a formula for scavenger receptor (SR) nomenclature was developed (as shown in *Figure 6*) [113]. Importantly, scavenger receptors were clearly defined as a cell surface receptor that typically bind to multiple ligands and capable of promoting the removal of non-self or altered-self targets [113]. Additionally, they have multiple functions that include endocytosis and phagocytosis as well as inducing cellular signalling downstream and act as immune pattern recognition receptors [113]. AGE receptors, belonging to the SR family, are expressed in various cells which include monocytes, macrophages, endothelial cells and adipocytes [99,111,112,115]. Endogenous AGEs and potentially dAGEs interact with AGE receptors, such as RAGE, Galectin-3, SR-A1, CD36, DC-SIGN and type I transmembrane mannose receptor (MMR), which can activate pro-inflammatory pathways including

nuclear factor kappa B (NF-κB) and induce production of reactive oxygen species (ROS), resulting in the enhanced production of pro-inflammatory cytokines, growth factors and adhesion molecules (as shown in *Figure 6*) [99,100,113,115]. Regarding human health, it has been widely reported that one of the major mechanisms by which dAGEs have a negative impact is via the AGE-receptor system [99-101,109-121]. Hence the need to clarify the affinity of dAGEs to bind with AGE receptors and their impact in health, including allergenicity.

The most widely research AGE receptor is the receptor for advanced glycation end-product (RAGE), which acts as a receptor for endogenous ligands such as the high-mobility group box 1 protein (HMGB1), S100 proteins, and *β*-amyloid as well as glycated proteins and AGE modified peptides [99,112]. It has been previously reported that the binding of RAGE to endogenous AGEs promotes the acceleration of oxidative stress and induces the expression of cytokines, which in turn induces intracellular inflammation [99,100,113,114]. The binding of glycated soy proteins to RAGE observed in Chapter 6 correlated positively with heating time and AGE formation (N^{ε}-carboxymethyllysine accumulation) which will be further discussed in the next section. Therefore, determining the binding of glycated proteins to RAGE in correlation with different stages of the MR and the different products formed should be investigated in order to design processing conditions which would minimize the formation of RAGE ligands. However, due to the large heterogenicity of dAGEs as well as the diversity in the potential receptor-ligand interaction, definition of common motifs of AGEs that result in binding to AGE receptors has not yet been done. Hence, the lack of knowledge regarding the structure-related binding of many dAGEs

to a certain receptor and its molecular consequences. This thesis can be considered the first step bridging this gap.

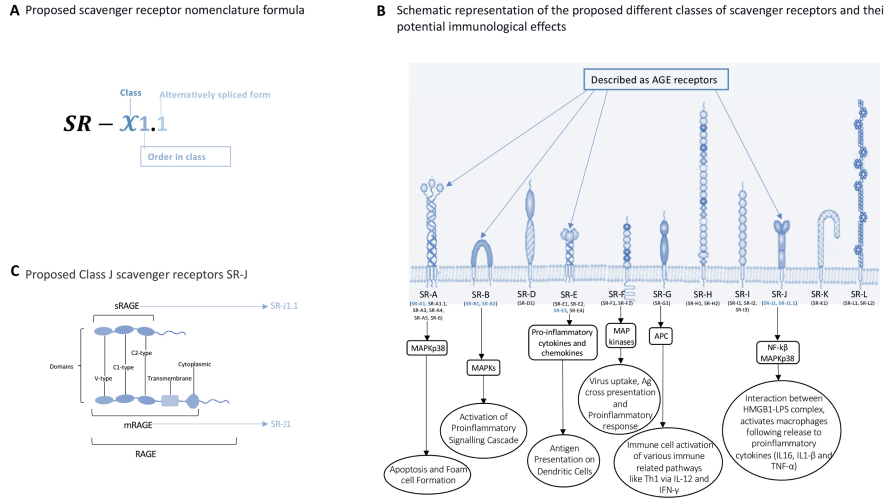

Figure 6. Summary of the Proposed Consensus Nomenclature of the Scavenger Receptors (SR); (A) Proposed SR by name, and structure [113], (B) Structure of RAGE (proposed name SR-J1), the evaluated AGE receptor in Chapter 6, (C) Schematic representation of the SR, for various SR relevant immune functions and pathways are named and several AGE receptors are identified (shown in blue) [99,111,112]: SR-A1, SR-B1, SR-B2 (also known as CD36), SR-E3 (also known as CD206 or human mannose receptor), SR-J1 (also known as membrane form of RAGE), SR-J1.1 (also known as soluble form of RAGE) [99,113]. Other identified AGE receptors, including oligosaccharyl transferase complex protein 48 (AGER1), 80 K-H protein (AGER2) and galectin-3 (AGER3) are yet to be named by the Consensus Nomenclature [113].

5.1 The Maillard reaction induces structural changes in soy proteins which enhance the binding of soy-derived Maillard reaction products to RAGE

The MR can be described to proceed in three steps (as shown in *Figure 7*) [115,122]. The early step, which is the reversible step of the MR, the carbonyl compounds reacts with the amino compounds forming an unstable

Schiff base and eventually forming the derivatives called Amadori products [10]. The intermediate step involves the degradation of the Amadori products and formation of various MRPs such as aldehydes, furans, pyrazines, etc [124]. In the advanced step, low molecular weight intermediates go through various reactions such as rearrangement, isomerization, and others that produce high molecular weight polymers color compounds e.g. melanoidins, (as shown in *Figure 7*) [122-124]. MRPs include Schiff bases, Amadori products and as the final products, AGEs which are produced via an oxidative or non-oxidative pathways [100]. N^{ε}-carboxymethyllysine (CML) and pentosidine are examples of the oxidative pathways, while pyrraline is a product of the non-oxidative pathway [99,100]. The antioxidant nature of soy protein is believed to neutralize free radicals and decrease inflammatory reactions [125]. In Chapter 6, in the advanced stages of the MR, antioxidants were formed which suggests that melanoidins could be part of the MRPs that have an antioxidant function. Recently, it has been recently reported that melanoidins may have an immune regulation effect in immunodeficient mice [126] and when exposed to an allergen (ovalbumin) they could induced IFN-γmRNA in spleen cells and IL-12 mRNA in macrophage like cells [127]; thus, suggesting that melanoidins may have an immunomodulatory effect as well. However, since the specific microstructure of melanoidins is not known (as shown in *Figure 7)*. However, further studies are needed to clarify this possible immunomodulatory effect.

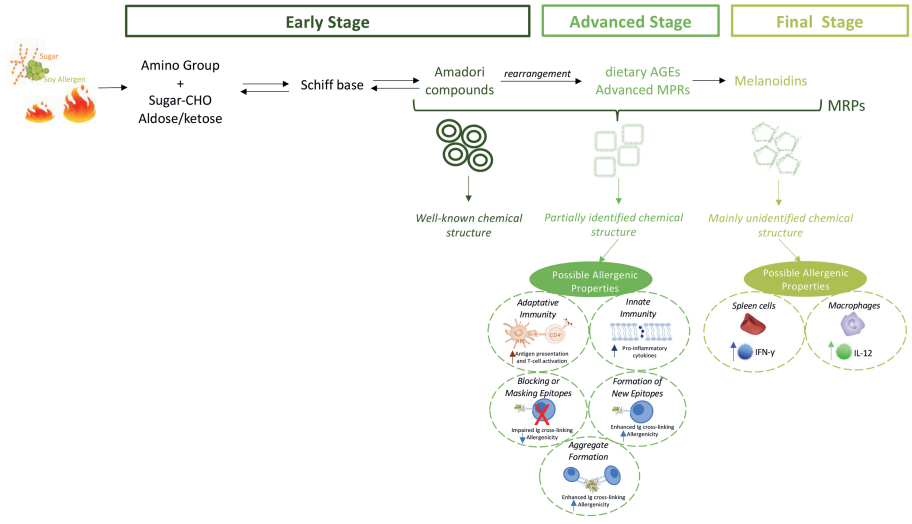

Figure 7. Overview of the stages of the Maillard reaction, the Maillard reaction products (MRPs) and reported effects of the Maillard reaction on allergenicity [99,111,127].

As discussed in Chapter 5, 6 and 7, the MR generates protein structural modifications which affect food allergenicity by masking, modifying or destroying sequential epitopes of an allergen (as shown in *Figure 7*); and is reflected in changes in sensitization capacity as well as specific IgE profile reflected in IgE binding [128-130]. Furthermore, it appears that the effect of the MR on food allergenicity is dependent on the type of protein and its physicochemical characteristics as well as the MR conditions such as the type of sugar, temperature, time of treatment, pH and water activity [100,109-111]. All those conditions contribute together to the type of final product; moreover, each condition separately is a variable that may influence the type of final product. As shown in the present thesis different heating times of soy proteins with glucose at stable temperature leads to the formation of different MRPs. The study presented in Chapter 6 shows the first time that the stages of MR at different heating times have been linked to their RAGE

binding capacity even though it has been previously reported that the interaction between RAGE and endogenous AGEs may be a way by which AGEs modulate the immune response [103,131-133].

The results in Chapter 6, showed that the formation of intermediate MRPs begins after the first minutes of heating, with their main activity in the first thirty minutes. The formation of advanced MRPs begun after ten minutes of heating, indicated by the presence of browning products and the formation of melanoidins. N^{ε}-carboxymethyllysine (CML) accumulation was observed to be time dependent, with the lowest amount for raw soy and the highest for the glycated soy sample for 120 minutes (G120). These results suggest that the types of MRPs are important factors are capable of influencing the binding capacity of RAGE which concurs with previous findings regarding AGE-modified proteins [29,61]. Additionally, the SDS-PAGE showed that both heating and glycation affected the structure of SPEs; however, the glycated SPEs showed the formation of high molecular weight aggregates were more intense when compared to the heated SPEs and appeared after 10 minutes of glycation. Moreover, since the binding capacity of the formed MRPs increased by heating soy protein for longer periods of time in the presence of glucose, it may be hypothesized that AGE formation could have been more important regarding soy immunogenicity than aggregate formation. Because AGE formation (CML) was time dependent as was the binding capacity to sRAGE in the glycated samples while in the glycated samples aggregate formation was constantly present from 10 minutes onwards. Additionally, in Chapter 7 the effects of the MR on the sera of six soy allergic patients was evaluated and high molecular weight material was observed in five out of six patients in the Western Blot. These results

indicate that there was an increased formation of aggregates from glycated proteins compared to only heated proteins that can be recognized by sIgE antibodies, which concur with the SDS-PAGE results of Chapter 6. Furthermore, in Chapter 7, the SDS-PAGE showed that upon treatment, some fraction of Gly m 5 and Gly m 6 disappeared, indicating aggregates visible on the top of the gel could contain the cross-linked fragments of these allergens.

Presently, there are no *in vivo* studies regarding soy protein glycation and changes in allergenicity [136]. Moreover, even though previous studies have suggested that aggregate formed during heating or glycation may have an impact on IgE binding capacity (as shown in *Figure 7*), the capacity of these aggregates to elicit basophil degranulation, a measure more indicative of a potential reduced or increased allergenic potency *in vivo*, has not been studied yet.

5.2. The potential impact of RAGE binding to soy-AGEs on allergic sensitization

The RAGE receptor interacts with MRPs, as it is expressed on different types of immune cells such as monocytes/macrophages, neutrophils, dendritic cells (DCs) as well as T and B lymphocytes [137-139]. Moreover, it is known that highly glycated proteins are often also aggregated which is essential for binding to RAGE [140]. Previously, it has been reported that both MR but also aggregation of proteins plays a role in generating of RAGE ligands in processed proteins [141]. This concurs with the results observed in Chapter 6 , where the binding capacity of SPEs increased with prolonged heating time in the presence of glucose (as shown in *Figure 8*) as well as the presence of aggregates in western blots performed with the sera of soy allergic patients

in Chapter 7. Roth-Walter et al described that compared to non-aggregated proteins the uptake of aggregated ß-lactoglobulin and α-lactalbumin by intestinal epithelial cells promoted significantly higher mucosal Th2-associated antibody responses and cytokine production profiles in a mouse model [142]. Liu and colleagues showed that the formation of aggregates is associated with the formation of ligands binding to sRAGE and the membrane form of RAGE; which would reflect an increased immunoreactivity of MR-modified agglomerates [143]. Moreover, due to the structural changes in the soy protein because of denaturation caused by heat, amyloid-like structures can be formed [144].

In Chapter 7, the formation of immunoreactive aggregates which were recognized by the sIgE present in the sera of soy allergic patients were observed. Along with the formation of aggregates, which were demonstrated in glycated soy samples presented in Chapter 7, the formation of these amyloid-like structures may be crucial factors for binding to the sRAGE receptor. In Chapter 6, it was observed that glycated soy samples for 20 and 30 minutes were not able to inhibit sRAGE binding while the aggregates were observed in both samples indicating that aggregates only do not determine the sRAGE binding; however, this effect was observed for the concentration of 25 µg/ml. This would be in-line with the hypothesis presented in Chapter 6 that AGE formation could have been more important regarding soy immunogenicity than aggregate formation.

Moreover, soy proteins glycated at prolonged heating times inhibited sRAGE binding already at low concentrations. Thus, the longer the heating time the higher the binding potential for the glycated SPEs; suggesting that prolonged glycation leads to the formation of more advanced AGEs with the

highest formation of AGEs at 90 and 120 minutes of heating (as shown in *Figure 8*). Mueller et al reported that RAGE did not interact with unmodified rAra h 1 but did interact with AGE-modified rAra h 1 [131]. Moreover, the authors described that significant AGE-modifications were identified on both raw and roasted allergens, suggesting that future studies on the effect of AGE modification should utilize a recombinant protein instead of raw extract as a control [131]. In Chapter 6, we confirmed specific binding between sRAGE and AGEs derived from soy proteins and its biological consequences measured as increased expression of pro-inflammatory cytokines by PBMCs (as shown in *Figure 8*). Moghaddam et al proposed that increased immunogenicity due to high-temperature antigen modification was mediated via enhanced targeting and presentation of AGEs by the receptors on the DCs [59]. The results observed in Chapter 6 showed that sRAGE was able to bind to the soy-AGE/MRPs; thus, this interaction suggest that these AGEs/MRPs can be recognized also by other by receptors present on DCs and described in detail in Chapter 5. As previously reviewed glycation generated AGEs are potent ligands of RAGE to activate downstream pathways that relate to immune response as well as inflammation and oxidative stress [99,108-110,121]. Moreover, it has been described that formed AGEs can interact with RAGE which is expressed on multiple immune cells and in turn promote downstream pro-inflammatory responses and cytokine secretions (as shown in *Figure 8*) [59-147]. IgE molecules produced by B cells; thus, IgE binding is part of the later adaptative immune response, and our data extend these notions to also include soy-derived AGEs.

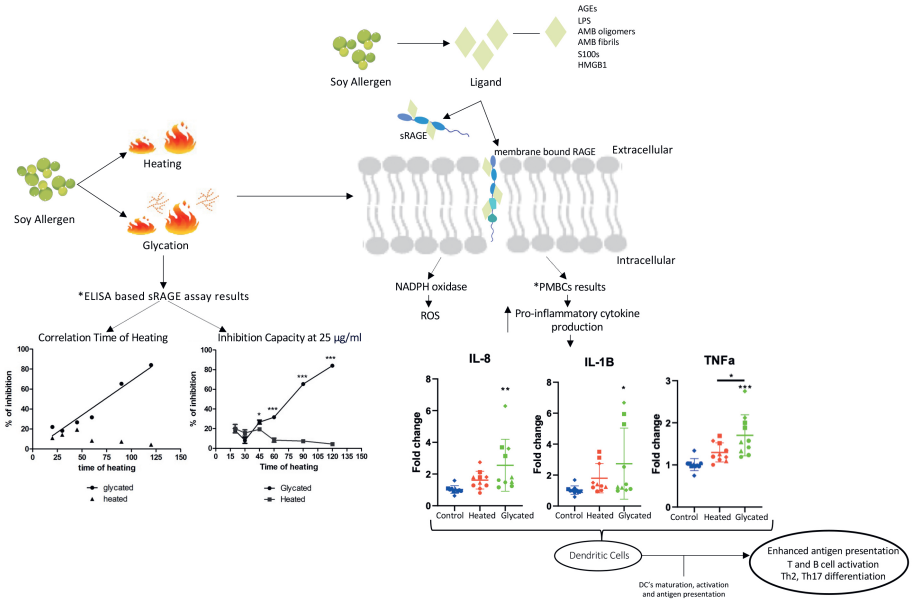

Figure 8. Chapter 6 results soluble RAGE binding to soy protein extracts (SPEs) after heating and glycation showed by ELISA assays and the PMBCs cytokine stimulation results

Nonetheless, currently the role that AGEs/MRPs play in food allergy is not completely understood and even though RAGE is the most widely researched AGE receptor more data is needed to understand its molecular routes of action. The results presented in Chapter 6 indicate that the binding capacity of glycated proteins to RAGE positively correlated with glycation time. Moreover, the binding capacity of the formed MRPs increased by heating soy protein for longer periods of time in the presence of glucose which suggests that RAGE binds to soy proteins that have been modified by MRPs/AGEs and doesn't interact with raw soy. Additionally, glycated SPEs were able to induce pro-inflammatory cytokines (IL-1ß, IL-8 and TNF- α), which suggests that the formed AGEs interact with immune cells possibly via

AGE receptors, including RAGE. Nonetheless, it remains unclear if stimulation of RAGE can potentially increase RAGE expression itself [149]. Hilmenyuk et al reported that AGE-stimulated dendritic cells produced more IL-6 as well as induced a stronger T helper 2 (Th2) response and an increased expression of RAGE [103]. Likewise, it has been described that HMGB1, the endogenous ligand of RAGE, has direct action on naïve CD4+ T cells inducing differentiation of Th2, Th17 cells via activation of the TRL2, TRL4 and RAGE-NF-κB pathways [148]. Thus, it has been suggested that RAGE can partly contribute to the polarization of CD4+ lymphocytes and the balance of Type 1 and 2 lymphocytes (as shown in *Figure 8*) [61,146,149]. Recently, Smith et al proposed the 'false alarm' hypothesis, establishing an indirect link between Western diet, AGEs, and the allergenic response by suggesting that RAGE activation by alarmins induces immunologic activation and multiple inflammatory responses [150]. Broadly, Smith et al proposes that the HMBG1/RAGE alarmin signaling is critical for allergic reactions and current western diet which are high in AGEs can induce alarming signaling [150]. Thus, western diets have the potential to mimic tissue damage through glycation/AGEs hence interventions are necessary to reduce AGE content and clearly understand the biological relevance of AGEs on the immune response with prospective epidemiological and *in vivo* studies [99,109,112,136,149,150].

5.3. Guidance for the future assessment on the effect of food processing on the allergenicity of soy

As stated previously, currently there is a lack of knowledge on the effects of the MR on immunogenic properties of soy allergens. Food processing

affects the structural integrity of various allergens; therefore, information regarding this changes will prove useful to food processors in selecting the processing technique that leads to an allergen safe food product. Moreover, since soy is hardly ever consumed raw it is important that when diagnosing a soy allergic patient, both raw and rather processed allergens are evaluated; thus, a more targeted dietary advice can be provided. Nonetheless, at present there are very few studies that evaluate the effects of food processing techniques on soy allergenicity *in vitro* and no reports on soy protein glycation and changes in allergenicity *in vivo* were found [109]. It has been reported that the MR, e.g., at 121°C degrees for 10 and 30 minutes, reduces the IgE-binding capacity of the analyzed soy proteins when compared to raw and heated due to structural changes [151,152,153]. A recent review by Gou et al, concluded that the MR was a good way to reduce the allergenicity of soy based on the various published studies; however, these studies assessed changes in allergenicity by evaluating the effects of the MR on IgE binding only [109]. In Chapter 6, loss of primary amino groups in the first 10 minutes of heating was not significant and the MR reached initial or intermediate phase rather than advanced (low fluorescence and browning index, low absorbance at 420 nm) and glycated SPEs for 10 minutes did not show significant binding to sRAGE even though the aggregates were already present on SDS-PAGE. Thus, it was proposed the AGE formation was more relevant for immunogenicity than aggregate formation. In Chapter 7, we observed the specific IgE binding to both raw and processed soy proteins especially with bigger aggregates containing sugar (MRPs). Moreover, the results of the Western Blot and IgE binding tests in Chapter 7 are in line, mostly showing strong binding to all processed proteins. These can indicate

that the early stages of the MR can mask epitopes and therefore reduce IgE binding as well as basophil activation, as proposed in Chapter 7 [153]. Therefore, as shown in Chapter 6 and 7, the stage of the MR and the type of product is crucial for the biological properties such as immunogenicity and allergenicity. Thus, when evaluating the effects of the MR on allergenicity of soy proteins it is crucial to include a few treatment conditions and to characterize the stages of the MR and the obtained products to be able to relate the results to other studies. Moreover, as the results of Chapter 7 showed there was a discrepancy between IgE binding (Competitive ELISA and Inhibition ImmunoCAP) and the functional assay (BAT) results when assessing the effect of soy processing on its allergenicity. Thus, since IgE-binding capacity does not always correlate to IgE cross-linking capacity, conclusions drawn from previous studies related to MR effects on allergenicity that are solely based on IgE binding tests should be viewed with care.

Furthermore, further studies on this topic would greatly benefit from the inclusion of a functional assay such as the BAT. As shown in Chapter 7, the BAT was used to analyze the sera of six soy allergic patients with positive sIgE to both Gly m 5 and Gly m 6. The AUC was calculated using multiple measuring points with different allergen concentrations (10, 300 and 1200 ng/ml as determined in previous optimization assays) [76]. The SPEs used in this study were raw, heated with glucose for 10 minutes (SH SPE + Glu) and for 30 minutes (LH SPE + Glu) plus SPEs heated without glucose for 10 minutes (SH SPE) and for 30 minutes (LH SPE) which were used as controls. The results from this small sample size suggests that along with allergen concentration, the effect of the MR on soy allergenicity is largely patient dependent. A clear trend line was observed for the SH SPE + Glu, were a

decrease in allergenicity was observed in 5 out of the 6 patients. This patient dependent effect is more evident when the ratio of the AUC is calculated (as shown in *Figure 9*), with this decreased allergenicity trend evident in *Figure 9C*.

This clear trend line of decrease in allergenicity was only seen for SH SPE + Glu. Thus, time-dependent structural changes of SPE caused by MR can result in epitopes changes reflected by lower or higher capacity of the same protein to degranulate basophils. Furthermore, it can be hypothesized that glycating soy for only 10 minutes stops the MR at an initial stage and thereby producing fewer MRPs and less structural changes to the protein. However, if continued glycation or heating is provided for a prolonged period of time (30 minutes), the MR continues to more advanced stages where the advanced MRPs are formed and more structural changes to the protein can occur with the possible formation of new allergic epitopes. The results both in Chapter 6 and 7 suggest that glycation of soy proteins for prolonged periods of time risk increasing the allergenicity more than glycation for shorter periods of time. This is clearly observed in the results presented in Chapter 7 which show that while short glycation may result in reduction of allergenicity as observed for SH SPE + Glu, the AGEs formed during prolonged heating may increase allergenicity (shown on *Figure 9B* and *9D*). However, the small number of patients included in the study due to the difficulty of recruitment of patients with this particular sensitization profile, limits a strong discussion on allergenicity trends. Therefore, although the results observed in Chapter 7 suggest that the effects of MR on soy allergenicity are patient-dependent and glycation for a short period of time (10 minutes) have the potential to reduce the allergenic capacity of soy proteins in the majority

of patients compared to raw or heated soy, this statement is not conclusive at present and larger studies must be conducted to reach an irrefutable conclusion.

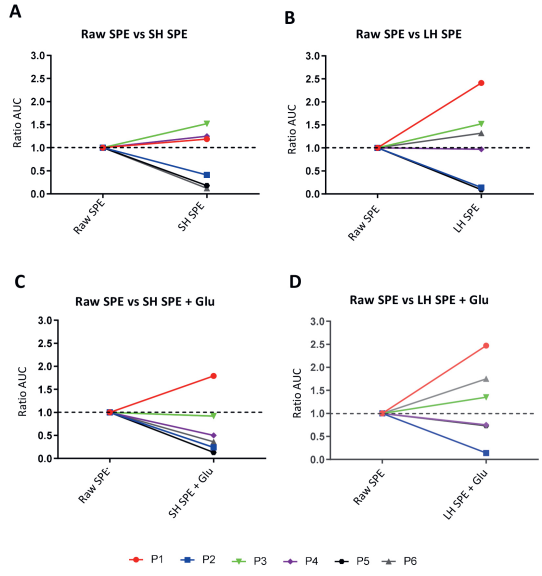

Figure 9. Results for AUC of the BAT results

Presently, it has been reported that IgE binding may be dependent on the sIgE epitope profile of the patient which is related to the sensitization phase [104,153]. Moreover, it appears that the IgE binding profile to glycated proteins can differ per protein [104,151-154]. Therefore, with the results presented in Chapter 6 and 7 it can be concluded that both changes in: (i) IgE binding as well as (ii) basophil activation are defined by the level of protein modification caused by temperature treatment itself and the stage of the MR. For some patients (P1, P3, P6) an enhanced basophil degranulation was observed by processed allergens compared to raw and for other patients' degranulation was reduced (P2, P5). This findings reveal an interesting

perspective of different individual profiles of sensitization which requires further studies on larger populations. Moreover, the inclusion of processed proteins might be beneficial in the standard diagnostic test diagnosis along with SPT, provocation test and/or BAT assay as indicated in Figure 4 were the algorithm for the future diagnosis for clinical soy allergy is presented.

6. Conclusions

In summary, this thesis has provided novel data on soy allergy, particularly the importance of co-sensitization and cross-reactive allergens when diagnosing soy allergic patients. Moreover, it has introduced and explored novel functional assays applicable for the diagnosis of soy allergy. Most importantly, the clinical applicability and considerations that can assist allergologists in properly diagnosing soy allergic individuals have been discussed. Briefly the following conclusions can be drawn from this work:

- Currently, one of the main challenges in diagnosing food allergies is to develop an easily accessible and reliable test, that can provide differentiation between sensitization and a clinical allergy; the BAT is a promising *ex vivo* diagnostic tool. However, universal applicability, several methodological aspects need to be investigated before a standardized protocol is available.
- In order to avoid over- or underestimation of the diagnostic value of sIgE sensitizations, it is crucial to analyze co-sensitization patterns in soy allergic populations and verify the clinical relevance of sensitization with the use of functional assays such as the BAT.

- Monitoring allergic food-related seasonal variations such as oral tolerance and gastrointestinal symptoms should not be limited to birch pollen-associated soy allergens; furthermore, future research should not be limited to seasonal measurements of allergic markers but add a functional assay like the BAT to evaluate clinical relevance of these seasonal changes.
- An important way that dietary AGEs (dAGEs) may influence innate immunity is by targeting AGE receptors via interaction with RAGE or by binding to AGE receptors and presentation of antigen to T-cells which facilitates T-cell activation and Th2 differentiation, possibly leading to production of specific IgE. Since, these AGE receptor system has been reported that one of the major mechanisms by which dAGEs have a negative it is important to clarify the affinity of dAGEs to bind with AGE receptor and their impact in health, including allergenicity.
- Glycated soy proteins are ligands of sRAGE and the binding capacity is positively correlated with time of heating of SPEs with glucose and formation of AGEs in the samples; thus, related to the stage of the MR. AGEs derived from soy proteins heated for 90 minutes in the presence of sugar can increase the expression of pro-inflammatory cytokines by PBMC which suggest that formed AGEs interact with immune cells possibly via various AGE receptors, including RAGE.
- A lack of correspondence between the IgE binding test and the functional assay was observed when evaluating the effects of heating and glycation on soy allergenicity, altered IgE binding

capacity could be due to the formation of new epitopes or by glucose favored recognition of IgE antibodies; thus, conclusions on the allergenic potential based solely on IgE binding tests should be drawn with care. Therefore, future research evaluating the effects of food processing techniques on food allergenicity would benefit from the inclusion of a functional assay such as the BAT.

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Chapter 9

Summary

Summary

The incidence of food allergies continue to rise at an alarming rate, particularly in the developed world. Moreover, food allergy represents a public health concern with a significant social and economic impact; thus, it is a topic of interest for scientists and clinicians alike. Presently, soy has been identified as one of the eight main food allergens. The consumption of soy has increased significantly since it is an ideal protein alternative and a variety of health benefits have been linked to its consumption. Therefore, soy protein is commonly used by the food industry and can be found in a wide variety of processed foods. The indicated treatment after a patient is diagnosed with soy allergy is strict avoidance; however, maintaining such elimination diet is a challenge not only because of the wide variety of foods containing soy but, more importantly, because in many cases such a restricted diet may not be necessary. Presently, the diagnosis of soy allergy requires the careful evaluation of the patient's clinical history plus a proper interpretation of the available diagnostic test results. However, the most common diagnostic tests (skin prick test, SPT and/or serum food specific IgE, sIgE) do not differentiate between sensitization and a clinical soy allergy; moreover, they do not account for allergen cross-reactivity or predict the patient's prognosis. Therefore, development and validation of more reliable diagnosis tools for soy allergy are crucial to avoid unnecessary elimination diets and to provide the information to formulate personalized dietary recommendations. Moreover, since soy is rarely consumed rare it is important to understand the protein changes that may occur during food processing (heating, glycation, etc.) and the effects that this techniques may have on soy allergenicity.

This thesis represented the first time, the basophil activation test (BAT) has been used to assess the allergenicity of soy 2S albumin and the effects of the Maillard reaction (MR) on soy allergenicity in a soy allergic adult population. The main aim of this thesis was to analyze the current challenges in clinical soy allergy diagnosis while taking into consideration the sensitization patterns to different soy allergens in accordance with their cross-reactive homologous allergens. Moreover, the addition of the BAT in diagnosis of clinical soy allergy was analyzed. Finally, the effects of the MR in soy allergenicity were evaluated.

The BAT is a *ex vivo* functional assay that measures the degree of degranulation after control or allergen stimulation by flow cytometry, since it assesses IgE cross-linking it has a more precise allergic readout than measuring the concentration of allergen specific IgE. Due to its diagnostic potential, in recent years, there has been an increased interest in use of the BAT in food allergy. Therefore, in *Chapter 2*, a review were the diagnostic potential of the BAT for the diagnosis of food allergies particularly those present in early life such as peanut, cow's milk and eggs was presented. The review summarized not only the current data regarding the application of BAT in food allergy diagnosis but the present hurdles for the widespread clinical use of the BAT.

In *Chapter 3*, thirty soy allergic adult patients were recruited with the aim to evaluate the diagnostic value of soy 2S albumin (Gly m 8) by determining the sensitization profiles based on the homologous soy allergens Bet v 1 (major birch pollen) plus Ara h 1, Ara h 2 and Ara h 3 (common peanut allergens). Additionally, the clinical relevance of sIgE was evaluated using the indirect BAT (iBAT) in a group of Gly m 8 sensitized patients. Two groups were

identified based on the sIgE sensitization patterns: (i) Peanut-associated soy allergy, all patients were sensitized to one or more of the peanut allergens) and (ii) Non-Peanut/PR-10-associated soy allergy, all patients were sensitized to the birch pollen allergen but not to any of the peanut allergens. The results showed that Gly m 8 was not a major allergen in the evaluated soy allergic population. Moreover, the iBAT results indicated that Gly m 8 was not able to induce basophil degranulation in the sIgE Gly m 8 sensitized soy allergic patients, indicating that the Gly m 8 sensitizations were not clinically relevant. Thus, the study in *Chapter 3* demonstrated how the correlation of the sIgE sensitization profile with clinical outcomes would benefit from the inclusion of a functional assays such as the iBAT since the rate of peanut co-sensitization in previous studies may have overestimated the diagnostic value of Gly m 8.

In the Netherlands it has been reported that most soy allergic adult patients are sensitized to Gly m 5 and Gly m 6, described as 'conventional soy allergy'. However, Gly m 4 mediated soy allergy is also very common as observed in the study population in *Chapter 3*, particularly in patients with birch pollen allergy. Moreover, due to the cross-reactivity of IgE antibodies induced by sensitization to the major birch pollen allergen Bet v 1 with homologous food allergens, birch pollen allergic patients show allergic symptoms after the ingestions of certain fruits and vegetables. Furthermore, this group of patients show an increase in food-triggered allergic symptoms during the pollen season, possibly due to season boosting of pollen-IgE levels. However, whether this increased pollen sensitization during the pollen season also affects the allergenicity of allergens that are non-cross-reactive with birch pollen, such as Gly m 5 and Gly m 6 , has not been evaluated until

now. In *Chapter 4* the first case of a patient with a 'conventional soy allergy' and pollinosis, who experiences worsening of gastrointestinal symptoms during the birch pollen season even though the eliciting food factor does not cross-react with birch pollen allergens was presented. The BAT results showed that both Gly m 5 and Gly m 6 were clinically relevant soy allergens, correlating with he reported clinical symptoms to processed soy. Moreover, the BAT against raw soy showed an increase in basophil activation during the birch pollen and a negative basophil activation result outside the birch pollen season. This case highlights the importance of adding a functional assay such as the BAT to evaluate clinical relevance when assessing birch pollen seasonality on soy allergenicity. Moreover, it showed it is not only birch pollen-associated soy allergy patients that may suffer an increase on gastrointestinal symptoms and changes in soy tolerance during the pollen season, and thus, clinicians may use the information provided in *Chapter 4* to adjust dietary advice accordingly.

The case presented in *Chapter 4* reflected a patient whose tolerance to soy processed products varied accordingly to the pollen season. One-fifth of the world's soy is used for direct human consumption and the majority of it is first processed since soy is rarely consumed raw. Various types of soybean flour are produced by different technological processes characterized by different degrees of thermal processing. Among them, the MR is frequently used since it provides food products with a different taste, color and aroma; however, it also produces advanced glycation end-products (AGEs) plus structural and functional protein modifications. In *Chapter 5*, a compressive review of the current literature regarding the immunogenicity of exogenous AGEs (obtained from food, known as dietary AGEs) in relation to endogenous

AGEs (formed within the body) was presented. The evidence showed that dietary AGEs (dAGEs) can act as signalling molecules modulating the innate and adaptative immunity; thus, possible contributing to the low-grade inflammation in certain non-communicable disease and food allergies. Moreover, dAGEs may activate the immune system in two different manners: via the receptor for advanced glycation end products (RAGE) and via binding of dAGEs to various AGE receptors such as RAGE, Galectin-3, CD-36 and SR-A. Thus, currently the evidence suggests that the interaction of dAGEs and specific AGE receptors on antigen presenting cells (APCs) play an important role in the immunological effects of dAGEs. Nonetheless, since not all studies use the same types of proteins and methods of glycation, specific characterization of AGEs and structural changes of proteins are still unknown; thus, further studies are needed to elucidate the effects of MR in the allergenicity of glycated proteins. Moreover, since information regarding the effects of human digestion on the glycated protein and their effects on APC is very limited at the moment, no definitive conclusions on the interaction of dAGEs with the immune system *in vivo* can be drawn at present. Furthermore, during the MR, protein structure and functionality changes are a consequence of the formation of Maillard reaction products (MRPs). At present, there is a knowledge gap between the structure-function dependency of MRPs formed during soy processing and their interaction with RAGE. Therefore, in *Chapter 6*, the MRPs formed during different heating times of soy proteins with glucose were characterized by analyzing the biochemical changes which were then related to functional changes such as antioxidant capacity, binding to soluble RAGE (sRAGE) and capacity to stimulate immune cells to secrete pro-inflammatory cytokines. The results

observed in *Chapter 6* showed that the type of MRPs/AGEs obtained strongly depended on heating time; moreover, the structural protein changes during heating without glucose had less binding potency to the sRAGE when compared to glycated proteins. Moreover, it was observed that the binding capacity of glycated proteins to sRAGE positively correlated with time of glycation; therefore, it can be suggested that time dependent differences in the biochemical characteristics of glycated soy when compared to heated soy could be attributed to the different stages of MR and thus, the diversity in the obtained MRPs. Moreover, the incubation of glycated soy sample for 90 minutes (G90) resulted in the increased expression of pro-inflammatory cytokines (IL-1ß, IL-8 and TNF- α), suggesting that formed AGEs interact with immune cells activating possibly via various AGE receptors, which includes RAGE.

At present, the majority of studies assess allergenicity via IgE immunoblotting and IgE binding tests, in *Chapter 7*, 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) and a Western Blot; however, unlike previous a functional assay (BAT) was added. Since IgE detection does not always signal the manifestation of a clinical allergy and the capacity of food allergens to trigger basophils makes it possible to use the *in vitro* functional BAT to assess allergenicity. The results observed in *Chapter 7* between the IgE binding tests and the functional assay were in-line for 2 of the 6 studied patients, with one patient showing no result correlation at all. These results show a discrepancy between IgE binding tests and basophil stimulation when assessing the effect of soy processing on its allergenicity. Thus, 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.

Finally, in *Chapter 8*, the previous chapters were discussed, the current pitfalls and future of clinical soy allergy diagnosis were furthered explored in this section. The possible improvement strategies in the diagnosis of clinical soy allergy used in the diagnostic studies presented in this thesis by adding a functional assay represented by the BAT were evaluated. Moreover, the factors that could influence soy diagnostic markers such as the patient selection, patient co-sensitization pattern as it regards to birch and peanut allergy and type of allergens (e.g., use of recombinant allergens) was discussed. Population selection was established as a crucial step to avoid under- or over-estimation in the diagnostic value of a soy allergenic marker (e.g., Gly m 8), selecting a soy allergic sub-population from an already selected allergic population such as peanut was strongly advised against. Furthermore, the advantages in the use of recombinant allergens was outlined; however, presently, steps are yet needed to standardize their use for many food allergies including soy allergy.

The discussion also analysed the role of cross-reactivity and co-sensitization in clinical soy allergy diagnosis comparing the implications of the reported findings to the current literature. Since currently cross-reactivity creates a dilemma when interpreting the clinical relevance of sIgE sensitization profiles, occasionally yielding a false positive result. Therefore, advice was provided for clinicians and researchers on how to correctly interpret the difference between sensitization and a clinical allergy when

evaluating soy allergy diagnostic test results. The benefits of adding a functional assay such as the BAT to assess co-sensitization and clinical reactivity was further examined.

Furthermore, since soy is rarely consumed raw, the implications of food processing techniques on the immunogenicity and allergenicity of soy proteins was reviewed; focusing on the structural changes induced by the Maillard reaction and increase RAGE binding to soy-AGEs. Additionally, advice for the future assessment on the effect of food processing on the allergenicity of soy was provided.

About the author

Daniela Briceno Noriega was born in Lima, Peru and was an avid reader from a young age. At 13 years old she read 'Death Be Proud', a memoir by John Gunther published in 1949 where he describes the illness and death of his son due to a brain tumor. This book touched her deeply describing the role of doctors when people need to confront a health crisis. Doctors are not only capable of healing the body but can aid in healing a soul. So, at 13, she decided to pursue a career in medicine.

She attended Universidad Cientifica del Sur and graduated with the degree of Medical Doctor. After graduation, she worked as a Pediatric Resident in the Anglo-American Hospital in Lima, Peru. To continue her medical education, she moved to the United States. While working in the Neonatal Intensive Care Unit at Rush Medical Centre in Chicago as a research assistant, she realized the importance of nutrition in order to maintain a lifestyle; thus, that the next step in her education was to combine her medical knowledge with higher nutritional education. She was accepted in Wageningen University in The Netherlands and completed a Master in nutrition and health with a specialization in Nutritional Physiology and Health Status. After graduation, life took her on a globetrotting adventure, moving first to Singapore, then to the Northern Territory in Australia and finally to Panama. In all this countries, she continued to gain medical and nutritional knowledge while working with different populations and institutions along the way.

After the years of travelling, experiencing different cultures and most importantly becoming a mother to two amazing children, she and her husband decided it was time to come back to Netherlands and settle down. She was then offered a PhD position with Huub Savelkoul at the Cell Biology and Immunology Group to study food allergies in an adult population focusing on the current challenges in diagnosing soy allergy in adults and the effects of food processing techniques, exemplified by the MR, in soy allergenicity (this thesis).

List of publications

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