

ADOPTED: 2 December 2021

doi: 10.2903/j.efsa.2022.7044

## Scientific Opinion on development needs for the allergenicity and protein safety assessment of food and feed products derived from biotechnology

EFSA Panel on Genetically Modified Organisms (GMO),  
Ewen Mullins, Jean-Louis Bresson, Tamas Dalmay, Ian Crawford Dewhurst,  
Michelle M Epstein, Leslie George Firbank, Philippe Guerche, Jan Hejatko, Hanspeter Naegeli,  
Fabien Nogué, Nils Rostoks, Jose Juan Sánchez Serrano, Giovanni Savoini, Eve Veromann,  
Fabio Veronesi, Antonio Fernandez Dumont and Francisco Javier Moreno

### Abstract

This Scientific Opinion addresses the formulation of specific development needs, including research requirements for allergenicity assessment and protein safety, in general, which is urgently needed in a world that demands more sustainable food systems. Current allergenicity risk assessment strategies are based on the principles and guidelines of the Codex Alimentarius for the safety assessment of foods derived from 'modern' biotechnology initially published in 2003. The core approach for the safety assessment is based on a 'weight-of-evidence' approach because no single piece of information or experimental method provides sufficient evidence to predict allergenicity. Although the Codex Alimentarius and EFSA guidance documents successfully addressed allergenicity assessments of single/stacked event GM applications, experience gained and new developments in the field call for a modernisation of some key elements of the risk assessment. These should include the consideration of clinical relevance, route of exposure and potential threshold values of food allergens, the update of *in silico* tools used with more targeted databases and better integration and standardisation of test materials and *in vitro/in vivo* protocols. Furthermore, more complex future products will likely challenge the overall practical implementation of current guidelines, which were mainly targeted to assess a few newly expressed proteins. Therefore, it is timely to review and clarify the main purpose of the allergenicity risk assessment and the vital role it plays in protecting consumers' health. A roadmap to (re)define the allergenicity safety objectives and risk assessment needs will be required to inform a series of key questions for risk assessors and risk managers such as 'what is the purpose of the allergenicity risk assessment?' or 'what level of confidence is necessary for the predictions?'

© 2022 Wiley-VCH Verlag GmbH & Co. KgaA on behalf of the European Food Safety Authority.

**Keywords:** Allergenicity assessment, protein safety, newly expressed proteins, innovative proteins, GMO, biotechnology

**Requestor:** EFSA internal mandate

**Question number:** EFSA-Q-2020-00316

**Correspondence:** GMO\_secretariat\_applications@efsa.europa.eu

**Panel members:** Ewen Mullins, Jean-Louis Bresson, Tamas Dalmay, Ian Crawford Dewhurst, Michelle M Epstein, Leslie George Firbank, Philippe Guerche, Jan Hejatko, Francisco Javier Moreno, Hanspeter Naegeli, Fabien Nogué, Nils Rostoks, Jose Juan Sánchez Serrano, Giovanni Savoini, Eve Veromann and Fabio Veronesi.

**Declarations of interest:** The declarations of interest of all scientific experts active in EFSA's work are available at <https://ess.efsa.europa.eu/doi/doiweb/doisearch>.

**Acknowledgements:** The Panel wishes to thank the members of the Working Group on Allergenicity, the members of the Stakeholder Consultative group 'Focus Group', the hearing experts Clare Mills and Henk van Loveren and the EFSA staff Riccardo Vrizz, Anna Lanzoni and Michele Ardizzone for the support provided to this scientific output.

**Suggested citation:** EFSA GMO Panel (FSA Panel on Genetically Modified Organisms), Mullins E, Bresson J-L, Dalmay T, Dewhurst IC, Epstein MM, George Firbank L, Guerche P, Hejatko J, Naegeli H, Nogué F, Rostoks N, Sánchez Serrano JJ, Savoini G, Veromann E, Veronesi F, Fernandez Dumont A and Moreno FJ, 2022. Scientific Opinion on development needs for the allergenicity and protein safety assessment of food and feed products derived from biotechnology. EFSA Journal 2022;20(1):7044, 38 pp. <https://doi.org/10.2903/j.efsa.2022.7044>

**ISSN:** 1831-4732

© 2022 Wiley-VCH Verlag GmbH & Co. KGaA on behalf of the European Food Safety Authority.

This is an open access article under the terms of the [Creative Commons Attribution-NoDerivs](https://creativecommons.org/licenses/by/4.0/) License, which permits use and distribution in any medium, provided the original work is properly cited and no modifications or adaptations are made.



The EFSA Journal is a publication of the European Food Safety Authority, a European agency funded by the European Union.



## Summary

This Scientific Opinion addresses the formulation of specific development needs, including research requirements for allergenicity assessment and protein safety, in general, which is urgently needed in a world that demands more sustainable food systems. Current allergenicity risk assessment strategies based on the principles and guidelines of the Codex Alimentarius for the safety assessment of foods derived from 'modern' biotechnology was initially published in 2003.

Due to the continuous scientific advances over the last two decades, there is a functional asynchrony between the availability of safety standards and concurrent scientific developments. The European Food Safety Authority (EFSA) has been proactive in this respect and has already invested resources to advance the allergenicity prediction field further. Likewise, EU-funded research programmes, such as the ImpARAS Cost Action, EuroPrevall, iFAAM and AllerScreening projects, among others, also provide insights on the use and improvement of existing and suggested assessment tools in the field of allergenicity assessment of foods. However, important knowledge gaps remain, and the development of novel approaches to deal with allergenicity assessment needs to be pursued further. This Scientific Opinion aims to: (i) define knowledge gaps on allergenicity prediction; (ii) identify specific research needs for improving the allergenicity risk assessment for products derived from biotechnology; (iii) determine how new basic research findings and technological developments can improve the current risk assessment methodology; and (iv) prioritise basic research funding.

By considering the complexity and variety of factors involved in food allergy and the current state-of-the-art, it is unrealistic that a single test in the short/medium term will be predictive of the allergenic potential of a protein. Therefore, the 'weight-of-evidence' approach for allergenicity assessment remains valid. However, the evidence needed might differ depending on whether a conventional GMO or another type of new biotech food is being assessed.

Although the Codex Alimentarius and EFSA guidance documents successfully addressed allergenicity assessments of single/stacked event GM applications, experience gained and new developments in the field call for a modernisation of some key elements, such as (i) better standardisation on the use of the available knowledge on the source of the gene and the protein itself – context of clinical relevance, route of exposure and potential threshold values of food allergens; (ii) modernisation of *in silico* tools used with more targeted databases; (iii) better integration of *in vitro* testing, with clear guidance on how protein stability and digestion informs the assessment and on the use of human sera; and (iv) better clarity on the use of the overall weight-of-evidence approach for protein safety and the aspects needed for expert judgement.

Furthermore, more complex future products will challenge the overall practical implementation of such guidelines, mainly targeted to assess few newly expressed proteins. More challenging applications are expected in the future with large numbers of diverse proteins, for instance, derived from new genome techniques and synthetic biology. Therefore, it is timely to review and clarify the main purpose of the allergenicity risk assessment overall and the vital role it plays in protecting consumers' health with existing food allergies and assessing the potential for foods to cause new food allergies.

Therefore, a draft of a roadmap that (re)defines the allergenicity safety objectives and risk assessment needs will be needed to address the key questions for risk assessors and risk managers, such as (1) what is the purpose of the allergenicity risk assessment?; (2) what should be assessed in the allergenicity assessment?; (3) what level of confidence is necessary for the predictions?; and (4) what is an unacceptable/acceptable risk in the allergenicity risk assessment?.

## Table of contents

|  |    |
|--|----|
| Abstract.....  | 1  |
| Summary.....   | 3  |
| 1. Introduction.....   | 5  |
| 1.1. Background.....   | 5  |
| 1.2. Terms of Reference.....   | 5  |
| 2. Data and methodologies.....   | 5  |
| 2.1. Data.....   | 5  |
| 2.2. Methodologies.....  | 5  |
| 3. Assessment.....   | 6  |
| 3.1. Allergenicity prediction in the safety assessment of foods derived from biotechnology.....      | 6  |
| 3.1.1. Clinical relevance of food allergens.....   | 7  |
| 3.1.2. Determinants of food protein allergenicity.....   | 8  |
| 3.1.2.1. Intrinsic and extrinsic properties of food allergens.....                                   | 8  |
| 3.1.2.2. Environmental and other factors influencing protein allergenicity.....                      | 9  |
| 3.1.2.3. Adjuvant properties of food components.....   | 10 |
| 3.1.2.4. Food matrix and processing.....   | 11 |
| 3.2. Risk assessment tools for allergenicity prediction.....   | 11 |
| 3.2.1. <i>In silico</i> tools.....   | 12 |
| 3.2.2. <i>In vitro</i> tools.....  | 14 |
| 3.2.2.1. Use of protein digestibility data in allergenicity risk assessment.....                     | 14 |
| 3.2.2.2. Use of human data in allergenicity risk assessment.....                                     | 17 |
| 3.2.2.3. <i>In vitro</i> tools to understand cellular and molecular mechanisms of sensitisation..... | 19 |
| 3.2.3. <i>In vivo</i> tools.....   | 20 |
| 3.2.4. Additional remarks on risk assessment tools.....  | 23 |
| 3.3. Other key elements in the allergenicity risk assessment.....                                    | 24 |
| 3.3.1. Acceptable levels and threshold values of food allergens.....                                 | 24 |
| 3.3.2. Post-market monitoring tools.....   | 25 |
| 4. Conclusions and Recommendations.....  | 26 |
| 5. Documentation provided to EFSA.....   | 27 |
| References.....  | 27 |
| Abbreviations.....   | 37 |

## 1. Introduction

### 1.1. Background

In 2017, the scientific Panel on Genetically Modified Organisms of the European Food Safety Authority (hereafter referred to as the 'GMO Panel') published a supplementary guidance document on allergenicity risk assessment of genetically modified (GM) plants addressing non-IgE-mediated adverse immune reactions to foods, *in vitro* protein digestibility tests and endogenous allergenicity of plant constituents (EFSA GMO Panel, 2017). The purpose of this guidance document was to incorporate new developments in allergenicity into the risk assessment process. For *in vitro* protein digestibility, the GMO Panel considered that additional investigations were needed before providing any further recommendations in the form of guidance to applicants. An EFSA external scientific report, where various proteins of plant and animal origin were tested under specific gastrointestinal conditions, was published in 2019 (Mackie et al., 2019).

Subsequently, an Ad hoc Allergenicity working group of the GMO Panel was established to address to what extent the *in vitro* digestion test adds value to the allergenicity risk assessment of GM plants and the protein safety assessment in general, and consequently, published a statement entitled '*in vitro* protein digestibility tests in allergenicity and protein safety assessment of genetically modified plants' (EFSA GMO Panel, 2021).

The GMO Panel guidance document of 2017 did not consider broader aspects relating to IgE-cross-reactivity and *de novo* sensitisation prediction. Based on current knowledge, experience gained, and their relevance for the assessment of GMOs and food and feed derived from biotechnology, it is important to address the issue of predicting IgE-cross-reactivity and *de novo* sensitisation. Therefore, the Ad hoc Allergenicity Working Group was asked to deliver a Scientific Opinion on current gaps and future development needs for the overall allergenicity and protein safety assessment, which is the aim of this document. To support the drafting of this scientific opinion, EFSA organised an Allergenicity Risk Assessment event, entitled 'Workshop on allergenicity assessment – prediction', in June 2021<sup>1</sup> and published an event report (EFSA, 2021).

### 1.2. Terms of Reference

The European Food Safety Authority (EFSA) asked the Panel on Genetically Modified Organisms (GMO Panel) to develop a GMO Panel Scientific Opinion on development needs in allergenicity and protein safety assessment of food and feed derived from biotechnology. No guidelines for applicants are provided in this document as it is not a follow-up of previous guidance documents.

## 2. Data and methodologies

### 2.1. Data

In delivering this scientific opinion, the EFSA GMO Panel considered information from relevant scientific publications retrieved from the public domain. However, this Scientific Opinion is not intended to be a comprehensive review of the field. The GMO Panel also considered comments raised by a Stakeholder Consultative Group following the activities of the GMO Panel Allergenicity Working Group and the main outcomes of the Allergenicity Risk Assessment Workshop in June 2021, organised by the Allergenicity Working Group of the EFSA GMO Panel in collaboration with the Stakeholder Group. The aim of the workshop was to set the scene on the current state-of-the-art in the science of allergenicity assessment and to define the specific elements of such an assessment to develop to move forward (EFSA, 2021).<sup>1</sup>

### 2.2. Methodologies

The GMO Panel considered the principles described on allergenicity in its guidance documents, statements, and scientific opinions (EFSA GMO Panel, 2010, 2011, 2017, 2021), Regulation (EU) No 503/2013 and other relevant international guidelines (Codex Alimentarius, 2003–2009).

<sup>1</sup> <https://www.efsa.europa.eu/en/events/gmo-workshop-allergenicity-assessment>

### 3. Assessment

The formulation of specific research requirements for allergenicity assessment and protein safety, in general, is urgently needed in a world that demands more sustainable food systems (EFSA, 2019). The European Commission targets food and nutrition security challenges with research and innovation policies designed to future-proof the food systems – to become more sustainable, resilient, responsible, inclusive, diverse and competitive. Consequently, the FOOD 2030<sup>2</sup> initiative should generate futureproofing of our currently unsustainable food systems supporting alternative proteins and innovative food sources. Before any food or feed derived from biotechnology can be introduced into the EU market, a premarket safety assessment is undertaken to ensure the product's wholesomeness. Evaluating adverse immune reactions to proteins (hereafter referred to as 'allergenicity') is a challenging aspect of this safety assessment. Adverse reactions to foods may involve IgE-mediated hypersensitivity reactions or non-IgE-mediated conditions, such as the T-cell-mediated gluten-sensitive enteropathy, also named coeliac disease (Sampson and Anderson, 2000; Johansson et al., 2001; Mills et al., 2013a; Valenta et al., 2015; Anvari et al., 2019).

Current allergenicity risk assessment strategies are based on the principles and guidelines of the Codex Alimentarius for the safety assessment of foods derived from 'modern' biotechnology, which was initially published in 2003 (Codex Alimentarius, 2003–2009). Subsequently, the GMO Panel published Guidance Documents for the allergenicity assessment of GM plants (EFSA GMO Panel, 2011, 2017) that follows the main principles laid down by Codex Alimentarius (2003–2009). As no single piece of information or experimental method provide sufficient evidence to predict allergenicity, the core approach for the safety assessment is based on a 'weight-of-evidence' approach, where information of different nature is considered for the assessment of allergenicity (Codex Alimentarius, 2003–2009; EFSA GMO Panel, 2011, 2017; Regulation (EU) No 503/2013).

According to the Codex Alimentarius, each step of the safety assessment aims to provide assurance, in the light of the best available scientific knowledge, that the food does not cause harm when prepared, used and/or eaten according to its intended use. Due to the continuous scientific advances over the last two decades, there is a functional asynchrony between the availability of safety standards and concurrent scientific developments. EFSA and other risk assessment bodies are mandated to mitigate these gaps as much as possible (EFSA, 2021). This is in line with the principles described in the Codex Alimentarius (2003–2009), which states that the safety assessment should be reviewed in the light of new scientific information calling into question the conclusions of the original safety assessment. EFSA has been proactive in this respect and has already invested resources to advance the allergenicity prediction further. A series of EFSA procurements were undertaken, which resulted in several publications representing significant steps forward (Mills et al., 2013a,b; Mackie et al., 2019; Parenti et al., 2019; EFSA GMO Panel, 2017, 2021). Likewise, EU-funded research programmes, such as the ImpARAS Cost Action, EuroPrevall, iFAAM and AllerScreening projects, among others, also provide insights on the use and improvement of existing and suggested assessment tools in the field of allergenicity assessment of foods. However, significant knowledge gaps remain, and the development of novel approaches to deal with allergenicity assessment needs to be pursued further (EFSA, 2021).

This Scientific Opinion aims to: (i) define knowledge gaps on allergenicity prediction; (ii) identify specific research needs for improving the allergenicity risk assessment for products derived from biotechnology; (iii) determine how new basic research findings and technological developments can improve the current risk assessment methodology; and (iv) prioritise basic research funding.

#### 3.1. Allergenicity prediction in the safety assessment of foods derived from biotechnology

The international consensus on the safety assessment approach of foods derived from biotechnology is based on the principle of a comparative safety assessment, where their equivalence to a conventional counterpart with a history of safe use should be established. Allergenicity risk assessment is part of the information required for the hazard identification and hazard characterisation steps and other aspects such as the molecular characterisation, comparative analysis, potential toxicity or nutritional value of the resulting food. The risk assessment is completed by an exposure assessment and, eventually, by a risk characterisation step, as needed (EFSA GMO Panel, 2011; European

<sup>2</sup> <https://fit4food2030.eu/food-2030/>

Commission, 2013). For the assessment of proteins, the current paradigm builds on classical principles and methodologies developed for assessing small molecules chemicals. However, proteins are large and complex biopolymers that challenge this paradigm and present different hazard and exposure assessments (Fernandez Dumont et al., 2018). Since the human body handles proteins in a very different manner to small molecules, the safety assessment relies on information of a different nature to provide the necessary weight-of-evidence to estimate potential risks. On a case-by-case basis, this information may include *in silico* bioinformatic analysis, *in vitro* tests on protein stability, *in vivo* studies and dietary exposure.

However, for the allergenicity assessment, key pieces of knowledge are lacking, including consensus lists of clinically relevant allergens that are structurally well-characterised and have demonstrable potency in eliciting an allergic reaction. The recently published FAO/WHO consultation has identified consensus on reference doses for many major allergenic foods based on published data (Taylor et al., 2002; Ballmer-Weber et al., 2015; Bluemchen and Eiwegger, 2019; Houben et al., 2020; Remington et al., 2020; FAO/WHO, 2021a,b), as shown in Section 3.3.1. However, significant data gaps remain regarding the allergenic potency of other allergenic foods, and there are no clinical data on threshold doses for individual allergenic protein molecules. These gaps in knowledge make it challenging to define strategies that consider the exposure in the risk characterisation step and increase the uncertainty in the overall risk assessment process.

The prediction of allergenicity is also challenging because an allergic reaction to a protein depends upon a complex interplay between an individual's immune system and the protein. Allergic disease develops in a process comprising sensitisation to the allergenic food and subsequent elicitation of the allergic reaction. The resulting symptoms occur upon re-exposure to the allergen when administered in sufficient amounts (Renz et al., 2018). The allergenicity risk assessment considers the risks that a newly expressed protein or whole food poses to the existing allergic population by virtue of showing IgE cross-reactivity. Existing methods are available for assessing the allergenic potential of new proteins for cross-reactivity with a reasonable level of confidence. However, there are limited options to assess the hazard and potential risks of new proteins due to *de novo* sensitisation (Remington et al., 2018; Mazzucchelli et al., 2018). This is because, contrary to other safety assessment areas, such as the toxicity assessment for which well-validated animal models have been in place for years (e.g. OECD protocols for small molecules), no single test or parameter is currently available which provides sufficient evidence to predict *de novo* sensitisation. Moreover, the methods included in the current weight-of-evidence approach for the allergenicity assessment were designed for the assessment of individual proteins and are not easily applicable to foods developed by introducing traits of many different newly expressed proteins (EFSA GMO Panel, 2022a,b) or to complex mixtures of proteins that often make up whole foods (e.g. insects).

The current paradigm, according to Codex Alimentarius (2003–2009), is that potential safety concerns on allergenicity are raised when, for example, (i) reasonable evidence of IgE-mediated oral, respiratory or contact allergy or non-IgE allergy is available on the source of the introduced protein or on the protein itself; (ii) a newly expressed protein has sequence similarities to known allergens higher than 35%; and/or (iii) highly stable proteins leading to resistant fragments following the classical pepsin resistance are separated and visualised by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE).

Over the years and following Codex Alimentarius principles (2003–2009), most of the tools used in the allergenicity risk assessment focus on understanding the potential IgE binding properties of allergens, leading to the typical classification of allergens as 'major' (> 50% IgE-binding) and 'minor' (< 50% IgE-binding) (Løwenstein, 1978). However, this classification does not carry any connotation of allergenic potency but rather relates to the proportion of an allergic population that are sensitised to a given molecule (Matricardi et al., 2016). This is because this classification is mainly based on the frequency of IgE-binding in the population, especially detected *in vitro*, irrespective of clinical impact. Thus, there is a need for a better approach to evaluate the clinical importance of allergens along with prevalence in a population.

### 3.1.1. Clinical relevance of food allergens

The characterisation of an allergen involves from the analysis of its IgE antibody binding capacity to the demonstration of clinical relevance. Moreover, the characterisation of all allergens is a challenging and comprehensive process that also includes physicochemical properties, biological function and

structure determination (Caraballo et al., 2020). An allergen becomes clinically relevant when it causes symptoms and is corroborated by medical history and/or provocation testing (Worm et al., 2021).

The clinical relevance of individual food allergens should be a key driver for developing new strategies and tools for allergenicity risk assessment (EFSA, 2021). To achieve this goal, it is necessary to rely on clinical data of good quality and to determine criteria for describing the allergenicity of single proteins. However, the factors that may determine a convincing history of an IgE-mediated allergic reaction to a specific food are still controversial. Likewise, it is challenging to define 'minimal criteria' for food allergy (Asai et al., 2020).

It is well accepted that individuals are often sensitised to a food or allergen molecule but are still able to consume food without experiencing an allergic reaction, and is one reason why double-blind placebo-controlled oral food challenges (DBPCFC) are considered the gold standard for a diagnosis of food allergy (Sicherer and Sampson, 2018). Consequently, criteria have been developed to identify allergenic foods of public health importance where oral food challenges play a crucial role in demonstrating clinical relevance, i.e. the capacity of a food to elicit an allergic reaction in an allergic individual (Björkstén et al., 2008; Chung et al., 2012). Thus, although sensitisation is a predisposing risk factor for IgE-mediated food allergy, neither a quantitative positive specific IgE test result nor a positive skin prick test can prove the clinical relevance of a food extract or purified molecule. The ultimate means of determining the clinical relevance of an allergen molecule would be to perform a provocation test with a purified allergen molecule, as is undertaken with inhalant allergens used for immunotherapy. However, data from such studies are lacking, and new alternatives are required. Therefore, there is a need for consensus definitions of clinically relevant allergens, and these should build on data available for component-resolved diagnostics in allergic patients, with some initiatives being recently proposed (Caraballo et al., 2020, 2021). A crucial aspect of such definitions relates to the source and quality of the diagnosis of the allergic population used to define an allergen.

The clinical relevance of allergens could include criteria such as (i) the severity (i.e. the proportion of severe objective allergic symptoms to the potential allergen); (ii) the potency (i.e. the amount of the potential allergen required to cause objective symptoms); (iii) the prevalence of immune-mediated hypersensitivity to the potential allergen source; and iv) the exposure route that the allergen presents to the immune system and the level of exposure. Recently, an Ad hoc Joint FAO/WHO Expert Consultation on Risk Assessment of Food Allergens reviewed and validated the Codex priority allergen list based on systematic and thorough assessments using prevalence, severity and potency as key criteria (FAO/WHO, 2021a).

In addition, the definition of a set of non/low-allergenic (control) proteins is needed. One initiative has been proposed by Krutz et al. (2019). Briefly, the main principle assumes that proteins to which humans are known to have significant exposure (such as proteins from spinach, corn, potato, rice, tomato or wheat), but that are not (or only rarely) associated with allergy, can be classified as having low (or even absent) sensitising potential.

Finally, in the last years, allergic diseases in animals have gained great prominence in veterinary practice. However, very few studies are currently available (mainly in dogs and horses), which provide evidence of the allergens involved, but it is unclear whether these allergens are similar to those in humans (Mueller et al., 2018). Furthermore, the prevalence of food allergy in animals is largely unknown, and additional efforts in this field are needed (Pali-Scholl et al., 2017, 2019).

### 3.1.2. Determinants of food protein allergenicity

#### 3.1.2.1. Intrinsic and extrinsic properties of food allergens

Despite many approaches aimed at understanding what makes a food protein an allergen (Huby et al., 2000; Helm, 2001; Bannon, 2004; Scheurer et al., 2015; Costa et al., 2020, 2021), the underlying reasons why proteins or peptides become allergenic in susceptible individuals is not fully understood (EFSA GMO Panel, 2010, 2011, 2017; EFSA NDA Panel, 2014). The molecular determinants of allergenicity depend on the protein sequence with contributions from protein structure and dynamics (James et al., 2018).

It has long been recognised that food and pollen allergens belong to a limited number of protein superfamilies (Jenkins et al., 2005; Radauer and Breiteneder 2006; Jenkins et al., 2007; Radauer et al., 2008). Although these protein family scaffolds are associated with allergenicity, there are no single common structural causes, features or sequence motifs identified that contribute to their overall allergenicity.

However, not all members of a certain protein family are allergens, and many allergens do not exhibit any known physicochemical, functional or structural properties that account for their allergenicity (Scheurer et al., 2015; Costa et al., 2020, 2021). Interestingly, recent studies reported differences in biophysical properties and structural dynamics between shrimp and pig tropomyosins, despite their high degree of conservation, which may explain differences in their allergenic potential (James et al., 2018; James and Nanda, 2020). Finally, although abundance might not be a universal characteristic of all food allergens, it seems to be a predisposing factor that enhances their chance to interact with the immune system, when coupled with other biochemical characteristics, that could produce a food allergen (Bannon, 2004; Foo and Mueller, 2021).

Nevertheless, there are possibly a few distinct biochemical characteristics associated with the different protein families that mainly correlate to the elicitation capacity of certain allergens. These characteristics are determined by the 3D structure of proteins, which confer the physicochemical and biological properties governing protein stability, such as the capacity to bind ligands (ranging from metal ions to lipids) and/or resistance to protease degradation and thermal stability (Radauer et al., 2008; EFSA GMO Panel, 2017, 2021; Foo and Mueller, 2021). Ligands generally increase the stability of allergens to thermal and/or proteolytic degradation (Moreno et al., 2005; Vassilopoulou et al., 2006; Bossios et al., 2011; Berecz et al., 2013; Petersen et al., 2014) and can also act as immunomodulatory agents that favour Th2 polarisation. However, some exceptions have been reported, as is the case of wheat LTP, whose ligand binding properties enhanced its conformational flexibility resulting in increased susceptibility to gastroduodenal proteolysis (Abdullah et al., 2016). Moreover, ligand-binding allergens expose the immune system to a variety of biologically active small molecules that could play important and still not well-understood roles in the sensitisation process in addition to the allergenic protein itself (Chruszcz et al., 2021).

Post-translational modifications (PTMs), such as disulphide bond formation (Apostolovic et al., 2016), have also been identified as additional important determinants for preserving allergenic properties in digestion-resulting peptides. Ideally, stable breakdown protein fragments should be characterised and evaluated with regard to the potential to cause adverse health effects linked to their biological activity (Bøgh and Madsen, 2016; EFSA GMO Panel, 2017). However, the appropriate methodology is currently unavailable (EFSA GMO Panel, 2021). Likewise, optimal IgE binding to linear epitopes of important allergens from timothy grass pollen (Phl p 1) (Petersen et al., 1998) and peanut (Ara h 2) (Bernard et al., 2015) requires post-translational hydroxylation of proline residues. The in-depth characterisation of potential PTMs on allergens warrants further research because the information is limited in some cases. This was the case of clinically relevant grass pollen and house dust mite allergens, which through a glycoproteomic analysis using a powerful analytical approach (i.e. orbitrap-based mass spectrometry with complementary fragmentation techniques for site-specific PTM characterisation) revealed novel PTMs. These were based on more complex glycan structures than previously reported and could play important roles in allergen recognition and response by the immune system (Halim et al., 2015). Nevertheless, according to the current state-of-the-art, PTMs are not a prerequisite for a high probability of allergenicity (Costa et al., 2020).

### 3.1.2.2. Environmental and other factors influencing protein allergenicity

In addition to the allergen itself, environmental factors may play a role. These include different routes of exposure, the timing of exposure, microbial exposure, oral and gut microbiota composition in case of oral exposure, epithelial barrier integrity and/or non-allergenic components of the food matrix such as immune-modulating components (adjuvants) of allergenic sources that facilitate T helper 2 (Th2) immune responses (Scheurer et al., 2015; Valenta et al., 2015). Human related factors (e.g. genetic factors such as mutations in the filaggrin genes, *SPINK5* and *SERPINB7*) and co-factors such as alcohol, anti-inflammatory drugs, infection, exercise or stress (Dua et al., 2020) could potentially reduce the barrier function of the intestinal epithelium and facilitate sensitisation and impact elicitation (Groschwitz and Hogan, 2009; Irvine et al., 2011; Perrier and Corthésy, 2011; Valenta et al., 2015; Breiteneder et al., 2020). More recently, glycosylation (specifically, sialylation) of IgE has been reported as an important regulator of allergic disease (Shade et al., 2020).

Possible links between the proteins' biological function/activity and their allergenicity are emerging (Ozias-Akins & Breiteneder, 2019; Foo and Mueller, 2021). For example, the proteolytic activity of some food allergens might contribute to the sensitisation process via different mechanisms such as the cleavage of certain proteinase-activated receptors leading to the release of pro-inflammatory cytokines (Cayrol et al., 2018; Scott et al., 2018; Dietz et al., 2019) or through the direct proteolysis of tight

junctions, and other extracellular structures enhancing the intestinal epithelial barrier permeability (Grozdanovic et al., 2016).

The route of allergen exposure may be an additional key driver in food allergy. Historically, the oral route of exposure has been the focus (Tordesillas and Berin, 2018). However, the other routes of exposure may also be relevant for sensitisation (Wavrin et al. 2015; du Toit et al., 2016; van Bilsen et al., 2017). For example, peanut exposure via impaired skin or the airway may lead to sensitisation (Kulis et al., 2021).

### 3.1.2.3. Adjuvant properties of food components

An adjuvant is a substance that augments the body's immune response to an antigen. They typically enhance the immunogenicity and/or allergenicity of unrelated proteins, but they are not usually immunogenic or allergenic. They are mainly lipids and glycans, sometimes minerals, and oils, and bacterial proteins, which enhances adaptive immune and allergic responses via the innate immune system.

In the clinical setting, vaccines and subcutaneous allergen immunotherapy products contain adjuvants. For allergen immunotherapy, the adjuvants are mainly aluminium phosphate, aluminium hydroxide (alum), aluminium monostearate (Jensen-Jarolim, 2015). While some vaccines also contain aluminium adjuvants, there are more options, including MF59 (derivative of squalene used as oil-in-water adjuvant), AS03 (squalene-base, DL- $\alpha$ -tocopherol, polysorbate 80), AS01 (liposome-based adjuvant containing 3-O-desacyl-4'-monophosphoryl lipid A from *Salmonella minnesota* and QS-21, saponins from *Quillaja saponaria Molina*), and AS04 (aluminium hydroxide and monophosphoryl lipid A), Pam3CSK4 (triacylated lipopeptide and TLR2/TLR1 ligand), Pam2CSK4 (diacylated lipopeptide), MPLA (monophosphoryl lipid A), saponins (plant-based), oligonucleotides – CpG, polyI:C and flagellin (globular protein in flagellated bacteria).

Adjuvants have been extensively used in basic immunology research to induce immune reactions in animals and skew these responses towards a Th1- or Th2-type pathway. Some examples include an emulsion of foreign protein with Freund's adjuvant to induce a Th1 response or precipitating a protein in aluminium hydroxide (alum) to generate a Th2 reaction. Researchers have also used bacterial products like pertussis and cholera toxins and lipopolysaccharide (LPS). Many *in vivo* food allergy animal models, most notably mice and rats, use adjuvants like cholera toxin to induce the disease.

There are also exogenous 'Th2 adjuvants' like glycans (e.g. N-glycans from *Schistosoma mansoni* egg antigens), lipids, mast cell and basophil-activating molecules, proteases, chitin, arachidonic acid metabolites. Other potential Th2 adjuvants are lectins, such as concanavalin A, colectins, adhesins, some galectins, selectins and mistletoe lectin I (ML-I), appear to enhance allergic responses *in vitro* and, in some cases, *in vivo* (Lavelle et al., 2001; Reyna-Margarita et al., 2019). The role of these adjuvants in food allergy, when present in foods and co-delivered with food proteins, is not currently well understood.

The role of intrinsic structural and functional features of some ingested food proteins that result in immune stimulation in the development of food allergy is also not well understood. Indeed, to date, there is little evidence that food proteins are adjuvants. There is some evidence that some food proteins have innate immune-stimulatory properties due to features such as glycosylation, lipid-binding and enzymatic activity (Ruiter and Shreffler, 2012). For instance, glycan structures on glycoproteins from peanuts, insects, and crustaceans *in vitro* can activate dendritic cells, enhance antigen uptake and potentially contribute to allergen sensitisation (Shreffler et al., 2006). However, it is not clear whether these immunostimulatory activities play a role *in vivo* at the concentrations present in ingested food.

Without evidence that ingested food proteins are adjuvants, the likelihood that a GMO protein or proteins have adjuvant properties is low. To date, there is no evidence that intact GMOs or isolated or recombinant GMO proteins at the levels expressed have adjuvant properties *in vivo*.

Overall, there are naturally occurring molecules found in whole foods like plant lectins, glycosylated proteins, lipids, proteases, phytoprostanes and chitin with potential adjuvant activity, though not confirmed as adjuvants that increase sensitisation to food allergens or symptom severity. However, there is a report illustrating that some allergenic foods (e.g. peanut, egg, and milk) bind and activate dendritic cells *in vitro* while other non-allergenic foods like chickpeas and corn do not (Kamalakkannan et al., 2016), suggesting that there are glycoproteins in food that might increase the allergenicity of the whole food.

### 3.1.2.4. Food matrix and processing

The EFSA GMO guidance document (EFSA GMO Panel, 2017) and the related statement on *in vitro* protein digestibility (EFSA GMO Panel, 2021) acknowledge the importance of the food matrix and food processing in the digestibility of food allergens and in the potential to trigger an immune response. However, the monitoring of individual newly expressed proteins in a matrix could be technically difficult because they are normally present at low levels. In addition, methods included in the current weight-of-evidence approach for allergenicity assessment were designed for the assessment of individual proteins and are not easy to apply to whole foods that may contain dozens to hundreds of different proteins (EFSA GMO Panel, 2022a,b). Furthermore, the safety assessment of GMOs normally covers any use of GM plants for food/feed purposes. This makes the overall assessment challenging because of the potential need to test all the possible food matrices and food processing conditions that the GM plants might undergo when released into the market.

The impact of processing, especially thermal treatments that most foods undergo, is important to understand the structural traits of food allergens at the molecular level (Nowak-Węgrzyn and Fiocchi, 2009; Wickham et al., 2009). Heat treatments induce chemical/physical modifications, which may affect the stability of enzymatic digestion and, consequently, the allergenicity of food proteins to a varying extent, depending on the time and temperature (Di Stasio et al., 2020). Physical stability (aggregation ability) of some allergens highly labile to digestion (e.g. bovine milk caseins, Ara h 1, etc.) is a key parameter that explains their allergenic capacity (Bøgh et al., 2009, 2012; Radosavljević et al., 2020). In addition, the homogenisation of milk could lead to an increase in allergic reactions because this non-thermal processing results in a large number of lipid droplets adsorbing caseins and whey proteins, as described by Poulsen et al. (1987), Høst and Samuelsson (1988) and Geiselhart et al. (2021). However, this effect could not be confirmed by other authors, indicating that the impact of homogenisation and other technological processes on the allergenic properties of milk proteins requires further clarification (Michalski and Januel, 2006; Michalski, 2007). Interestingly, adjuvant effects in food could arise from the Maillard reaction. Cooking or heating food may lead to the production of advanced glycation end-products of food proteins. In a food allergy model, increased expression of the receptor for advanced glycation end-products on dendritic cells enhanced T-cell responses (Hilmenyuk et al., 2010), thus, suggesting that cooking or heating may increase the allergenicity of ingested food proteins.

Unfortunately, most of the investigations have been limited to single purified allergens (Koppelman et al., 2010; Bøgh and Madsen, 2016; Pekar et al., 2018), pointing out that the stability of allergens within their natural matrix upon heat treatments and the elicitation properties of the resulting digestion products have been poorly explored (Prodic et al., 2018; Di Stasio et al., 2020; Mattar et al., 2021). In addition, the assessment process mainly focuses on the properties of the intact proteins, even though they change during passage through the gastrointestinal tract (GIT). Moreover, certain food protein fragments that are stable to digestion, like gluten proteins, might be even more hazardous than the intact protein. Coeliac disease is activated when intact gluten peptides pass through the intestinal epithelium into the lamina propria where they are deamidated by tissue transglutaminase, which activates the peptides for CD4<sup>+</sup> T-cell binding via the human leukocyte antigen (HLA)-DQ 2 or 8 cell surface receptors (Shan et al., 2002; Fernandez et al., 2019; Pilolli et al., 2019). Other studies such as that by Prodic et al. (2018) showed that a peptide's ability (e.g. LTPs) to hold together and adopt a three-dimensional (3D) structure, similar to the native protein under certain conditions, allows them to retain their *in vivo* allergenic activity (Vassilopolou et al., 2006).

## 3.2. Risk assessment tools for allergenicity prediction: current stage and improvement needs

The purpose of the allergenicity assessment for products derived from biotechnology mainly focuses on the assessment of newly expressed proteins. For the risk assessment, it is necessary to include information on the source of the gene/protein (history of use), the amino acid sequence for performing similarity searches, and on structural properties such as susceptibility to enzymatic degradation. For the latter, although the isolation or purification of the newly expressed protein is needed, it might not be possible or practical because of the presence of a large number of proteins or technical difficulties of intractable proteins (Bushey et al., 2014; Eaton et al., 2017). Synthetic biology-derived plants (and their derived food and feed products) may arrive on the market in the near future

with an increased level of complexity compared to conventional GM plants (e.g. composition, number of newly proteins expressed) (EFSA GMO Panel, 2022a).

For non-IgE-mediated adverse immune reactions to foods, detailed risk assessment considerations were provided by the EFSA GMO Panel on the safety profiles of the protein or peptide under assessment with regard to its potential to cause coeliac disease. This assessment includes available information on the source of the transgene, on the protein itself, and *in silico* and *in vitro* data, when appropriate (EFSA GMO Panel, 2017).

An additional aspect considered in the allergenicity assessment is the evaluation of the whole food and feed to ensure that the genetic modification does not affect the levels or characteristics of endogenous compounds that would adversely impact human and animal health (König et al., 2004; EFSA GMO Panel, 2011; Fernandez et al., 2013). The latest EFSA GMO Panel guidance on allergenicity provides detailed information on the current stage and improvement needs for this topic (EFSA GMO Panel, 2017).

The following sections below will address the current allergenicity risk assessment tools in place for the safety assessment of newly expressed proteins, providing insights on their usefulness and relevance within the current weight-of-evidence approach, as well as the identification of potential improvement needs in terms of alternative and/or complementary tools.

### 3.2.1. *In silico* tools

The current practice for the *in silico* assessment of a protein consists of an amino acid sequence similarity search against an allergen database and a sliding window analysis designed to evaluate the extent to which the protein under assessment is similar in structure to a known allergen. The amino acid sequence homology comparison is performed using publicly available search engines such as the FASTA local alignment algorithm (Pearson and Lipman, 1988) or the Basic Local Alignment Search Algorithm (BLAST) (Altschul et al., 1990) and a default threshold value of 35% identity over at least 80 amino acids established by an FAO/WHO scientific advisory panel in 2001. Such an approach was adopted by Codex Alimentarius (2003–2009) and, subsequently, by EFSA (EFSA GMO Panel, 2010, 2011). This strategy is highly conservative and untargeted for current assessment purposes, also considering the follow-up actions required in case of relevant hits with known allergens are identified. This is because the original *in silico* approach was defined for the assessment of few individual proteins, and it was mainly based on knowledge about birch pollen homologues belonging to the same protein family, i.e. the pathogenesis-related proteins 10 family (PR-10). Furthermore, this approach has been considered inadequate when broadly applied to a large number of protein sequences, such as for the assessment of putative open reading frames (Harper et al., 2012).

The highly conservative current approach appears to lead to a high number of false positives (Ladics et al., 2007; Abdelmoteleb et al., 2021; Herman et al., 2021). A full FASTA approach with appropriate match criteria has claimed to be as sensitive as the 35% identity over an 80-aa sliding window approach, while the specificity is significantly higher (Ladics et al., 2007; Silvanovich et al., 2009; Abdelmoteleb et al., 2021). Conversely, there are studies reporting experimental IgE cross-reactivity between proteins despite a low sequence identity (i.e. below 35% sequence identity) (D'Avino et al., 2011; Guhsl et al., 2014; Dubiela et al., 2018).

Two important additional considerations for homology comparisons are as follows:

- i) The *in silico* approaches are used as a first step in identifying relevant identity between a newly expressed protein and a known allergen before other confirmatory but more laborious testing are required, such as *in vitro* and/or *in vivo* studies. However, the *in silico* tool only informs about the capacity of a protein to cross-react with IgE directed towards a known allergen. Briefly, if relevant shared sequence identity is observed with a known allergen (currently a sequence identity higher than 35% over at least 80 amino acids as defined by FAO/WHO in 2001), subsequent serum IgE binding studies using sera from individuals with a specific, relevant type of allergy would likely follow, as established by Codex Alimentarius (2003–2009). The absence of sequence homology indicates that a newly expressed protein is unlikely to be cross-reactive with IgE directed towards known allergens. However, current *in silico* tools used in the allergenicity assessment does not provide information on the capacity of proteins for *de novo* sensitisation. By considering the current framework, the amino acid sequence homology comparison does not possess the capacity to predict on its own for the allergenicity risk assessment of newly expressed proteins, and additional pieces of information are needed to conclude the allergenicity assessment.

- ii) The allergen sequence databases<sup>3</sup> used for sequence comparison have a strong influence on the outcome of the *in silico* analysis. The allergen sequence databases currently in use for the allergenicity risk assessment do not all provide systematic information on the allergenic potential of entries, and the inclusion criteria used are often different between databases (Mazzucchelli et al., 2018; Radauer and Breiteneder, 2019). Discrepancies in the quantity and quality of entries between existing databases are documented evidence of the lack of consensus on the inclusion criteria for building a reliable database. This aspect might be a source of inconsistent opinions depending on the database used for the sequence identity search or resources available for data curation and maintenance. Following current approaches, whenever a relevant hit with a known allergen is identified, the follow-up risk assessment strategy analyses the quality of the pairwise sequence alignment, and testing using human sera is also required. The clinical relevance of the known allergen is usually considered only as an additional element in the overall evaluation. The current approach relies heavily on expert judgement to interpret *a posteriori* the outcome of the bioinformatic analysis, which can lead to a lack of harmonisation, reproducibility, and transparency of the risk assessment.

Other bioinformatic approaches for predicting the allergenic potential of proteins have been developed that differ from those defined by Codex and which might provide higher sensitivity, specificity, and accuracy than the classical FASTA algorithm. These also include alternative or complementary approaches beyond sequence alignment principles as defined by Codex. Some selected examples of alternative *in silico* approaches are (i) increasing the match criteria above 35% identity and decreasing the *E*-score below  $1e^{-7}$  or smaller (Abdelmoteleb et al., 2021); (ii) numerical descriptors representing the physicochemical properties of the amino acid in protein sequence and machine learning approach for classification of allergens (Dimitrov et al., 2013, 2014a); (iii) similarity of their 3D protein structure as well as their amino acid sequence (Maurer-Stroh et al., 2019); (iv) similarity search to a data set of allergenic and non-allergenic proteins represented as binary fingerprints (Dimitrov et al., 2014b); (v) machine learning approaches based on mapping of IgE epitope, motif search and/or other selected variables (Westerhout et al., 2019; Sharma et al., 2021); (vi) as well as novel approaches considering human leucocyte antigens (HLA) binders from known allergens for the *in silico* assessment of the sensitisation potential of innovative/novel proteins (Dimitrov & Atanasova, 2020).

These advanced bioinformatic tools provide new opportunities to develop novel approaches that reduce uncertainties and improve allergenicity prediction. However, further work is needed to validate these new approaches by using an appropriate set of positive and negative control allergens. Therefore, the definition of control proteins that can be used to test specific hypotheses relevant for allergenicity assessment is of paramount importance (Table 1). In July 2021, EFSA launched a procurement<sup>4</sup> focusing the attention in this direction.

The *in silico* criteria for the risk assessment of new proteins and their potential to cause coeliac disease were delineated in the most recent guidance on the allergenicity of the EFSA GMO Panel (2017). These were based on searches for sequence identity (e.g. searches with known coeliac disease peptide sequences and motif searches) and, if concerns from the sequence identity search were raised, in a second step, *in silico* peptide modelling can be applied. New recent approaches have been developed based on: (i) the definition of clear inclusion criteria for database formation (Sollid et al., 2020; Fernandez et al., 2021)<sup>5</sup>; (ii) the ranking of T-cell epitopes according to their clinical relevance and related features (Vriz et al., 2021); and (iii) the development of a software tool for peptide binding prediction to HLA-DQ2 and/or HLA-DQ8 proteins and to predict their binding affinities, specially designed and developed for EFSA.<sup>6</sup> These elements could be useful in the future, when proven predictive, for reshaping the risk assessment strategy of innovative proteins and their potential to trigger coeliac disease.

<sup>3</sup> For example: <http://www.allergenonline.org/>; <https://www.allergome.org/>; <http://www.allermatch.org/>; <https://comparedatabase.org/>; <http://allergen.org/>

<sup>4</sup> <https://etendering.ted.europa.eu/cft/cft-display.html?cftId=8829>

<sup>5</sup> <https://croplife.org/plant-biotechnology/celiac-peptide-database>

<sup>6</sup> <https://ted.europa.eu/udl?uri=TED:NOTICE:129869-2020:TEXT:EN:HTML>

**Table 1:** Challenges and research needs identified by the EFSA GMO Panel for *in silico* tools used in the allergenicity risk assessment of foods derived from biotechnology

| <b>Challenges necessary to improve the reliability and predictability of the allergenicity risk assessment</b>  | <b>Research needs</b>  |
|---|--|
| <p>To identify the relevant <i>in silico</i> approaches to improve sensitivity, specificity and accuracy compared with the classical sequence alignment algorithms for assessing the allergenic potential of food proteins (using IgE cross-reactivity).</p> <p>To develop <i>in silico</i> methods with the capacity to assess the hazard and potential risks of new proteins resulting from <i>de novo</i> sensitisation.</p> | <p>To validate alternative bioinformatics approaches using a series of well-defined positive and negative control allergens.</p> <p>To determine if cut-off values or ranges can be set for risk assessment purposes and fit into the sensitisation and elicitation scenarios.</p>                 |
| <p>To refine and harmonise the existing allergen databases to create more targeted/fit-for-purpose databases for the allergenicity risk assessment. To ensure data curation and maintenance.</p>  | <p>To only include well-defined and characterised allergens in the allergen databases following reliable and consensual inclusion criteria. To introduce follow up actions when specific hits upon identification of known allergens. To identify resources for data curation and maintenance.</p> |

### 3.2.2. *In vitro* tools

The *in vitro* tools currently in place in the weight-of-evidence approach for allergenicity assessment include the classical pepsin resistance test and immunological assays (e.g. immunoblots) if sera are available (Codex Alimentarius 2003–2009; EFSA GMO Panel, 2010, 2011).

The pepsin resistance test is performed regularly, although several studies have demonstrated that there is a poor correlation between resistance to pepsin digestion and allergenicity (Kenna and Evans, 2000; Fu et al., 2002; Takagi et al., 2003; Thomas et al., 2004; Herman et al., 2007; Ofori-Anti et al., 2008; Costa et al., 2020). In contrast, other studies show that the classical pepsin resistance assay and simple SDS–PAGE analysis, as developed by Astwood et al. (1996), can distinguish between pepsin susceptible and resistant proteins and remains as the most useful assessment of the potential exposure of an intact newly expressed protein as part of product safety assessment within a weight-of-evidence approach (Wang et al., 2017, 2020). However, these studies only used small sets of proteins and a larger reference set is needed to make definite conclusions on the predictability of digestion tests. Furthermore, Foster et al. (2013) reported that analysis of pepsin-resistant fragments could improve the power of the pepsin test to discriminate between allergens and non-allergens when studied in their native form. This controversy was previously pointed out in the statement on *in vitro* protein digestibility tests published by the EFSA GMO Panel (2021).

More recently, a series of *in vitro* models to assess antigen uptake via the intestinal mucosal barrier, epithelium and dendritic cell activation and migration, and T- and B-cell differentiation, have been identified to evaluate the potential sensitising capacity of food proteins (Lozano-Ojalvo et al., 2019).

Finally, the types of test items used in *in vitro* studies performed for regulatory purposes are important. For example, in the GMO area, *in vitro* studies are mostly carried out on purified newly expressed proteins because their expression levels *in planta* are usually very low. In addition, the safety assessment of these products should cover any use of GM plants for food/feed purposes, which makes the overall assessment a challenge (EFSA GMO Panel, 2021).

#### 3.2.2.1. Use of protein digestibility data in allergenicity risk assessment

In January 2021, the GMO Panel delivered a statement addressing the usefulness of *in vitro* protein digestion in allergenicity and protein safety assessment (EFSA GMO Panel, 2021). The highlights were:

- the classical pepsin resistance test, as currently used, is not an *in vitro* digestibility test designed to mimic the physiologic conditions of gastric digestion.
- the evidence supporting the resistance to degradation by pepsin as a direct predictor of allergy is weak.
- the information that the classical pepsin resistance test can provide is on the stability of the proteins under acidic conditions. However, there are other methods that can be used to obtain data on a protein's structural and/or functional integrity.

- for future development, there is a need for more reliable systems to predict digestion, to better understand the fate of the protein/fragments in the GIT and how they interact with the relevant cells in the human body.

A series of general and specific research questions were formulated in the statement on *in vitro* protein digestion in allergenicity and protein safety assessment of the EFSA GMO Panel (2021). This Scientific Opinion provides additional suggestions to the general questions whereas the specific questions would require dedicated research programs/procurements to be fully addressed.

#### General questions

- *What is the usefulness of in vitro digestion in the overall protein safety assessment?*

Digestibility studies may provide useful data regarding the properties and characteristics of proteins. This is important for understanding their presentation to the gastrointestinal mucosal immune system (gastrointestinal luminal processing) and uptake into the body (Akkerdaas et al., 2018; EFSA GMO Panel, 2021). Both processes can affect the generation of specific IgE-sensitisation and elicitation of reactions in allergic individuals. In addition to resistance to extracellular digestion by gastrointestinal proteases, the resistance to endosomal degradation (i.e. digestion within the antigen-processing and presenting cells (APC) of the immune system, such as dendritic cells) and its relationship with a protein's capability to act as an allergen has been less studied (Foster et al., 2013; Machado et al., 2016; Soh et al., 2019; Kamath et al., 2020). To be recognised as an allergen, exogenous antigens must first be internalised into the endosome of APC and then are subjected to endosomal degradation, where they are exposed to cathepsin proteases under increasingly acidic and reducing conditions. The resulting peptide fragments are loaded onto the class two major histocompatibility complex (MHCI) and presented on the cell surface for recognition by T-cell receptors (Foo and Mueller, 2021). Moreover, the intestinal barrier has a crucial role in protecting the organism against pathogens and possibly harmful substances derived from the external environment (Cardoso-Silva et al., 2019). A dysfunctional GIT barrier makes a key contribution to food allergic reactions, and, more concretely, the physiological gastrointestinal barrier seems to play an essential role in food allergy (Samadi et al., 2018). Thus, factors such as food processing, digestion, and transport (including internal processing and presentation to the immune cells) should be ideally included in an allergenicity assessment assay; however, it is crucial to consider the feasibility and practicality of including these factors (EFSA GMO Panel, 2017, 2021; Smits et al., 2021). Likewise, new data have indicated that the GIT is a reservoir of IgE<sup>+</sup> B lineage cells in food allergy in peanut-allergic patients, whereas mice cannot switch from IgA to IgE due to the ordering of isotypes in their IgH locus (Hoh et al., 2020). These data suggest that B cell differentiation pathways in patients who develop food allergy differ from those in patients with aeroallergies, and potentially that food allergy sensitisation or allergen-specific B cell clonal expansion may occur in oral or gastrointestinal mucosa (Hoh et al., 2020), supporting the relevance of the gastrointestinal environment in food allergy.

Protein digestibility plays a central role for the risk assessment of coeliac disease, where gastrointestinal digestion is important in the delivery of immunologically active fragments to gastrointestinal mucosal segments (Shan et al., 2002; Pilolli et al., 2019; Vriz et al., 2021). The proline-rich nature of gluten renders these proteins resistant to degradation by enzymes in the GIT resulting in the generation of relatively long, persistent gluten peptide fragments in the small intestine. Thus, the resistance to proteolytic degradation contributes to the allergenic nature of gluten peptides (Shan et al., 2002) together with specific recognition by the transglutaminase 2 present in the GIT and peptide-binding properties of HLA-DQ2.5 and HLA-DQ8 (EFSA GMO Panel, 2017).

- *What are the most suitable in vitro digestion models?*

Gastrointestinal digestion is a dynamic, complex, highly integrated and regulated process, which makes it challenging to replicate *in vitro*. The pepsin resistance test is a biochemical surrogate of 'protein stability' under acidic conditions and does not provide sufficient information on gastric digestion. It is well known that variations in assay conditions (pH and pepsin:protein ratio values) have a large impact on the digestibility of proteins *in vitro*, and a ring-trial validated protocol for pepsin resistance assays has subsequently become a *de facto* standard method (Thomas et al., 2004). This method does not (and does not seek to) replicate human *in vivo* digestion but serves as a standardised method for comparing the pepsin resistance of proteins in a well-understood context (Pickles et al., 2014). Interestingly, data from the pepsin resistance test measuring resistance to degradation by pepsin is currently used in a weight-of-evidence approach to assessing not only the potential allergenicity but also the potential toxicity of newly expressed proteins in GM plants (EFSA

GMO Panel, 2021). However, according to the Codex Alimentarius (2003–2009), the assessment of potential toxicity should consider, among other aspects, the stability of the protein to degradation in suitable representative gastric and intestinal model systems.

*In vitro* gastroduodenal digestion methods that use physiological conditions may reveal more information about protein presentation to the gastrointestinal epithelium in a physiologically relevant context (EFSA GMO Panel, 2021). However, there are gaps in gastroduodenal *in vitro* digestibility protocols that prevent their potential application at short-term in a risk assessment context:

- There has been little work on the applicability of these assays to new proteins, and the number of control proteins included in these studies is low.
- Validation required to suit the needs of foods derived from modern biotechnology risk assessment regarding (i) levels and type of enzymes and biosurfactants (these change with age, health status, food composition) (EFSA GMO Panel, 2017); (ii) type of material to be tested; and (iii) read-out to be used (SDS-PAGE, chromatographic and spectrometric techniques to monitor peptide profile, bioactivity measurements).
- Their reliability of predictions in the allergenicity assessment remains to be determined.
- *What are the optimal items to test in such models?*

The *in vitro* pepsin resistance test was initially developed to assess individual and abundant proteins (Astwood et al., 1996; Metcalfe et al., 1996). The use of test materials of higher complexity than that of purified single proteins could require the fine-tuning of the read-outs because more sensitive and higher resolution detection analytical methods could be needed to monitor the *in vitro* digestion tests. Ideally, the more representative test material, the better the results of the test. However, the test material could vary depending on the nature of the product to be assessed. For instance, in the case of intractable proteins or transcription factors expressed at a very low level, an extract from edible plant tissues could be a more appropriate material to have functional and active proteins than the use of heterologous expression systems.

Food matrix and processing may play an important role in modulating the digestibility rate of proteins. For example, *in vitro* digestion studies of purified Ara h 3 allergen revealed that this allergen is labile to pepsin-digestion and, therefore, it is unlikely to sensitise via the GIT and cause systemic food allergy symptoms (van Boxtel et al., 2008). However, the harmonised *in vitro* INFOGEST oral-gastro-duodenal digestion sequential model found contradictory results. This model was complemented with a brush border membrane step proteomics and immunochemical assays to track the metabolic fate of allergens in a food matrix. It showed that the food matrix impacts enzymatic degradation of peanuts with digestion leading to previously undetected large fragments of Ara h 3 (ranging from 7 to 21 kDa by western blotting and from 0.8 to 5 kDa by mass spectrometry) that survived *in vitro* human digestion and still harboured IgE-binding sequences (Di Stasio et al., 2017). A possible explanation of this finding is a 'masking effect' of the peanut matrix that delays or impairs protein degradation and alters the pattern of the peptide fragments released by proteolysis. However, Ara h 3 is one of the most abundant proteins in peanuts, while the interpretation of data derived from *in vitro* digestion studies of proteins expressed at low levels within a complex food matrix is technically difficult, as explained above (Section 3.1.2.4). A similar effect is observed with peanuts baked in a muffin matrix, although when presented in a cookie or chocolate dessert matrix used in the iFAAM project for food challenges, the peanut allergens are highly digestible (Rao et al., 2020; Mattar et al., 2021). Similar results are seen with gluten proteins which, when presented in a purified solubilised gliadin fraction, provide highly digestible, but when baked their digestibility was radically reduced (Smith et al., 2015). How food is processed and prepared for consumption is important when preparing material for testing in experimental studies when investigating hazard identification, characterising new or modified proteins and determining the extent of exposure. Ideally, all possible processing methods should be considered in the assessment (Remington et al., 2018); this approach could be feasible in a product-based risk safety assessment or in more targeted applications but not in full scope applications covering any potential use of for food/feed purposes (Section 3.1.2.4).

- *What would follow-up actions be required to assess the relevant proteins/fragments identified in previous steps?*

There is no consensus about how to interpret specific characteristics of digestion products (e.g. molecular size, persistence and abundance) within the context of the safety assessment of newly expressed proteins. Moreover, the criteria for classifying a protein as resistant or labile to digestion as

well as the risk implications (which may be different when considering sensitisation or elicitation) of such data are not defined, which impairs the setting of appropriate limits for digestibility in assessing the safety of a protein (EFSA GMO Panel, 2021). It is evident that more targeted research is needed to link *in vitro* data analysis of digesta to the probability of allergenicity. For instance, the use of multivariate analytic and machine learning approaches to provide statistical analysis of all persistent peptides and using a broad range of known allergens and their epitopes as training sets has been proposed (Mackie et al., 2019). Therefore, European Commission mandates and research projects may be needed for establishing: (i) the most appropriate standardised and harmonised test conditions and items to test which could better elucidate the interaction between proteins/fragments and the GIT/immune system in a risk assessment context; (ii) the most suitable methodology for fragment profiling; (iii) the criteria to identify digestion fragments as relevant for risk assessment of sensitisation and/or elicitation (i.e. abundance, persistence size, and/or others); and (iv) an appropriate set of reference control proteins ('allergenic' and 'nonallergenic') (Table 2). All this gathered information will likely be needed before a consensus can be agreed upon as to what is meant by 'resistance' to digestion (Mills et al., 2013b).

– *How can this information be integrated into a weight-of-evidence approach?*

Measurement of protein digestibility should not be regarded as a stand-alone endpoint for the safety assessment of novel proteins (Ladics, 2019). Therefore, the weight-of-evidence approach for allergenicity assessment remains valid. What is needed is to develop specifically how much weight each method provides, including *in vitro* digestion.

### 3.2.2.2. Use of human data in allergenicity risk assessment

The human-specific immunoglobulins E (sIgE) present in the sera of allergic patients can be used as molecular probes to detect allergenic proteins intended for human consumption (e.g. newly expressed proteins in GMOs or in new foods). However, this is not a first-line screening tool. In GMOs, specific serum tests should be performed whenever sera are available, (i) if the source of the introduced gene is considered allergenic or (ii) if the source is not known to be allergenic, but there is an indication that the newly expressed protein presents a sequence homology (> 35%)/structure similarity with a known allergen (EFSA GMO Panel, 2011). This strategy is much more difficult to apply to a whole food, because they may contain many proteins and some without known gene sequences. Generally, experience has revealed some practical and technical problems with this procedure, also because Codex Alimentarius and other guidance documents do not provide information on:

- i) why human sera testing is always required independently of the clinical relevance of the known allergen to which the hit was identified;
- ii) how the testing is performed – is not clearly outlined; and
- iii) what other testing might be required to conclude the allergic potential of the protein in question.

#### IgE binding to allergens

To fulfil regulatory requirements, sera should be collected from very well-characterised allergic individuals. These individuals should present a convincing clinical history of allergy against a specific food and a cause-and-effect relationship between the consumption of the food, and the elicitation of allergic symptoms should be established by a DBPCFC.

Individual sera, rather than pooled sera, should be used (EFSA GMO Panel, 2010, 2011). According to an FAO/WHO expert consultation (FAO/WHO, 2001), a minimum of eight relevant sera is required to achieve a 99% certainty that a new protein is not an allergen in the case of a major allergen. Similarly, a minimum of 24 relevant sera is required to achieve the same level of certainty in the case of minor allergens. These numbers of sera requested when performing such studies might not always be available. Furthermore, an important additional consideration is that food allergies may vary depending on the population studied and, that should be considered when performing such tests.

IgE binding assays, such as radio or enzyme allergosorbent assays (RAST or EAST), enzyme-linked immunosorbent assay (ELISA) or electrophoresis combined to immunoblotting with sIgE sera, are considered adequate (EFSA GMO Panel, 2011). Immunoblots have the advantage to test more than one protein. So, the simultaneous use of other IgE binding tests (RAST or ELISA) or a better test for functional IgE binding (Basophil activation) is advisable (Verhoeckx et al., 2016). Also, sera from

individuals with allergies to non-phylogenetically related organisms (negative controls) should be used to exclude non-specific IgE binding (Verhoeckx et al., 2016; Remington et al., 2018).

However, a single serum represents a heterogeneous repertoire, even when considering only the IgE isotype. Mutagenesis studies showed that multiple amino acids could be critical for IgE binding to a single epitope (Coco et al., 2003). Furthermore, a single serum may contain a mixture of antibodies with different isotypes recognising clinically irrelevant epitopes. Thus, serum-based assessment could be significantly improved by defining epitope specificities and affinities of the selected sera (Ehlers et al., 2019). However, the collection of significant volumes of serum in allergic patients, notwithstanding ethical considerations, constitutes a major bottleneck, particularly for rare allergens.

From a future perspective, these practical and methodological obstacles could be overcome by using human-derived monoclonal IgE antibodies. A first step could be the isolation of patients' allergen-specific B cells through antigen-specific flow-cytometry. This technique allows the study of the B-cell repertoire against specific allergens, confirming that, within a single patient, numerous B-cell clones may recognise a narrowly defined epitope (Hoh et al., 2016). Once these B cells are isolated, it is possible to generate monoclonal antibodies from a single B-cell RT-PCR to clone into a eukaryotic expression vector (Tiller et al., 2008). Another possibility is to select allergen-specific IgE B cells in allergic patients and to fuse them with myeloma cells to create hybridomas, which will produce human monoclonal IgE antibodies (Wurth et al., 2018).

Allergen-specific monoclonal IgE antibodies allow to map epitopes, assess cellular activation in response to allergen exposure (Madritsch et al., 2011; Hecker et al., 2011; Ehlers et al., 2019) and even study key structural aspects of allergen-sIgE interaction (Pomés et al., 2020).

As for IgG, IgE can be produced using a range of expression systems and with sufficient yields to allow translation into clinical applications (Sutton et al., 2019). Thus, ideally, the building up of a bank of monoclonal sIgE, which could be used to detect allergenic proteins, is possible. This could be achieved through international cooperation like the human genome project, with more clinically relevant results. These methods should be validated before any application in risk assessment.

Similar reasoning may be applied to other immunoglobulin isotypes, which could also be considered valuable probes for the detection of allergenic epitopes. Indeed, some isotypes (e.g. IgG4) recognise the same epitope patterns, as do IgE, in allergic patients, probably due to developmental IgG4-IgE filiation (isotype switching) (Gould and Wu, 2018; Saunders et al., 2019).

#### Functional assessment of IgE binding

As stated in Section 3.1.1, IgE binding as such does not indicate that a clinically relevant reaction will take place. The presence of specific IgE in plasma reflects sensitisation to a given allergen but does not predict that an allergic reaction will occur if the subject is exposed again to this allergen. Therefore, a subsequent step might be needed to evaluate the clinical relevance of the *in vitro* IgE binding with *ex vivo/in vitro* functional testing strategies (Codex Alimentarius, 2003–2009; EFSA GMO Panel, 2010,2011; Verhoeckx et al., 2016).

IgE binds to two principal receptors FcεRI and FcεRII/CD23. FcεRI is expressed on tissue mast cells, blood basophils, intestinal epithelial cells and various antigen-presenting cells. IgE has a very high affinity for FcεRI ( $K_a 10^{10} \text{ M}^{-1}$ ), at least two orders of magnitude higher than that of IgG for any of its receptors. Thus, most IgE is cell bound (Sutton et al., 2019). The allergic reaction is triggered by the binding of a multivalent allergen to its specific, cell-bound IgE, thereby cross-linking the FcεRI receptors, initiating signal transduction leading to basophil/mast cell activation, which results in the explosive release of preformed mediators and concurrent synthesis of inflammatory lipid mediators with pleiotropic effects (Gould and Wu, 2018). Reproducing this chain of events would demonstrate that the specific IgE/serum tested do recognise clinically relevant allergens.

#### Basophil activation test

Activation of basophils can be detected through upregulation of selected surface proteins measured by flow cytometry. CD63 is the most used activation marker. Its expression on the surface of the basophils is tightly correlated with histamine release in the medium (Knol et al., 1991). The response to more than four sequential dilutions of allergen should be determined. In allergic patients, the percentage of CD63 basophils (%CD63<sup>+</sup>) follows a sigmoidal dose–response curve, with a plateau at high allergen concentrations. BAT was consistently proven to be highly specific and highly sensitive, particularly in food allergies (Santos et al., 2021). Thus, its use can dispense patients from a risky and stressful exposure to allergens during oral food challenges (Santos and Lack, 2016). Indeed, BAT can correctly predict the clinical outcome following exposure of allergic patients to specific allergens

(elicitation) (Santos et al., 2021). This technique could be further refined and standardised by using the microfluidic immunoaffinity basophil activation test (miBAT) (Aljadi et al., 2019). However, BAT also presents limitations. For instance, analyses should preferably be performed within 4 h after sampling. Despite many efforts, BAT remains difficult to standardise. Furthermore, it does not allow large scale analyses, and its results may be biased by the presence of non-responding basophils.

### Mast cell activation test

It is important to consider the use of tissue-resident mast cells (MC), long considered as the primary effector cells in IgE-dependent allergies. However, it is very difficult to isolate viable and functional MC in sufficient numbers. To circumvent this difficulty, it is possible to generate human-derived MC (hMC) from peripheral blood precursors (CD117+CD34+ cells) from healthy donors. hMC can be passively sensitised by incubation with patients' sera (or monoclonal IgE), then challenged with various purified or recombinant allergens and their activation monitored by the upregulation of membrane activation markers (CD63 or CD107a). This human-derived mast cell activation test (hMAT) presented the best discriminating power compared with all other tests, including BAT. hMAT also displayed a very high sensitivity which would be very useful in assessing the unintended presence of allergen during food production (Bahri et al., 2018). This technique may be superior to other MAT using other sources of MC or LAD-2 cell lines (Elst et al., 2021), and its development will require collaboration and funding support.

Thus, it is possible to conceive an *in vitro* platform to screen the presence of an allergen in foods by using a bank of human-derived monoclonal sIgE (and IgG4) specific for a vast array of allergens, then to evaluate the clinical relevance of its sIgE detection through the activation of human-derived MC (Table 2).

### **3.2.2.3. *In vitro* tools to understand cellular and molecular mechanisms of sensitisation**

Molecular and cellular events potentially involved in food sensitisation are studied using *in vitro* and *in vivo* data. This information has been collected, organised and evaluated applying the concept of adverse outcome pathway (AOP) (van Bilsen et al., 2017; Lozano-Ojalvo et al., 2019). The proposed AOP framework describes the events of an adverse outcome at a biological level of organisation with relevance for risk assessment.

Briefly, the AOP for food sensitisation starts with a molecular initiating event involving the allergen uptake over the mucosal barrier of the digestive tract. The food protein passage may induce the activation of intestinal epithelial cells, followed by the local activation of dendritic cells and their migration to the mesenteric lymph nodes. Subsequently, dendritic cells present processed allergen to naive T cells, priming them towards a Th2 response.

Thus, these events may cause the activation of B cells and also the production of specific IgE by plasma cells, being the adverse outcome clinical symptoms upon repeated exposure to the offending food (Lozano-Ojalvo et al., 2019). The data gaps identified by these authors could drive future research needs that might be directed into developing *in vitro* microfluidics systems, human gut-on-a-chip devices (Kim et al., 2012), intestinal organoids (Leushacke and Baker, 2014), *ex vivo* models (Westerhout et al., 2014), among others (Table 2). Notably, this AOP mainly focuses on the oral route of exposure and other routes of exposure such as the skin should be further investigated.

Integration and comparability between experiments and the need of setting the panel of tested food proteins by including also low/non-allergenic proteins was considered an important breakthrough within the weight-of-evidence approach to determine the sensitising potential of food proteins. It was postulated that when applied in the context of an integrated testing strategy, such an AOP approach could reduce and ideally replace current animal testing (Lozano-Ojalvo et al., 2019).

**Table 2:** Challenges and research needs identified by the EFSA GMO Panel for *in vitro* tools used in the allergenicity risk assessment of foods derived from biotechnology

| <b>Challenges necessary to improve the reliability and predictability of the allergenicity risk assessment</b> | <b>Research needs</b>  |
|--|--|
| To determine optimal protocols for digestibility assays.   | To standardise and harmonise test conditions and items to investigate the interaction between proteins/fragments and the gastrointestinal tract/immune system for a risk assessment. |

| Challenges necessary to improve the reliability and predictability of the allergenicity risk assessment   | Research needs  |
|---|---|
| To determine which endpoints, fragments versus intact protein, should be used to assess <i>in vitro</i> digestibility.  | To correlate <i>in vitro</i> analysis of digesta with the probability of allergenicity using multivariate data analysis, statistical analysis and machine learning. |
| To determine the criteria to identify digestion fragments as relevant for risk assessment of sensitisation and/or elicitation (i.e. abundance, persistence size, and/or others).    | To investigate the optimal methodology for fragment profiling.  |
| To determine which follow-up actions are required to assess the relevant proteins/fragments identified in <i>in vitro</i> digestibility test.                                       | To determine the feasibility of setting acceptable/unacceptable limits for digestibility for assessing the safety of a protein.                                     |
| To set up a bank of well-characterised sIgE and to design <i>ex vivo/in vitro</i> functional test strategies to evaluate the clinical relevance of sIgE binding to a given protein. | To further investigate the use of sera of allergic patients as molecular probes.  |
| To unravel mechanisms of pathogenesis leading to food allergy.  | To build upon existing AOPs for food sensitisation and to develop an integrated strategy of tests for allergenicity prediction.                                     |

### 3.2.3. *In vivo* tools

*In vivo* animal models of food allergy typically focus on mechanistic insights and are not used in risk assessment because they are not yet predictive. The main reasons for using *in vivo* models are for: elucidating disease pathogenesis of IgE-mediated food allergy, mechanisms governing allergic sensitisation to food proteins, and testing prophylactic and therapeutic strategies. Ideally, if *in vivo* models are ever used for risk assessment, they would need to induce disease that (1) mimics clinical symptoms with measurable allergic responses, (2) uses inbred animals, (3) distinguishes low from high allergenic proteins, (4) are translational, (5) can test individual proteins and/or whole foods for allergenicity and adjuvanticity, (6) have varying disease susceptibility, and (7) are robust, sensitive, fast, cost-effective, easy and reliable. However, several limitations have hindered developing a standardised and validated animal model used to predict proteins allergenicity and adjuvanticity, including a lack of predictive biomarkers for disease development and because they do not wholly reproduce clinical disease.

Provided that food allergy models are developed in the future that is predictive and translatable to humans, and allergenicity risk assessment would benefit. The models must be validated and predictive for individual proteins and/or whole foods and could be potentially considered for the following, (1) for proteins or foods without a history of safe human dietary consumption; (2) for testing proteins with a high risk for sensitising and causing allergic reactions; or (3) a food matrix that can potentially alter tolerance, cause sensitisation, elicitation or has adjuvant properties. Novel *in vivo* models would also be potentially useful for determining threshold doses for sensitisation or elicitation using different exposure routes and determining whether proteolysis and heat processing modifies allergy sensitisation or elicitation (e.g. more severe symptoms). Any model should predict the clinical outcomes of sensitisation to individual proteins and/or whole foods, predict sensitisation, IgE reactivity, clinically relevant sensitising proteins and adjuvants.

The most frequently used models are with mice and rats. However, to date, the immune responses in rodents are not predictive for allergenicity, adjuvanticity or for the ranking of the strength of allergenic responses against proteins (Ladics et al., 2010). Though food allergy models are not predictive, rodent models have elucidated many underlying pathophysiological processes in the allergic response to food, including immune responses, roles of IgE and IgA, multiple mediators, e.g. cytokines, thymic stromal lymphopoietin, granulocyte-macrophage colony-stimulating factor, IL-25 and IL-33 and cells, e.g. T-helper Th2 cells, ILC2s, regulatory T (Treg) cells, natural killer T cells, basophils, mast cells and dendritic cells. Furthermore, *in vivo* models shed light on the tolerance of cross-reactive allergens and the role of bystander effects from other proteins and contaminants. Integrating basic immunological data from *in vivo* models to fully understand the sensitisation potential of new food

proteins is essential. Moreover, sensitisation mechanisms go beyond the immune system with crucial knowledge from *in vivo* models on the GIT pathophysiology such as digestion, pH, motility, barriers, mucin, tight junctions and GIT permeability.

Despite many available *in vivo* models, the basic model to mimic IgE-mediated allergic disease to food proteins requires two steps. Step 1: Sensitisation – administer the allergen by ingestion, inhalation, dermal application or systemically with intraperitoneal (i.p.) injection; Step 2: Elicitation – administer the same allergen after the immune response develops via the GIT by feeding or intragastric administration. However, protocols may differ for both steps as follows: Exposure time, dose and frequency of the food or protein, the allergen (e.g. milk, egg, peanut), the nature of the allergen tested (e.g. purified proteins, processed or non-processed whole food matrix with possible contaminants, cooked or heated), allergen administration route (e.g. oral, dermal, i.p., inhalation), use and type of adjuvant (e.g. cholera toxin, lipids, alum, lectins), and use of negative and positive controls. The models may also utilise different animals and genetic backgrounds (e.g. mice (BALB/c, C3H), rats (Brown Norway), genetically modified mouse strains or humanised mice (human immune cells seeded into immunodeficient mice) and animals with differing microbiomes and environmental conditions (e.g. diet, housing).

Once sensitised and challenged, there are several measured endpoints for disease (Castan et al., 2020), such as the quantification of serum immunoglobulins (e.g. allergen-specific IgE), inflammation – circulating and local tissue (e.g. location, cellular homing, cell numbers, phenotypes), cytokine production (e.g. Th2 cytokines, mast cell, basophil and eosinophil mediators), response to allergen rechallenge, basophil and mast cell degranulation (e.g. basophil activation test), and clinical parameters (e.g. body temperature/hypothermia, active or passive-cutaneous anaphylaxis, ear swelling, diarrhoea).

For non-IgE-mediated diseases, e.g. coeliac disease, an *in vivo* model is unnecessary because of the extensive knowledge on the underlying mechanisms (Koning et al., 2015) and an array of non *in vivo* methods to predict disease development.

Although the different IgE-mediated *in vivo* models are beneficial for generalising results about underlying disease mechanisms, it makes using them challenging for the allergenicity risk assessment. It will only be possible to use an *in vivo* model when it is well defined, validated and subsequently standardised, as with other *in vivo* toxicity studies used for risk assessment (EFSA GMO Panel, 2011).

Several studies have tested GMOs in various animals, including pigs, salmon, sheep, cattle, zebrafish, rats or mice (El Sanhoty et al., 2004; Prescott et al., 2005; Custodio et al., 2006; Finamore et al., 2008; Paul et al., 2008; Trabalza-Marinucci et al., 2008; Adel-Patient, et al., 2011; Walsh et al., 2012; Gu et al., 2013; Sanden et al., 2013; Zeljenkova et al., 2014; Andreassen et al., 2015). Some experimental models have addressed potential immunogenicity, allergenicity, or adjuvanticity of GMOs, including *Bacillus thuringiensis* (Bt) Cry1 proteins, alpha-amylase inhibitor (aAI) peas, PHA-E lectin in rice, sunflower seed albumin in narrow leaf lupin and lactoferrin GMOs (Marsteller et al., 2015).

More specifically, for assessing adjuvanticity, most studies focused on *Bacillus thuringiensis* (Bt)-maize Cry1 proteins or including the whole food matrix (Vázquez-Padrón et al., 1999; Vázquez et al., 1999; Guimaraes et al., 2008; Reiner et al., 2014; Andreassen et al., 2016; Tulinska et al., 2018). However, other studies addressed the potential adjuvant effect of the bean alpha-amylase inhibitor in GM peas (Prescott et al., 2005; Lee et al., 2013). A few of these studies reported adjuvant effects upon co-administration of the ingested protein or food matrix with an unrelated protein from peanut or chicken egg, but with Cry1 protein doses much higher than what is found in Bt-maize. Notably, there was no adjuvant effect upon short-term feeding of mice with a diet containing 33% of Bt-maize (MON810), showing that a diet with physiological levels of GM protein did not enhance allergic responses (Reiner et al., 2014). In studies reporting adjuvant effects, it was not clear whether the results were related to the high dose of the administered study protein.

Overall, such studies evaluate the GMO effects on animals that consumed GM, near-isogenic or non-GM materials or recombinant, purified, isolated or extracted GM proteins. Using *in vivo* models for GMOs and also for novel food allergenicity risk assessment is difficult due to many challenges (Table 3).

To date, the usefulness of *in vivo* models for predictive allergenicity risk assessment is uncertain because of the current lack of validated, predictive models for allergenicity in humans. However, once available, a validated predictive animal model could help identify biotechnology products, mainly because of the complex pathophysiology. However, it is essential to consider their use in the context of the current limitations and following a search for a history of safe use, sequence homology, serum testing, protein characterisation, and pepsin digestibility and potentially other physiological *in vitro*

digestion studies. To effectively utilise *in vivo* models in the safety assessment of genetically modified crops, it is necessary to address critical knowledge gaps (Table 3).

*In vivo* models could potentially improve risk assessment and facilitate the introduction of innovative/novel protein sources with a low risk of allergic sensitisation. However, it is currently impossible to use them in the allergenicity risk assessment because there are no standardised predictive models. Additionally, it would be ideal to avoid animals for the allergenicity risk assessment. However, if animal models are ever to be used in allergenicity risk assessment in the future, following the 3Rs principles, a consensus approach is necessary with the predictive power to mimic human allergic risks. For now, *in vivo* food allergy models could be further developed into an established useful tool for testing individual proteins and whole foods for allergenicity used to potentially validate *in vitro* models if beneficial and to elucidate unknown mechanisms underlying disease.

**Table 3:** Challenges and research needs identified by the EFSA GMO Panel for *in vivo* tools used in the allergenicity risk assessment of foods derived from biotechnology

| Challenges necessary to improve the reliability and predictability of the allergenicity risk assessment   | Research needs   |
|---|--|
| <b>Animal models</b>  |  |
| Overall, need to select an optimal experimental design with standardised protocols – animal, allergen, dose, route, dose–response relationship, adjuvant, and appropriate positive and negative controls.   | To establish an optimal experimental design with standardised protocols – animals, allergens, doses, routes, dose–response relationships, adjuvants, and appropriate positive and negative controls. |
| Outcomes of experiments with models with different protocols, endpoints and test materials may differ.  | To establish an optimal experimental design with standardised protocols, endpoints and test materials.   |
| Assays and endpoints from animal experiments may differ – clinical signs, immune markers, or protein-specific functionally active IgE making comparisons difficult (Bøgh et al. 2016; Castan et al., 2020)  | To establish an optimal experimental design with standardised endpoints.   |
| Contradictory data from different laboratories or models make it difficult to assess the risk for human food safety.  | To establish an optimal experimental design with standardised protocols – animals, allergens, doses, routes, dose–response relationships, adjuvants, and appropriate positive and negative controls. |
| Determining which type of model for risk assessment is optimal – a model for sensitisation, elicitation or cross-reactivity.  | To establish an optimal experimental design for sensitisation, elicitation and cross-reactivity to determine which is more predictive.   |
| Environmental conditions may alter the response to GM proteins, including diet, housing conditions, microbiota and contaminated test materials, e.g. GM food with fungal contamination – aflatoxin or other mycotoxins).  | To report on environmental conditions for the experiment, e.g. housing, diet, microbiome, and full assessment of test materials.   |
| Experimental reproducibility may differ within and between laboratories making intra- and inter-laboratories comparisons with the same test materials difficult to compare – demonstrated by two reports of labs testing the same GM, using the same materials, protocol, and mouse strain and yet, the results were contradictory, emphasising the importance of repeated experiments in independent laboratories (Prescott et al., 2005; Lee et al., 2013). |  |
| <b>Types of test materials</b>  |  |
| Presence of cross-reactive proteins in a GM material or novel food might interfere with the results – alpha-amylase inhibitor GM peas contain a pea lectin that is cross-reactive (Lee et al., 2013).   | To test whole food matrix where possible with appropriate extracts and purified proteins for protein-specific responses, e.g. <i>in vivo</i> challenge and <i>ex vivo</i> analysis, serum IgE.       |

| <b>Challenges necessary to improve the reliability and predictability of the allergenicity risk assessment</b>  | <b>Research needs</b>  |
|---|--|
| Appropriate controls for a GMO, an isogenic line are necessary – a strongly allergenic positive control, a non-allergenic protein/material, and the vehicle alone. For novel foods, it could be even more challenging to select the correct controls.   | To establish standardised positive and negative controls.  |
| Standardisation of the test materials are dependent on the test material and the type of cooking and/or processing methods used.  | To determine the best approaches for processing and preparation of test materials.   |
| Protein concentration of a GMO differs, making comparisons difficult – Mon810 contains 0.01% of the total protein (Steinke et al., 2010) and whether protein quantities change in processed food and feed end products unless testing whole food.   | To establish protein content of test materials for comparative analyses.   |
| Differential post-translational modifications in the host plant (Campbell et al., 2011) compared with recombinant test proteins may lead to new conformational allergenic epitopes resulting in potential allergenicity identified <i>in vitro</i> and <i>in vivo</i> .   | To consider post-translational modifications in test materials when comparing experiments.   |
| Recombinant proteins may contain contaminants lipopolysaccharide (LPS), which might explain observed differences in reports (e.g. Reiner et al., 2014; Andreassen et al., 2015).  | To test, remove and report on potential contaminants in test materials.  |
| Determining the effect of added (e.g. cholera toxin) and intrinsic adjuvants (LPS) in the test materials. GMOs contain lectins and carbohydrates, which could stimulate antigen uptake and influence immune responses to unrelated proteins (Takata et al., 2000; Cardone et al., 2014). Other innovative or novel foods also may have adjuvant properties. | To establish and standardise protocols containing adjuvants and to assess the content of potential contaminants in whole food matrix test materials. |
| <b>Translation to humans – prediction and validation</b>  |  |
| Determining and mimicking human consumption of a GMO or novel food in animal experiments challenging because of the difficulty of translation from humans to animals.   | To establish standardised approaches for quantity and frequency of consumption based on the test material and human consumption patterns.            |
| Determining how to validate the model, e.g. against pepsin resistance or <i>in vitro</i> digestion studies.   | To establish standardised models used to validate <i>in vitro</i> assays.  |
| Reliable ranking of allergen allergenicity in animal models would enable predictability and could be compared to the clinical relevance of the particular allergen.   | To establish a reference set of proteins for gauging allergenicity- low to high responsiveness that mirrors clinical relevance of the allergen.      |

### 3.2.4. Additional remarks on risk assessment tools

In addition to the identification of improvement needs dealing with specific issues related to *in silico*, *in vitro* and *in vivo* tools, this document also collects a pool of overarching challenges whose solutions could help to reduce the knowledge gaps on allergenicity prediction for risk assessment of food/feed derived from modern biotechnology (Table 4).

Ideally, the development needs to predict allergenicity would include the following: (i) models for sensitisation, elicitation, adjuvanticity and cross-reactivity; (ii) a comprehensive evaluation of tools with extensive validation testing with allergenic and non-allergenic materials under carefully controlled experimental conditions, ensuring appropriate statistical power under standardised conditions and proper controls; (iii) tools for use in risk assessment that are simple, reproducible, specific and sensitive; (iv) tools that can predict the threshold and magnitude of the allergic potential of an allergen; and (v) tools for ranking proteins that correlate with clinical relevance.

**Table 4:** Challenges and research needs identified by the EFSA GMO Panel for the allergenicity risk assessment of foods derived from biotechnology. Overarching issues

| <b>Challenges necessary to improve the reliability and predictability of the allergenicity risk assessment</b>  | <b>Research needs</b>   |
|---|---|
| To elaborate a consensus list of clinically relevant allergens with demonstrable potency in eliciting allergic reactions in humans and animals.   | <p>To build on data available for component-resolved diagnostics in allergic patients.</p> <p>To collect data regarding the allergenic potency of certain allergenic foods and identify genetic differences between allergic and non-allergic individuals.</p> <p>To collect data on the prevalence of food allergy in animals (e.g. companion animals, farm) and determine the allergens involved.</p> |
| To establish a reference set of proteins with varying allergenic potential for the development of improved predictive models for risk assessment.   | To collect and analyse data for the generation of a database on scaling and comparison of the allergenic potential for allergenic foods and individual allergens.   |
| To fully understand the interaction between allergenic proteins with other components in food that influences their potency and stability and their potential as adjuvants.   | To develop reliable, accurate and sensitive methods to assess the potency, stability and potential adjuvant activity of allergens.  |
| To identify new <i>in silico</i> , <i>in vitro</i> , <i>ex vivo</i> and <i>in vivo</i> approaches able to predict allergenicity of food proteins.   | <p>To develop new tools as predictive methods for the allergenicity risk assessment will require validation and standardisation of methodology, experimental design, and read-outs.</p> <p>To determine if adverse outcome pathway (AOP) can be applied to food sensitisation and/or elicitation to support new allergenicity risk assessment strategies.</p>   |
| <p>To establish standardised test materials for the prediction of allergenicity, such as individual proteins and extracts (raw or processed), whole food matrix or a combination of all these.</p> <p>To determine which characteristics of test materials, e.g. post-translational modifications, other biochemical and/or physicochemical properties, are related to protein stability.</p> | To determine standardised, relevant strategies for processing and preparation of test materials and if those are compatible with full scope applications (i.e. covering any potential use for food/feed purposes) or if they should be circumscribed to product-based risk safety assessment.   |
| Comparative analysis and data integration between experiments to allow for the extrapolation of broader conclusions than those from a single study.   | To standardise the experimental design to validate clinical context. To integrate all data sets using multivariate models   |

### 3.3. Other key elements in the allergenicity risk assessment

#### 3.3.1. Acceptable levels and threshold values of food allergens

Establishing thresholds constitutes a critical first step to assessing the risk from allergens, as they are a characteristic of the hazard that allergenic foods present to the food-allergic population. Their establishment is, thus, essential to the evidence-based application of risk management and mitigation strategies, such as Precautionary Allergen Labelling (PAL) (FAO/WHO, 2021b). However, no threshold values applicable to food allergens are currently available for risk assessment purposes (EFSA NDA Panel, 2014). A key question going beyond scientific issues would be 'what level of risk is acceptable?'. Although there is a consensus that zero risk is not realistic or achievable (Madsen et al., 2012; DunnGalvin et al., 2015), the level of risk that is acceptable to consumers and regulators remains unclear (Madsen et al., 2020).

Recently, the FAO/WHO Expert Committee on risk assessment of food allergens has agreed that, for a series of priority allergenic food sources, the objective of minimising 'to a point where further

refinement does not meaningfully reduce health impact, the probability of any clinically relevant objective allergic response' could be met by defining reference doses (RfDs) based on dose distribution modelling of minimum eliciting doses (MEDs) and supported by data on the severity of symptoms. Using this approach, the Committee agreed the safety objective could be met for RfD's corresponding to eliciting doses predicted to result in objective reactions in no more than 5% (ED<sub>05</sub>) of the allergic population, as evaluated using the data from Remington et al. (2020) and Houben et al. (2020). Recommended RfD values (as mg of protein from the allergenic source) have been established for several tree nuts and peanut (ranging from 1 to 3 mg of total protein from the allergenic source), egg (2 mg), wheat (5 mg), fish (5 mg) and shrimp (200 mg) as a result of a high level of quality, quantity, availability and accessibility of data for these priority allergenic food sources and also supported by data on health manifestations (severity) at the proposed RfD (FAO/WHO, 2021b). However, it has been recently reported that around 5% of individuals reacting to an ED<sub>01</sub> or ED<sub>05</sub> level of exposure to peanuts might develop anaphylaxis in response to that dose. This equates to 1 and 6 anaphylaxis events per 2,500 patients exposed to an ED<sub>01</sub> or ED<sub>05</sub> dose, respectively, in the broader population of individuals with peanut allergy (Patel et al., 2021), illustrating that zero risk is not a realistic goal.

Therefore, the use of data on eliciting doses and co-factors affecting these is considered of great potential use for its incorporation into an allergenicity risk assessment, although some challenges have first to be overcome (i.e. information supporting some or all of the above considerations are lacking for individual allergens and less commonly allergenic food sources, or how to deal with interindividual variability or low level of quality of clinical data, etc.). However, there could be sufficient knowledge on the most common and potent allergens that could be used to cover those for which there is less available and robust data and, therefore, to implement a framework with threshold levels that may be realistic, attainable, and provide optimal protection for people with food allergy (Madsen et al., 2020).

### 3.3.2. Post-market monitoring tools

Post-market monitoring is a risk management measure that may assist the risk assessment process (Codex Alimentarius, 2003–2009). According to the EFSA GMO Panel and Regulation (EU) No 503/2013, when there is a likelihood of allergenicity, the food derived from the GM plant should be further characterised in the light of anticipated intake and appropriate conditions for placing on the market, including labelling (EFSA GMO Panel, 2011).

EFSA has previously published an external report reviewing the existing post-market monitoring strategies developed for the safety assessment of human and animal health useful for GM food and feed (ADAS, 2015). Other EU projects such as MARLON investigated the possibility of measuring health indicators during post-market monitoring for potential effects of feeds, particularly GMOs, on livestock animal health (de Santis et al., 2018).

Despite having been identified as a gap many years ago (Hepburn et al., 2008), little progress has been made on how to undertake post-market monitoring to provide useful insights into adverse reactions to novel foods beyond fat replacers (Slough et al., 2001). However, online surveys have been used in Japan to re-evaluate the safety of nutritional supplements (Nishijima et al., 2019). Registries have been developed to collect data on severe, anaphylactic reactions (Worm et al., 2014), which could be adapted to allow clinicians or patients to report adverse reactions to provide a signal of potential adverse effects. Innovative approaches may be required to provide a cost-effective solution and could draw on those being developed to improve reporting and analysis of adverse events caused by drugs. For example, text mining of social media has provided new insights into adverse events for widely prescribed drugs such as steroids which could have applicability to identifying adverse events to foods (Vivekanantham et al., 2020). The current tools for text mining are imperfect, and many challenges remain for such approaches as identified by the Innovative Medicines Initiative (IMI) WEB-RADR (Recognising Adverse Drug Reactions) project where a need for coordination to facilitate development, adoption and acceptance of such technology was clearly identified (Vivekanantham et al., 2020). Social media networks for health already exist, such as Health Unlocked,<sup>7</sup> but their use needs to be undertaken in a sensitive manner as studies have already shown that consumer trust is greater in activities undertaken for research or by regulatory authorities (Bulcock et al., 2021). In addition, some initial activities have been piloted as a reporting system.<sup>8</sup>

<sup>7</sup> <https://healthunlocked.com/>

<sup>8</sup> <https://www.food.gov.uk/report-a-food-allergy-intolerance-reaction>

Future assessment of complex foods will benefit from a developed post-market monitoring system, paying attention to the reliability, sensitivity and specificity of the proposed methods, which should answer specific questions such as uncertainties in the pre-market assessment phase. Such initiatives could be linked with others ongoing in the healthcare sector.

#### 4. Conclusions and Recommendations

This Scientific Opinion has: (i) defined knowledge gaps on allergenicity prediction; (ii) identified specific research needs for improving the allergenicity risk assessment for products derived from biotechnology; (iii) determined how new basic research findings and technological developments can improve the current risk assessment methodology; and (iv) prioritised basic research funding (Tables 1–4).

By considering the complexity and variety of factors involved in food allergy, as well as the current state-of-the-art, it is unrealistic that a single test will, in short/medium term, be predictive of allergenicity. Therefore, the 'weight-of-evidence' approach for allergenicity assessment is still valid, although the evidence needed might differ depending on whether a conventional GMO or another type of new biotech food is being assessed (Figure 1).

On the one hand, it is necessary to progress in this field as the current guidelines in the Codex Alimentarius, initially published in 2003, focused on food derived from existing 'modern' biotechnology available at the time and requires updating. Although the Codex Alimentarius and EFSA guidance documents successfully addressed allergenicity assessments of single/stacked event GM applications, experience gained and new developments in the field call for a modernisation of some key elements such as (i) better standardisation on the use of the available knowledge on the source of the gene and the protein itself – context of clinical relevance, route of exposure and potential threshold values of food allergens; (ii) modernisation of *in silico* tools used with more targeted databases; (iii) better integration of *in vitro* data with clear guidance on how protein stability and digestion inform the assessment and on the use of human sera; and (iv) clarity on the use of the overall weight-of-evidence approach for protein safety and the aspects needed for expert judgement. This could benefit from being set within the risk assessment frameworks used in other aspects of public health with clearly defined terminologies relating to the level of potential risk at a population level. This framework can support greater transparency in the way conclusions are drawn from the weight-of-evidence approach.

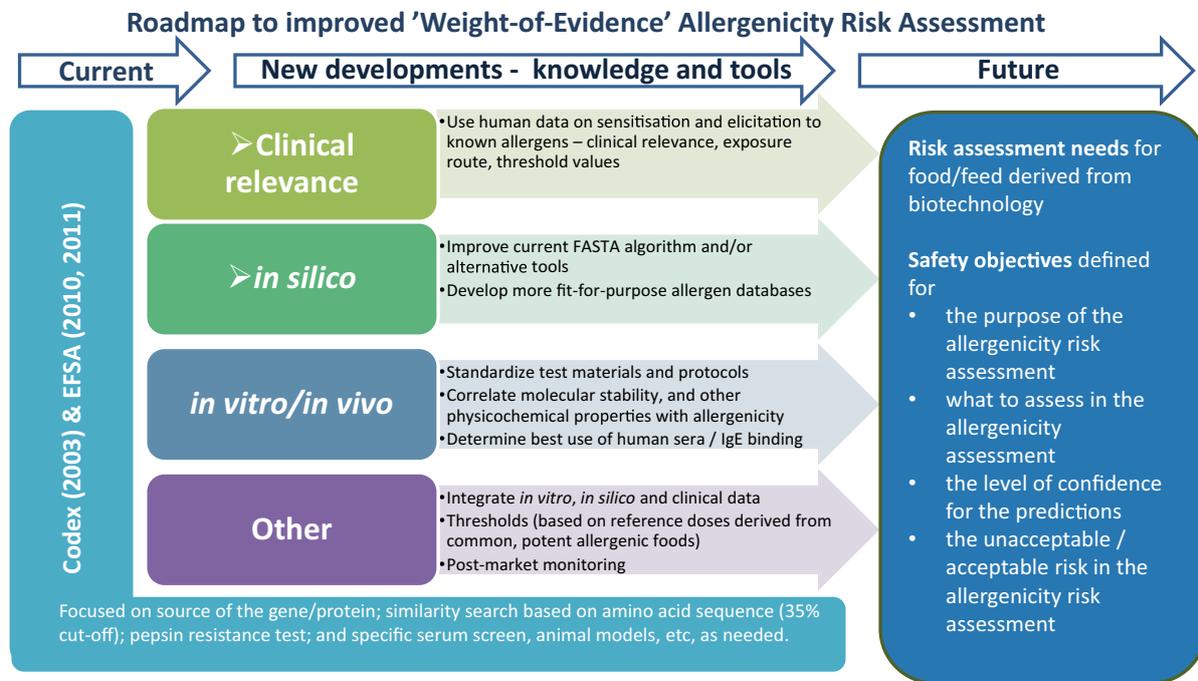
On the other hand, the pace of innovation in the Agri-Food arena needs to meet the challenges facing society in the 21st century and will increasingly challenge the allergenicity risk assessment process. The risk assessment process, which started in the 1990s in the wake of the release of transgenic crops, now needs to be extended to meet the challenges of innovations from genome editing to synthetic biology. The recent FAO/WHO expert consultation provided safety objectives and guidance on the aspects relating to managing existing, known allergenic foods to ensure allergic consumer safety. Therefore, it is timely to review and clarify the main purpose of the allergenicity risk assessment and the vital role it plays in protecting consumers' health with existing food allergies and assessing the potential for foods to cause new food allergies.

The setting of clear safety objectives that address new technologies are needed as a backdrop to inform the safety assessment and to ensure that allergenic risks of foods are assessed in an appropriate, consistent and proportionate manner across all the many different technologies for their production. Therefore, the draft of a roadmap to (re)define the allergenicity safety objectives and risk assessment will be needed to address the key questions for risk assessors and risk managers: (1) what is the purpose of the allergenicity risk assessment?; (2) what is to be assessed in the allergenicity assessment?; (3) what level of confidence do we need for the predictions?; (4) what is considered an unacceptable/acceptable risk in the allergenicity risk assessment? (Figure 1).

EFSA GMO Panel recommends:

- to continue investing resources in the modernisation of available tools to consider experience gained, current and new knowledge that could lead to increase the robustness, avoid inconsistencies and lack of reproducibility of the assessments. For such purposes, a series of research priorities are proposed in Tables 1–4 to advance the allergenicity risk assessment field in a systematic, interdisciplinary and coordinated approach. The outcome of present and future EFSA procurements, European Commission mandates, as well as EU and other projects, will guide the EFSA Panels.

- progresses along the lines outlined in Figure 1 will be possible only if European wide multicentre collaborations are established which imply the development of standardised tools and quality control to reduce redundancies and increase data reliability. To investigate possibilities of EFSA facilitating or acting as an international focal point to find a consensus addressing the questions above to prepare for the future. This activity will require broad and transdisciplinary participation where collaboration with the Member States, Stakeholders and the international community at large will be needed.



**Figure 1:** Roadmap to improved 'Weight-of-Evidence' Allergenicity Risk Assessment

## 5. Documentation provided to EFSA

- 1) Proposal for a self-task mandate of the EFSA GMO Panel to establish a Working Group to develop supplementary guidelines for the allergenicity assessment of GM plants to incorporate new developments. May 2014. Submitted by the Chair of the EFSA GMO Panel.
- 2) Acceptance of the self-task mandate of the EFSA GMO Panel to establish a Working Group to develop supplementary guidelines for the allergenicity assessment of GM plants to incorporate new developments. July 2014. Submitted by EFSA Executive Director.
- 3) Acceptance of the self-task mandate of the EFSA GMO Panel to establish a Working Group to develop a statement of the GMO Panel updating its latest guidance document on Allergenicity assessment of GM plants (EFSA GMO Panel, 2017). May 2020. Submitted by EFSA Executive Director.

## References

- Abdelmoteleb M, Zhang C, Furey B, Kozubal M, Griffiths H, Champeaud M and Goodman RE, 2021. Evaluating potential risks of food allergy of novel food sources based on comparison of proteins predicted from genomes and compared to. *Food and Chemical Toxicology*, 147, 111888.
- Abdullah SU, Alexeev Y, Johnson PE, Rigby NM, Mackie AR, Dhaliwal B and Mills ENC, 2016. Ligand binding to an allergenic lipid transfer protein enhances conformational flexibility resulting in an increase in susceptibility to gastroduodenal proteolysis. *Scientific Reports*, 26, 30279.
- ADAS, 2015. Strategy support for the Post-Market Monitoring (PMM) of GM Plants: review of existing PMM strategies developed for the safety assessment of human and animal health. EFSA Supporting Publication 2014;EN739, 117 pp. <https://doi.org/10.2903/sp.efsa.2014.739>
- Adel-Patient K, Guimaraes VD, Paris A, Drumare MF, Ah-Leung S, 2011. Immunological and metabolomic impacts of administration of Cry1Ab protein and MON 810 maize in mouse. *PLoS One*, 6, e16346.

- Akkerdaas J, Totis M, Barnett B, Bell E, Davis T, Edrington T, Glenn K, Graser G, Herman R, Knulst A, Ladics G, McClain S, Poulsen LK, Ranjan R, Rasclé J-B, Serrano H, Speijer D, Wang R, Pereira Mouries L, Capt A and van Ree R, 2018. Protease resistance of food proteins: a mixed picture for predicting allergenicity but a useful tool for assessing exposure. *Clinical and Translational Allergy*, 8, 30.
- Aljadi Z, Kalm F, Nilsson C, Windquist O, Russom A, Lundahl J, Nopp A, 2019. A novel tool for clinical diagnosis of allergy operating microfluidic immunoaffinity basophil activation test technique. *Clinical Immunology*, 209, e108268.
- Altschul SF, Gish W, Miller W, Myers EW and Lipman DJ, 1990. Basic local alignment search tool. *Journal of Molecular Biology*, 215, 403–410.
- Andreassen M, Rocca E, Bøhn T, Wikmark OG, van den Berg J, Løvik M, Traavik T and Nygaard UC, 2015. Humoral and cellular immune responses in mice after airway administration of *Bacillus thuringiensis* Cry1Ab and MON810 *cry1Ab*-transgenic maize. *Food and Agricultural Immunology*, 26, 521–537.
- Andreassen M, Bøhn T, Wikmark O-G, Bodin J, Traavik T, Løvik M and Nygaard UC, 2016. Investigations of immunogenic, allergenic and adjuvant properties of Cry1Ab protein after intragastric exposure in a food allergy model in mice. *BMC Immunology*, 17, 10.
- Anvari S, Miller J, Yeh CY and Davis CM, 2019. IgE-mediated food allergy. *Clinical Reviews in Allergy and Immunology*, 57, 244–260.
- Apostolovic D, Stanic-Vucinic D, de Jongh HH, de Jong GA, Mihailovic J, Radosavljevic J, Radibratovic M, Nordlee JA, Baumert JL, Milcic M, Taylor SL, Garrido Clua N, Cirkovic VT and Koppelman SJ, 2016. Conformational stability of digestion-resistant peptides of peanut conglutins reveals the molecular basis of their allergenicity. *Scientific Reports*, 6, 29249.
- Asai Y, Martino D, Eiwegger T, Nadeau K, Koppelman GH, Clarke AE, Lee YA, Chan ES, Simons E, Laprise C, Mazer B, Marenholz I, Royce D, Elliott SJ, Hampson C, Gerdtts J, Eslami A, Soller L, Hui J, Azad M, Sandford A and Daley D, 2020. Phenotype consensus is required to enable large-scale genetic consortium studies of food allergy. *Allergy*, 75, 2383–2387.
- Astwood JD, Leach JN and Fuchs RL, 1996. Stability of food allergens to digestion *in vitro*. *Nature Biotechnology*, 14, 1269–1273.
- Bahri R, Custovic A, Korosec P, Tsoumani M, Barron M, Wu J, Sayers R, Weimann A, Ruiz-Garcia M, Patel N, Robb A, Shamji MH, Fontanella S, Silar M, Mills EN, Clare, Simpson A, Turner PJ and Bulfone-Paus S, 2018. Mast cell activation test in the diagnosis of allergic disease and anaphylaxis. *The Journal of Allergy and Clinical Immunology*, 142, 485–496.
- Ballmer-Weber BK, Fernandez-Rivas M, Beyer K, Defernez M, Sperrin M, Mackie AR, Salt LJ, Hourihane JO, Asero R, Belohlavkova S, Kowalski M, de Blay F, Papadopoulos NG, Clausen M, Knulst AC, Roberts G, Popov T, Sprickelman AB, Dubakiene R, Vieths S, van Ree R, Crevel R and Mills ENC, 2015. How much is too much? Threshold dose distributions for 5 food allergens. *The Journal of Allergy and Clinical Immunology*, 135, 964–971.
- Bannon GA, 2004. What makes a food protein an allergen? *Current Allergy and Asthma Reports*, 4, 43–46.
- Berez B, Clare Mills EN, Parádi I, Láng F, Tamás L, Shewry PR and Mackie AR, 2013. Stability of sunflower 2S albumins and LTP to physiologically relevant *in vitro* gastrointestinal digestion. *Food Chemistry*, 138, 2374–2381.
- Bernard H, Guillon B, Drumare MF, Paty E, Dreskin SC, Wal JM, Adel-Patient K and Hazebrouck S, 2015. Allergenicity of peanut component Ara h 2: contribution of conformational versus linear hydroxyproline-containing epitopes. *Journal of Allergy and Clinical Immunology*, 135, 1267–1274.
- Björkstén B, Crevel R, Hischenhuber C, Løvik M, Samuels F, Strobel S, Taylor SL, Wal JM and Ward R, 2008. Criteria for identifying allergenic foods of public health importance. *Regulatory Toxicology and Pharmacology*, 51, 42–52.
- Bluemchen K and Eiwegger T, 2019. Oral peanut immunotherapy How much is too much? How much is enough? *Allergy*, 74, 220–222.
- Bøgh KL, Kroghsbo S, Dahl L, Rigby NM, Barkholt V, Mills EN and Madsen CB, 2009. Digested Ara h 1 has sensitizing capacity in Brown Norway rats. *Clinical and Experimental Allergy*, 39, 1611–1621.
- Bøgh KL, Barkholt V, Rigby NM, Mills EN and Madsen CB, 2012. Digested Ara h 1 loses sensitizing capacity when separated into fractions. *Journal of Agricultural and Food Chemistry*, 60, 2934–2942.
- Bøgh KL, van Bilsen J, Głogowski R, López-Expósito I, Bouchaud G, Blanchard C, Bodinier M, Smit J, Pieters R, Bastiaan-Net S, de Wit N, Untersmayr E, Adel-Patient K, Knippels L, Epstein MM, Noti M, Nygaard UC, Kimber I, Verhoeckx K and O'Mahony L, 2016. Current challenges facing the assessment of the allergenic capacity of food allergens in animal models. *Clinical and Translational Allergy*, 6, 21.
- Bøgh KL and Madsen CB, 2016. Food allergens: is there a correlation between stability to digestion and allergenicity? *Critical Reviews in Food Science and Nutrition*, 56, 1545–1567.
- Bossios A, Theodoropoulou M, Mondoulet L, Rigby NM, Papadopoulos NG, Bernard H, Adel-Patient K, Wal JM, Mills CE and Papageorgiou P, 2011. Effect of simulated gastro-duodenal digestion on the allergenic reactivity of beta-lactoglobulin. *Clinical Translational Allergy*, 1, 6.

- Breiteneder H, Peng YQ, Agache I, Diamant Z, Eiwegger T, Fokkens WJ, Traidl-Hoffmann C, Nadeau K, O’Hehir RE, O’Mahony L, Pfaar O, Torres MJ, Wang DY, Zhang L and Akdis CA, 2020. Biomarkers for diagnosis and prediction of therapy responses in allergic diseases and asthma. *Allergy*, 75, 3039–3068.
- Bulcock A, Hassan L, Giles S, Sanders C, Nenadic G, Campbell S and Dixon W, 2021. Public perspectives of using social media data to improve adverse drug reaction reporting: a mixed-methods study. *Drug Safety*, 44, 553–564.
- Bushey DE, Bannon GA, Delaney BF, Graser G, Hefford M, Jiang X, Lee TC, Madduri KM, Pariza M, Privalle LS, Ranjan R, Saab-Rincon G, Schafer BW, Thelen JJ, Zhang JXQ and Harper MS, 2014. Characteristics and safety assessment of intractable proteins in genetically modified crops. *Regulatory Toxicology and Pharmacology*, 69, 154–170.
- Campbell PM, Reiner D, Moore AE, Lee RY, Epstein MM and Higgins TJ, 2011. Comparison of the alpha-amylase inhibitor-1 from common bean (*Phaseolus vulgaris*) varieties and transgenic expression in other legumes—post-translational modifications and immunogenicity. *Journal of Agriculture and Food Chemistry*, 59, 6047–6054.
- Caraballo L, Valenta R, Puerta L, Pomés A, Zakzuk J, Fernandez-Caldas E, Acevedo N, Sanchez-Borges M, Ansotegui I, Zhang L, van Hage M, Abel-Fernández E, Arruda LK, Vrtala S, Curin M, Gronlund H, Karsonova A, Kilimajer J, Riabova K, Trifonova D and Karaulov A, 2020. The allergenic activity and clinical impact of individual IgE-antibody binding molecules from indoor allergen sources. *World Allergy Organization Journal*, 13, 100118.
- Caraballo L, Valenta R, Acevedo N and Zakzuk J, 2021. Are the terms major and minor allergens useful for precision allergology? *Frontiers in Immunology*, 12, 651500.
- Cardone M, Ikeda KN, Varano B, Belardelli F, Millefiorini E, Gessani S, 2014. Opposite regulatory effects of IFN-beta and IL-3 on C-type lectin receptors, antigen uptake, and phagocytosis in human macrophages. *Journal of Leukocyte Biology*, 95, 161–168.
- Cardoso-Silva D, Delbue D, Itzlinger A, Moerkens R, Withoff S, Branchi F and Schumann M, 2019. Intestinal barrier function in gluten-related disorders. *Nutrients*, 11, 2325.
- Castan L, Bøgh KL, Maryniak NZ, Epstein MM, Kazemi S, O’Mahony L, Bodinier M, Smit JJ, Bilsen JHM, Blanchard C, Głogowski R, Kozáková H, Schwarzer M, Noti M, Wit N, Bouchaud G and Bastiaan-Net S, 2020. Overview of in vivo and ex vivo endpoints in murine food allergy models: Suitable for evaluation of the sensitizing capacity of novel proteins? *Allergy*, 75, 289–301.
- Cayrol C, Duval A, Schmitt P, Roga S, Camus M, Stella A, Burlet-Schiltz O, Gonzalez-de-Peredo A and Girard J-P, 2018. Environmental allergens induce allergic inflammation through proteolytic maturation of IL-33. *Nature Immunology*, 19, 375–385.
- Chruszcz M, Chew FT, Hoffmann-Sommergruber K, Hurlburt BK, Mueller GA, Pomés A, Rouvinen J, Villalba M, Wöhrl BM and Breiteneder H, 2021. Allergens and their associated small molecule ligands—their dual role in sensitization. *Allergy*, 76, 2367–2382.
- Chung YJ, Ronsmans S, Crevel RW, Houben GF, Rona RJ, Ward R and Baka A, 2012. Application of scientific criteria to food allergens of public health importance. *Regulatory Toxicology and Pharmacology*, 64, 315–323.
- Codex Alimentarius, 2003–2009. Foods derived from modern biotechnology. Codex Alimentarius Commission, Joint FAO/WHO Food Standards Programme, Rome.
- Costa J, Bavaro SL, Benedé S, Diaz-Perales A, Bueno-Diaz C, Gelencser E, Klueber J, Larré C, Lozano-Ojalvo D, Lupi R, Mafra I, Mazzucchelli G, Molina E, Monaci L, Martín-Pedraza L, Piras C, Rodrigues PM, Roncada P, Schrama D, Cirkovic-Velickovic T, Verhoeckx K, Villa C, Kuehn A, Hoffmann-Sommergruber K and Holzhauser T, 2020. Are physicochemical properties shaping the allergenic potency of plant allergens? *Clinical Reviews in Allergy and Immunology*, <https://doi.org/10.1007/s12016-020-08810-9>
- Costa J, Villa C, Verhoeckx K, Cirkovic-Velickovic T, Schrama D, Roncada P, Rodrigues PM, Piras C, Martín-Pedraza L, Monaci L, Molina E, Mazzucchelli G, Mafra I, Lupi R, Lozano-Ojalvo D, Larré C, Klueber J, Gelencser E, Bueno-Diaz C, Diaz-Perales A, Benedé S, Bavaro SL, Kuehn A, Hoffmann-Sommergruber K and Holzhauser T, 2021. Are physicochemical properties shaping the allergenic potency of animal allergens? *Clinical Reviews and Allergy Immunology*, <https://doi.org/10.1007/s12016-020-08826-1>
- Custodio MG, Powers WJ, Huff-Lonergan E, Faust MA and Stein J, 2006. Growth, pork quality, and excretion characteristics of pigs fed Bt corn or non-transgenic corn. *Canadian Journal of Animal Science*, 86, 461–469.
- D’Avino R, Bernardi ML, Wallner M, Palazzo P, Camardella L, Tuppo L, Alessandri C, Breiteneder H, Ferreira F, Ciardiello MA and Mari A, 2011. Kiwifruit Act d 11 is the first member of the ripening-related protein family identified as an allergen. *Allergy*, 66, 870–877.
- de Santis B, Stockhofe N, Wal JM, Weesendorp E, Lallès JP, van Dijk J, Kok E, De Giacomo M, Einspanier R, Onori R, Brera C, Bikker P, van der Meulen J and Kleter G, 2018. Case studies on genetically modified organisms (GMOs): potential risk scenarios and associated health indicators. *Food and Chemical Toxicology*, 117, 36–65.
- Di Stasio L, Picariello G, Mongiello M, Nocerino R, Berni Canani R, Bavaro S, Monaci L, Ferranti P and Mamone G, 2017. Peanut digestome: identification of digestion resistant IgE binding peptides. *Food and Chemical Toxicology*, 107, 88–98.
- Di Stasio L, Tranquet O, Picariello G, Ferranti P, Morisset M, Denery-Papini S and Mamone G, 2020. Comparative analysis of eliciting capacity of raw and roasted peanuts: the role of gastrointestinal digestion. *Food Research International*, 127, 108758.

- Dietz CJ, Sun H, Yao WC, Citardi MJ, Corry DB and Luong AU, 2019. *Aspergillus fumigatus* induction of IL-33 expression in chronic rhinosinusitis is PAR2-dependent. *Laryngoscope*, 129, 2230–2235.
- Dimitrov I and Atanasova M, 2020. AllerScreener – a server for allergenicity and cross-reactivity prediction. *Cybernetics and Information Technologies*, 20, 175–184.
- Dimitrov I, Flower DR, Doytchinova I, 2013. AllerTOP - a server for in silico prediction of allergens. *BMC Bioinformatics*, 14(Suppl. 6), S4, 2013. *Protein Eng. Des. Sel.*, 26(10), 631–634.
- Dimitrov I, Bangov I, Flower DR and Doytchinova I, 2014a. AllerTOP vol 2 - a server for in silico prediction of allergens. *Journal of Molecular Modeling*, 20, 2278.
- Dimitrov I, Naneva L, Bangov I and Doytchinova I, 2014b. AllergenFP: allergenicity prediction by descriptor fingerprints. *Bioinformatics*, 30, 846–851.
- Dua S, DOWey J, Garcia MR, Bond S, Durham S, Kimber I, Mills C, Roberts G, Skypala I, Wason J, Ewan P, Boyle R and Clark A, 2020. How reaction severity is affected by cofactors and repeat challenges: a prospective study of peanut allergic adults. *Journal of Allergy and Clinical Immunology*, 145, AB182.
- Dubiela P, Kabasser S, Smargiasso N, Geiselhart S, Bublin M, Hafner C, Mazzucchelli G and Hoffmann-Sommergruber K, 2018. Jug r 6 is the allergenic vicilin present in walnut responsible for IgE cross-reactivities to other tree nuts and seeds. *Scientific Reports*, 8, 11366.
- DunnGalvin A, Chan CH, Crevel R, Grimshaw K, Poms R, Schnadt S, Taylor SL, Turner P, Allen KJ, Austin M, Baka A, Baumert JL, Baumgartner S, Beyer K, Bucchini L, Fernández-Rivas M, Grinter K, Houben GF, Hourihane J, Kenna F, Kruizinga AG, Lack G, Madsen CB, Clare Mills EN, Papadopoulos NG, Aldrick A, Regent L, Sherlock R, Wal JM and Roberts G, 2015. Precautionary allergen labelling: perspectives from key stakeholder groups. *Allergy*, 70, 1039–1051.
- du Toit G, Tsakok T, Lack S and Lack G, 2016. Prevention of food allergy. *The Journal of Allergy and Clinical Immunology*, 137, 998–1010.
- Eaton AD, Zimmermann C, Delaney B and Hurley BP, 2017. Primary human polarized small intestinal epithelial barriers respond differently to a hazardous and an innocuous protein. *Food and Chemical Toxicology*, 106, 70–77.
- EFSA (European Food Safety Authority), Bronzwaer S, Kass G, Robinson T, Tarazona J, Verhagen H, Verloo D, Vrbos D and Hugas M, 2019. Editorial on food Safety Regulatory Research Needs 2030. *EFSA Journal* 2019;17(7):e170622, 8 pp. <https://doi.org/10.2903/j.efsa.2019.e170622>
- EFSA (European Food Safety Authority), 2021. Workshop on allergenicity assessment – prediction. EFSA Supporting publication 2021;EN-6826, 16 pp. <https://doi.org/10.2903/sp.efsa.2021.EN-6826>
- EFSA GMO Panel (EFSA Panel on Genetically Modified Organisms), 2010. Scientific Opinion on the assessment of allergenicity of GM plants and microorganisms and derived food and feed. *EFSA Journal* 2010;8(7):1700, 168 pp. <https://doi.org/10.2903/j.efsa.2010.1700>
- EFSA GMO Panel (EFSA Panel on Genetically Modified Organisms), 2011. Scientific Opinion on guidance for risk assessment of food and feed from genetically modified plants. *EFSA Journal* 2011;9(5):2150, 37 pp. <https://doi.org/10.2903/j.efsa.2011.2150>
- EFSA GMO Panel (EFSA Panel on Genetically Modified Organisms), 2017. Guidance on allergenicity assessment of genetically modified plants. *EFSA Journal* 2017;15(5):4862, 49 pp. <https://doi.org/10.2903/j.efsa.2017.4862>
- EFSA GMO Panel (EFSA Panel on Genetically Modified Organisms), Naegeli H, Bresson JL, Dalmay T, Dewhurst IC, Epstein MM, Firbank LG, Guerche P, Hejatko J, Moreno FJ, Mullins E, Nogué F, Rostoks N, Sánchez Serrano JJ, Savoini G, Veromann E, Veronesi F, Dumont AF, 2021. Statement on *in vitro* protein digestibility tests in allergenicity and protein safety assessment of genetically modified plants. *EFSA Journal* 2021;19(1):6350, 16 pp. <https://doi.org/10.2903/j.efsa.2021.6350>
- EFSA GMO Panel (EFSA Panel on Genetically Modified Organisms), 2022a. Evaluation of existing guidelines for their adequacy for the food and feed risk assessment of genetically modified plants obtained through synthetic biology. EFSA draft Opinion for Public Consultation, in progress.
- EFSA GMO Panel (EFSA Panel on Genetically Modified Organisms), 2022b. Evaluation of existing guidelines for their adequacy for the food and feed risk assessment of microorganisms obtained through synthetic biology. EFSA draft Opinion for Public Consultation, in progress
- EFSA NDA Panel (EFSA Panel on Dietetic Products, Nutrition and Allergies), 2014. Scientific Opinion on the evaluation of allergenic foods and food ingredients for labelling purposes. *EFSA Journal* 2014;12(11):3894, 286 pp. <https://doi.org/10.2903/j.efsa.2014.3894>
- Ehlers AM, Blankestijn MA, Knulst AC, Klinge M and Otten HG, 2019. Can alternative epitope mapping approaches increase the impact of B-cell epitopes in food allergy diagnostics? *Clinical and Experimental Allergy*, 49, 17–26.
- El Sanhoty R, El-Rahman AA and Bogl KW, 2004. Quality and safety evaluation of genetically modified potatoes spunta with Cry V gene: compositional analysis, determination of some toxins, antinutrients compounds and feeding study in rats. *Nahrung*, 48, 13–18.
- Elst J, van der Poorten M-L, Van Gasse AL, De Puyssseleir L, Hagendorens MM, Faber MA, Van Houdt M, Passante E, Bahri R, Walschot M, Mertens C, Bridts CH, Sabato V and Ebo DG, 2021. Mast cell activation tests by flow cytometry: a new diagnostic asset? *Clinical and Experimental Allergy*, 51, 1482–1500.

- European Commission, 2013. Commission Implementing Regulation (EU) No. 503/2013 of 3 April 2013 on applications for authorisation of genetically modified food and feed in accordance with Regulation (EC) No. 1829/2003 of the European Parliament and of the Council and amending Commission Regulations (EC) No. 641/2004 and (EC) No. 1981/2006. Official Journal of European Union L157, 1–48.
- FAO and WHO, Codex Alimentarius Commission, 2021b. Summary report of the Ad hoc Joint FAO/WHO Expert Consultation on Risk Assessment of Food Allergens. Part 2: Review and establish threshold levels in foods of the priority allergens. FAO, Rome. Available online: [https://cdn.who.int/media/docs/default-source/food-safety/jemra/2nd-allergen-summary-report-20aug2021.pdf?sfvrsn=915a8417\\_8](https://cdn.who.int/media/docs/default-source/food-safety/jemra/2nd-allergen-summary-report-20aug2021.pdf?sfvrsn=915a8417_8)
- FAO and WHO, Codex Alimentarius Commission, 2021a. Summary report of the Ad hoc Joint FAO/WHO Expert Consultation on Risk Assessment of Food Allergens. Part 1: Review and validation of Codex priority allergen list through risk assessment. FAO, Rome. Available online: [https://cdn.who.int/media/docs/default-source/food-safety/jemra/1st-allergen-summary-report-10may2021.pdf?sfvrsn=c505375a\\_7](https://cdn.who.int/media/docs/default-source/food-safety/jemra/1st-allergen-summary-report-10may2021.pdf?sfvrsn=c505375a_7)
- FAO/WHO, 2001. Evaluation of allergenicity of genetically modified foods. Report of a Joint FAO, WHO Expert Consultation on Allergenicity of Food Derived from Biotechnology, 22–25, January 2001. Food and Agriculture organisation of the United Nations (FAO), Italy, Rome.
- Fernandez A, Mills EN, Lovik M, Spoek A, Germini A, Mikalsen A and Wal JM, 2013. Endogenous allergens and compositional analysis in the allergenicity assessment of genetically modified plants. Food and Chemical Toxicology, 62, 1–6.
- Fernandez A, Mills ENC, Koning F and Moreno FJ, 2019. Safety assessment of immune-mediated adverse reactions to novel food proteins. Trends in Biotechnology, 37, 796–800.
- Fernandez A, Mills ENC, Koning F and Moreno FJ, 2021. Allergenicity assessment of novel food proteins: what should be improved? Trends in Biotechnology, 39, 4–8.
- Fernandez Dumont A, Paoletti C, Ruiz JG, Ardizzone M and Lanzoni A, 2018. The safety assessment of proteins in food: where do we stand? Toxicology Letters, 295, S140–S141.
- Finamore A, Roselli M, Britti S, Monastra G, Ambra R, Turrini A and Mengheri E, 2008. Intestinal and peripheral immune response to MON810 maize ingestion in weaning and old mice. Journal of Agriculture and Food Chemistry, 56, 11533–11539.
- Foo ACY and Mueller GA, 2021. Abundance and stability as common properties of allergens. Frontiers Allergy, 2, 769728.
- Foster ES, Kimber I and Dearman RJ, 2013. Relationship between protein digestibility and allergenicity: comparisons of pepsin and cathepsin. Toxicology, 308, 30–38.
- Fu T-J, Abbott UR and Hatzos C, 2002. Digestibility of food allergens and nonallergenic proteins in simulated gastric fluid and simulated intestinal fluid. A comparative study. Journal of Agricultural and Food Chemistry, 50, 7154–7160.
- Geiselhart S, Podzhilkova A and Hoffmann-Sommergruber K, 2021. Cow's milk processing-friendly or foe in food allergy? Foods, 10, 572.
- Gould HJ and Wu YCB, 2018. IgE repertoire and immunological memory: compartmental regulation and antibody function. Intern Immunology, 30, 403–412.
- Groschwitz KR and Hogan SP, 2009. Intestinal barrier function: molecular regulation and disease pathogenesis. The Journal of Allergy and Clinical Immunology, 124, 3–20.
- Grozdanovic MM, Čavić M, Nešić A, Andjelković U, Akbari P, Smit JJ and Gavrović-Jankulović M, 2016. Kiwifruit cysteine protease actinidin compromises the intestinal barrier by disrupting tight junctions. Biochimica Et Biophysica Acta - General Subjects, 1860, 516–526.
- Gu JN, Krogdahl A, Sissener NH, Kortner TM, Gelencser E, Hemre GI, 2013. Effects of oral Bt-maize (MON810) exposure on growth and health parameters in normal and sensitized Atlantic salmon. Salmo Salar L. Br J Nutr, 109, 1408–1423.
- Guhsl EE, Hofstetter G, Hemmer W, Ebner C, Vieths S, Vogel L, Breiteneder H and Radauer C, 2014. Vig r 6, the cytokinin-specific binding protein from mung bean (*Vigna radiata*) sprouts, cross-reacts with Bet v 1-related allergens and binds IgE from birch pollen allergic patients' sera. Molecular Nutrition and Food Research, 58, 625–634.
- Guimaraes VD, Drumare MF, Ah-Leung S, Lereclus D, Bernard H, Créminon C, Wal JM and Adel-Patient K, 2008. Comparative study of the adjuvanticity of *Bacillus thuringiensis* Cry1Ab protein and cholera toxin on allergic sensitisation and elicitation to peanut. Food and Agricultural Immunology, 19, 325–337.
- Halim A, Carlsson MC, Madsen CB, Brand S, Møller SR, Olsen CE, Vakhrushev SY, Brimnes J, Wurtzen PA, Ipsen H, Petersen BL and Wandall HH, 2015. Glycoproteomic analysis of seven major allergenic proteins reveals novel post-translational modifications. Molecular and Cellular Proteomics: MCP, 14, 191–204.
- Harper B, McClain S and Ganko EW, 2012. Interpreting the biological relevance of bioinformatic analyses with T-DNA sequence for protein allergenicity. Regulatory Toxicology and Pharmacology, 63, 426–432.
- Hecker J, Diethers A and Etzold S, 2011. Generation and epitope analysis of human monoclonal antibody isotypes with specificity for the timothy grass major allergen Phl p 5a. Molecular Immunology, 48, 1236–1244.
- Helm RM, 2001. Topic 5: Stability of Known Allergens (Digestive and Heat Stability). Report of a Joint FAO, WHO Expert Consultation on Allergenicity of Food Derived from Biotechnology, 22–25 January 2001. Food and Agriculture organisation of the United Nations (FAO), Italy, Rome.

- Hepburn P, Howlett J, Boeing H, Cockburn A, Constable A, Davi A, de Jong N, Moseley B, Oberdörfer R, Robertson C, Wal JM and Samuels F, 2008. The application of post-market monitoring to novel foods. *Food and Chemical Toxicology*, 46, 9–33.
- Herman RA, Song P, Mirsky HP and Roper JM, 2021. Evidence-based regulations for bioinformatic prediction of allergen cross-reactivity are needed. *Regulatory Toxicology and Pharmacology*, 120, 104841.
- Herman RA, Woolhiser MM, Ladics GS, Korjagin VA, Schafer BW, Storer NP, Green SB and Kan L, 2007. Stability of a set of allergens and non-allergens in simulated gastric fluid. *International Journal of Food Sciences and Nutrition*, 58, 125–141.
- Hilmenyuk T, Bellinghausen I, Heydenreich B, Ilchmann A, Toda M, Grabbe S and Saloga J, 2010. Effects of glycation of the model food allergen ovalbumin on antigen uptake and presentation by human dendritic cells. *Immunology*, 129, 437–445.
- Hoh RA, Joshi SA, Liu Y, Wang C, Roskin KM, Lee JY, Pham T, Looney TJ, Jackson KJL, Dixit VP, King J, Lyu SC, Jenks J, Hamilton RG, Nadeau KC and Boyd SD, 2016. Single B-cell deconvolution of peanut-specific antibody responses in allergic patients. *The Journal of Allergy and Clinical Immunology*, 137, 157–167.
- Hoh RA, Joshi SA, Lee JY, Martin BA, Varma S, Kwok S, Nielsen SCA, Nejad P, Haraguchi E, Dixit PS, Shutthanandan SV, Roskin KM, Zhang W, Tupa D, Bunning BJ, Manohar M, Tibshirani R, Fernandez-Becker NQ, Kambham N, West RB, Hamilton RG, Tsai M, Galli SJ, Chinthrajah RS, Nadeau KC and Boyd SD, 2020. Origins and clonal convergence of gastrointestinal IgE<sup>+</sup> B cells in human peanut allergy. *Science Immunology*, 5, eaay4209.
- Høst A and Samuelsson EG, 1988. Allergic reactions to raw, pasteurized, and homogenized/pasteurized cow milk: a comparison. *Allergy*, 43, 113–118.
- Houben GF, Baumert JL, Blom WM, Kruizinga AG, Meima MY, Remington BC, Wheeler MW, Westerhout J and Taylor SL, 2020. Full range of population Eliciting Dose values for 14 priority allergenic foods and recommendations for use in risk characterization. *Food and Chemical Toxicology*, 146, 111831.
- Huby RD, Dearman RJ and Kimber I, 2000. Why are some proteins allergens? *Toxicological Sciences*, 55, 235–246.
- Irvine AD, McLean WH and Leung DY, 2011. Filaggrin mutations associated with skin and allergic diseases. *New England Journal of Medicine*, 365, 1315–1327.
- James JK and Nanda V, 2020. Comparative dynamics of tropomyosin in vertebrates and invertebrates. *Proteins*, 88, 265–273.
- James JK, Pike DH, Khan IH and Nanda V, 2018. Structural and dynamic properties of allergen and non-allergen forms of tropomyosin. *Structure*, 26, 997–1006.
- Jenkins JA, Breiteneder H and Mills EN, 2007. Evolutionary distance from human homologs reflects allergenicity of animal food proteins. *The Journal of Allergy and Clinical Immunology*, 120, 1399–1405.
- Jenkins JA, Griffiths-Jones S, Shewry PR, Breiteneder H and Mills EN, 2005. Structural relatedness of plant food allergens with specific reference to cross-reactive allergens: an in silico analysis. *The Journal of Allergy and Clinical Immunology*, 115, 163–170.
- Jensen-Jarolim E, 2015. Aluminium in Allergies and Allergen immunotherapy. *World Allergy Organization Journal*, 8, 7.
- Johansson SGO, Hourihane Jo'b, Bousquet J, Brujnzeel-Koomen C, Dreborg S, Haahtela T, Kowalski ML, Mygind N, Ring J, van Cauwenberge P, van Hage-Hamsten M and Wuthrich B, 2001. A revised nomenclature for allergy. An EAACI position statement from the EAACI nomenclature task force. *Allergy*, 56, 813–824.
- Kamalakaran M, Chang LM, Grishina G, Sampson HA and Masilamani M, 2016. Identification and characterization of DC-SIGN-binding glycoproteins in allergenic foods. *Allergy*, 71, 1145–1155.
- Kamath SD, Scheibhofer S, Johnson CM, Machado Y, McLean T, Taki AC, Ramsland PA, Iyer S, Joubert I, Hofer H, Wallner M, Thalhamer J, Rolland J, O'Hehir R, Briza P, Ferreira F, Weiss R and Lopata AL, 2020. Effect of structural stability on endolysosomal degradation and T-cell reactivity of major shrimp allergen tropomyosin. *Allergy*, 75, 2909–2919.
- Kenna JG and Evans RM, 2000. Digestibility of proteins in simulated gastric fluid. *Toxicologist*, 54, 141.
- Kim HJ, Huh D, Hamilton G and Ingber DE, 2012. Human gut-on-a-chip inhabited by microbial flora that experiences intestinal peristalsis-like motions and flow. *Lab on a Chip*, 12, 2165–2174.
- Knol EF, Mul FP, Jansen H, Calafat J and Roos D, 1991. Monitoring human basophil activation via CD63 monoclonal antibody 435. *The Journal of Allergy and Clinical Immunology*, 88(3 Pt 1), 328–338.
- König A, Cockburn A, Crevel RW, Debruyne E, Grafstroem R, Hammerling U, Kimber I, Knudsen I, Kuiper HA, Peijnenburg AA, Penninks AH, Poulsen M, Schauzu M and Wal JM, 2004. Assessment of the safety of foods derived from genetically modified (GM) crops. *Food and Chemical Toxicology*, 42, 1047–1088.
- Koning F, Thomas R, Rossjohn J and Toes RE, 2015. Coeliac disease and rheumatoid arthritis: similar mechanisms, different antigens. *Nature Reviews Rheumatology*, 11, 450–461.
- Koppelman SJ, Hefle SL, Taylor SL and de Jong GA, 2010. Digestion of peanut allergens Ara h 1, Ara h 2, Ara h 3, and Ara h 6: a comparative in vitro study and partial characterization of digestion-resistant peptides. *Molecular Nutrition and Food Research*, 54, 1711–1721.
- Krutz NL, Winget J, Ryan CA, Wimalasena R, Maurer-Stroh S, Dearman RJ, Kimber I and Gerberick GF, 2019. Proteomic and bioinformatic analyses for the identification of proteins with low allergenic potential for hazard assessment. *Toxicological Sciences*, 170, 210–222.

- Kulis MD, Smeekens JM, Immormino RM and Moran TP, 2021. The airway as a route of sensitization to peanut: an update to the dual allergen exposure hypothesis. *The Journal of Allergy and Clinical Immunology*, 148, 689–693.
- Ladics GS, 2019. *Journal of Immunotoxicology*, 16 pp. 43–53.
- Ladics GS, Bannon GA, Silvanovich A and Cressman RF, 2007. Comparison of conventional FASTA identity searches with the 80 amino acid sliding window FASTA search for the elucidation of potential identities to known allergens. *Molecular Nutrition Food Research*, 51, 985–998.
- Ladics GS, Knippels LMJ, Penninks AH, Bannon GA, Goodman RE and Herouet-Guichenev C, 2010. Review of animal models designed to predict the potential allergenicity of novel proteins in genetically modified crops. *Regulatory Toxicology and Pharmacology*, 56, 212–224.
- Lavelle EC, Grant G, Pusztai A, Pfüller U and O'hagan DT, 2001. The identification of plant lectins with mucosal adjuvant activity. *Immunology*, 102, 77–86.
- Lee RY, Reiner D, Dekan G, Moore AE, Higgins TJV, 2013. Genetically modified  $\alpha$ -amylase inhibitor peas are not specifically allergenic in mice. *PLoS One*, 8, e52972.
- Leushacke M and Barker N, 2014. Ex vivo culture of the intestinal epithelium: strategies and applications. *Gut*, 63, 1345–1354.
- Løwenstein H, 1978. Quantitative immunoelectrophoretic methods as a tool for the analysis and isolation of allergens. *Prog Allergy*, 25, 1–62.
- Lozano-Ojalvo D, Benedé S, Antunes CM, Bavaro SL, Bouchaud G, Costa A, Denery-Papini S, Díaz-Perales A, Garrido-Arandia M, Gavrovic-Jankulovic M, Hayen S, Martínez-Blanco M, Molina E, Monaci L, Pieters RHH, Villemin C, Wichers HJ, Wróblewska B, Willemsen LEM, Roggen EL and van Bilsen JHM, 2019. Applying the adverse outcome pathway (AOP) for food sensitization to support in vitro testing strategies. *Trends in Food Science and Technology*, 85, 307–319.
- Machado Y, Freier R, Scheibhofer S, Thalhamer T, Mayr M, Briza P, Grutsch S, Ahammer L, Fuchs JE, Wallnoefer HG, Isakovic A, Kohlbauer V, Hinterholzer A, Steiner M, Danzer M, Horejs-Hoeck J, Ferreira F, Liedl KR, Tollinger M, Lackner P, Johnson CM, Brandstetter H, Thalhamer J and Weiss R, 2016. Fold stability during endolysosomal acidification is a key factor for allergenicity and immunogenicity of the major birch pollen allergen. *The Journal of Allergy and Clinical Immunology*, 137, 1525–1534.
- Mackie A, Dupont D, Torcello-Gómez A, Jardín J and Deglaire A, 2019. Report on EFSA project OC/EFSA/GMO/2017/01. "In vitro protein digestibility" (Allergestion). EFSA Supporting Publication 2019:EN-1765, 82 pp. <https://doi.org/10.2903/sp.efsa.2019.EN-1765>
- Madritsch C, Flicker S, Scheibhofer S, Zafred D, Pavkov-Keller T, Thalhamer J, Keller W and Valenta R, 2011. Recombinant monoclonal human immunoglobulin E to investigate the allergenic activity of major grass pollen allergen Phl p 5. *Clinical and Experimental Allergy*, 41, 270–280.
- Madsen CB, Hattersley S, Allen KJ, Beyer K, Chan CH, Godefroy SB, Hodgson R, Mills EN, Muñoz-Furlong A, Schnadt S, Ward R, Wickman M and Crevel R, 2012. Can we define a tolerable level of risk in food allergy? Report from a EuroPrevall/UK Food Standards Agency workshop. *Clinical and Experimental Allergy*, 42, 30–37.
- Madsen CB, van den Dungen MW, Cochrane S, Houben GF, Knibb RC, Knulst AC, Ronsmans S, Yarham RAR, Schnadt S, Turner PJ, Baumert J, Cavandoli E, Chan CH, Warner A and Crevel RWR, 2020. Can we define a level of protection for allergic consumers that everyone can accept? *Regulatory Toxicology and Pharmacology*, 117, 104751.
- Marsteller N, Bøgh KL, Goodman RE and Epstein MM, 2015. A review of animal models used to evaluate potential allergenicity of genetically modified organisms (GMOs). *Drug Discovery Today: Disease Models*, 17, 81–88.
- Matricardi PM, Kleine-Tebbe J, Hoffmann HJ, Valenta R, Hilger C, Hofmaier S, Aalberse RC, Agache I, Asero R, Ballmer-Weber B, Barber D, Beyer K, Biedermann T, Bilò MB, Blank S, Bohle B, Bosshard PP, Breiteneder H, Brough HA, Caraballo L, Caubet JC, Cramer R, Davies JM, Douladiris N, Ebisawa M, Eigenmann PA, Fernandez-Rivas M, Ferreira F, Gadermaier G, Glatz M, Hamilton RG, Hawranek T, Hellings P, Hoffmann-Sommergruber K, Jakob T, Jappe U, Jutel M, Kamath SD, Knol EF, Korosec P, Kuehn A, Lack G, Lopata AL, Mäkelä M, Morisset M, Niederberger V, Nowak-Węgrzyn AH, Papadopoulos NG, Pastorello EA, Pauli G, Platts-Mills T, Posa D, Poulsen LK, Raulf M, Sastre J, Scala E, Schmid JM, Schmid-Grendelmeier P, van Hage M, van Ree R, Vieths S, Weber R, Wickman M, Muraro A, Ollert M, 2016. EAACI molecular allergology user's guide. *Pediatric Allergy and Immunology*, 27(Suppl. 23), 1–250.
- Mattar H, Padfield P, Simpson A and Mills ENC, 2021. The impact of a baked muffin matrix on the bioaccessibility and IgE reactivity of egg and peanut allergens. *Food Chemistry*, 15, 129879.
- Maurer-Stroh S, Krutz NL, Kern PS, Gunalan V, Nguyen MN, Limviphuvadh V, Eisenhaber F and Gerberick GF, 2019. AllerCatPro-prediction of protein allergenicity potential from the protein sequence. *Bioinformatics*, 35, 3020–3027.
- Mazzucchelli G, Holzhauser T, Cirkovic Velickovic T, Diaz-Perales A, Molina E, Roncada P, Rodrigues P, Verhoeckx K and Hoffmann-Sommergruber K, 2018. Current (food) allergenic risk assessment: is it fit for novel foods? Status quo and identification of gaps. *Molecular Nutrition and Food Research*, 62, 1700278.
- Metcalfe DD, Astwood JD, Townsend R, Sampson HS, Taylor SL and Fuchs RL, 1996. Assessment of the allergenic potential of foods derived from genetically engineered crop plants. *Critical Reviews in Food Science and Nutrition*, 36, S165–S186.

- Michalski M-C, 2007. On the supposed influence of milk homogenization on the risk of CVD, diabetes and allergy. *British Journal of Nutrition*, 97, 598–610.
- Michalski M-C and Januel C, 2006. Does homogenization affect the human health properties of cow's milk? *Trends in Food Science and Technology*, 17, 423–437.
- Mills ENC, Marsh JT, Boyle R, Hoffmann-Sommergruber K, DuPont D, Bartra J, Bakalis S, McLaughlin J and Shewry PR, 2013a. Literature review: 'non-IgE-mediated immune adverse reactions to foods'. EFSA Supporting Publication 2013;EN-527, 24 pp. <https://doi.org/10.2903/sp.efsa.2013.EN-527>
- Mills ENC, Marsh JT, Johnson PE, Boyle R, Hoffmann-Sommergruber K, DuPont D, Bartra J, Bakalis S, McLaughlin J and Shewry PR, 2013b. Literature review: 'in vitro digestibility tests for allergenicity assessment'. EFSA Supporting Publication 2013;EN-529, 52 pp.
- Moreno FJ, Mackie AR and Mills EN, 2005. Phospholipid interactions protect the milk allergen alpha-lactalbumin from proteolysis during in vitro digestion. *Journal of Agriculture and Food Chemistry*, 53, 9810–9816.
- Mueller RS, Jensen-Jarolim E, Roth-Walter F, Marti E, Janda J, Seida AA and DeBoer D, 2018. Allergen immunotherapy in people, dogs, cats and horses – differences, similarities and research needs. *Allergy*, 73, 1989–1999.
- Nishijima C, Chiba T, Sato Y and Umegaki K, 2019. Nationwide online survey enables the reevaluation of the safety of *Coleus forskohlii* extract intake based on the adverse event frequencies. *Nutrients*, 11, 866.
- Nowak-Węgrzyn A and Fiocchi A, 2009. Rare, medium, or well done? The effect of heating and food matrix on food protein allergenicity. *Current Opinion in Allergy and Clinical Immunology*, 9, 234–237.
- Ofori-Anti AO, Ariyaratna H, Chen L, Lee HL, Pramod SN and Goodman RE, 2008. Establishing objective detection limits for the pepsin digestion assay used in the assessment of genetically modified foods. *Regulatory Toxicology and Pharmacology*, 52, 94–103.
- Ozias-Akins P and Breiteneder H, 2019. The functional biology of peanut allergens and possible links to their allergenicity. *Allergy*, 74, 888–898.
- Pali-Schöll I, De Lucia M, Jackson H, Janda J, Mueller RS and Jensen-Jarolim E, 2017. Comparing immediate-type food allergy in humans and companion animals—revealing unmet needs. *Allergy*, 72, 1643–1656.
- Pali-Schöll I, Blank S, Verhoeckx K, Mueller RS, Jada J, Marti E, Seida AA, Rhyner C, DeBoer DJ and Jensen-Jarolim E, 2019. EAACI position paper: Comparing insect hypersensitivity induced by bite, sting, inhalation or ingestion in human beings and animals. *Allergy*, 74, 874–887.
- Parenti MD, Santoro A, Del Rio A and Franceschi C, 2019. Literature review in support of adjuvanticity/immunogenicity assessment of proteins. EFSA Supporting Publication 2019;EN-1551, 68 pp. <https://doi.org/10.2903/sp.efsa.2019.EN-1551>
- Patel N, Adelman DC, Anagnostou K, Baumert JL, Blom WM, Campbell DE, Chinthrajah RS, Mills ENC, Javed B, Purington N, Remington BC, Sampson HA, Smith AD, Yarham RAR and Turner PJ, 2021. Using data from food challenges to inform management of consumers with food allergy: a systematic review with individual participant data meta-analysis. *The Journal of Allergy and Clinical Immunology*, 147, 2249–2262.e7.
- Paul V, Steinke K and Meyer HH, 2008. Development and validation of a sensitive enzyme immunoassay for surveillance of Cry1Ab toxin in bovine blood plasma of cows fed Bt-maize (MON810). *Analytica Chimica Acta*, 607, 106–113.
- Pearson WR and Lipman DJ, 1988. Improved tools for biological sequence comparison. *Proceedings of the National Academy of Sciences*, 85, 2444–2448.
- Pekar J, Ret D and Untersmayr E, 2018. Stability of allergens. *Molecular Immunology*, 100, 14–20.
- Perrier C and Corthésy B, 2011. Gut permeability and food allergies. *Clinical and Experimental Allergy*, 41, 20–28.
- Petersen A, Schramm G, Schlaak M and Becker WM, 1998. Post-translational modifications influence IgE reactivity to the major allergen Phl p 1 of timothy grass pollen. *Clinical and Experimental Allergy*, 28, 315–321.
- Petersen A, Rennert S, Kull S, Becker WM, Notbohm H, Goldmann T and Jappe U, 2014. Roasting and lipid binding provide allergenic and proteolytic stability to the peanut allergen Ara h 8. *Biological Chemistry*, 395, 239–250.
- Pickles J, Rafiq S, Cochrane SA and Lalljie A, 2014. *In vitro* pepsin resistance of proteins: effect of non-reduced SDS-PAGE analysis on fragment observation. *Toxicology Reports*, 16, 858–870.
- Piloli R, Gadaleta A, Mamone G, Nigro D, De Angelis E, Montemurro N and Monaci L, 2019. Scouting for naturally low-toxicity wheat genotypes by a multidisciplinary approach. *Scientific Reports*, 9, 1646.
- Pomés A, Mueller GA and Chruszcz M, 2020. Structural aspects of the allergen-antibody interaction. *Frontiers in Immunology*, 2, 2067.
- Poulsen OM, Hau J and Kollerup J, 1987. Effect of homogenization and pasteurization on the allergenicity of bovine milk analysed by a murine anaphylactic shock model. *Clinical and Experimental Allergy*, 17, 449–458.
- Prescott VE, Campbell PM, Moore A, Mattes J, Rothenberg ME, Foster PS, 2005. Transgenic expression of bean alpha-amylase inhibitor in peas results in altered structure and immunogenicity. *Journal of Agriculture and Food Chemistry*, 53, 9023–9030.
- Prodic I, Stanic-Vucinic D, Apostolovic D, Mihailovic J, Radibratovic M, Radosavljevic J, Burazer L, Milcic M, Smiljanic K, van Hage M and Cirkovic Velickovic T, 2018. Influence of peanut matrix on stability of allergens in gastric-simulated digesta: 2S albumins are main contributors to the IgE reactivity of short digestion-resistant peptides. *Clinical and Experimental Allergy*, 48, 731–740.

- Radauer C and Breiteneder H, 2006. Pollen allergens are restricted to few protein families and show distinct patterns of species distribution. *The Journal of Allergy and Clinical Immunology*, 117, 141–147.
- Radauer C and Breiteneder H, 2019. Allergen databases – a critical evaluation. *Allergy*, 74, 2057–2060.
- Radauer C, Bublin M, Wagner S, Mari A and Breiteneder H, 2008. Allergens are distributed into few protein families and possess a restricted number of biochemical functions. *The Journal of Allergy and Clinical Immunology*, 121, 847–52.e7.
- Radosavljević J, Apostolović D, Mihailović J, Atanasković-Marković M, Burazer L, van Hage M and Ćirković Veličković T, 2020. Digestomics of cow's milk: short digestion-resistant peptides of casein form functional complexes by aggregation. *Foods*, 9, 1576.
- Rao H, Baricevic I, Bernard H, Smith F, Sayers R, Balasundaram A, Costello CA, Padfield P, Semic-Jusufagic A, Simpson A, Adel-Patient K, Xue W and Mills ENC, 2020. The effect of the food matrix on the in vitro bio-accessibility and ige reactivity of peanut allergens. *Molecular Nutrition & Food Research*, 64, 1901093.
- Reiner D, Lee R-Y, Dekan G and Epstein MM, 2014. No adjuvant effect of *Bacillus thuringiensis*-maize on allergic responses in mice. *PLoS One*, 9, e103979.
- Remington B, Broekman HCH, Blom WM, Capt A, Crevel RWR, Dimitrov I, Faeste CK, Fernandez-Canton R, Giavi S, Houben GF, Glenn KC, Madsen CB, Kruizinga AK and Constable A, 2018. Approaches to assess IgE mediated allergy risks (sensitization and cross-reactivity) from new or modified dietary proteins. *Food and Chemical Toxicology*, 112, 97–107.
- Remington BC, Westerhout J, Meima MY, Blom WM, Kruizinga AG, Wheeler MW, Taylor SL, Houben GF and Baumert JL, 2020. Updated population minimal eliciting dose distributions for use in risk assessment of 14 priority food allergens. *Food and Chemical Toxicology*, 139, 111259.
- Renz H, Allen KJ, Sicherer SH, Sampson HA, Lack G, Beyer K and Oettgen HC, 2018. Food Allergy. *Nature Reviews Disease Primers*, 4, 17098.
- Reyna-Margarita HR, Irais CM, Mario-Alberto RG, Agustina RM, Luis-Benjamin SG and David PE, 2019. Plant phenolics and lectins as vaccine adjuvants. *Current Pharmaceutical Biotechnology*, 20, 1236–1243.
- Ruiter B and Shreffler WG, 2012. Innate immunostimulatory properties of allergens and their relevance to food allergy. *Seminars in Immunopathology*, 34, 617–632.
- Samadi N, Klems M and Untersmayr E, 2018. The role of gastrointestinal permeability in food allergy. *Annals of Allergy, Asthma and Immunology*, 121, 168–173.
- Sampson HA and Anderson JA, 2000. Summary and recommendations: Classification of gastrointestinal manifestations due to immunologic reactions to foods in infants and young children. *Journal of Pediatric Gastroenterology and Nutrition*, 30, S87–S94.
- Sanden M, Ormsrud R, Sissener NH, Jorgensen S, Gu JN, Bakke AM, 2013. Cross-generational feeding of Bt (*Bacillus thuringiensis*)-maize to zebrafish (*Danio rerio*) showed no adverse effects on the parental or offspring generations. *British Journal of Nutrition*, 110, 2222–2233.
- Saunders SP, Ma EGM, Aranda CJ and Curotto de Lafaille MA, 2019. Non-classical B cell memory of allergic IgE responses. *Frontiers in Immunology*, 10, 715–730.
- Santos AF and Lack G, 2016. Basophil activation test: food challenge in a test tube or specialist research tool? *Clinical Translation Allergy*, 15, 10.
- Santos AF, Alpan O and Hoffmann HJ, 2021. Basophil activation test: mechanisms and considerations for use in clinical trials and clinical practice. *Allergy*, 76, 2420–2432.
- Scheurer S, Toda M and Vieths S, 2015. What makes an allergen? *Clinical and Experimental Allergy*, 45, 1150–1161.
- Scott IC, Majithiya JB, Sanden C, Thornton P, Sanders PN, Moore T, Guscott M, Corkill DJ, Erjefält JS and Cohen ES, 2018. Interleukin-33 is activated by allergen- and necrosis-associated proteolytic activities to regulate its alarmin activity during epithelial damage. *Scientific Reports*, 8, 3363.
- Shade KC, Conroy ME, Washburn N, Kitaoka M, Huynh DJ, Laprise E, Patil SU, Shreffler WG and Anthony RM, 2020. Sialylation of immunoglobulin E is a determinant of allergic pathogenicity. *Nature*, 582, 265–270.
- Shan L, Molberg Ø, Parrot I, Hausch F, Filiz F, Gray GM, Sollid LM and Khosla C, 2002. Structural basis for gluten intolerance in celiac sprue. *Science*, 297, 2275–2279.
- Sharma N, Patiyal S, Dhall A, Pande A, Arora C and Raghava GPS, 2021. AlgPred 2.0: an improved method for predicting allergenic proteins and mapping of IgE epitopes. *Briefings in Bioinformatics*, 22, bbaa294.
- Shreffler WG, Castro RR, Kucuk ZY, Charlop-Powers Z, Grishina G, Yoo S, Burks AW and Sampson HA, 2006. The major glycoprotein allergen from *Arachis hypogaea*, Ara h 1, is a ligand of dendritic cell-specific ICAM-grabbing nonintegrin and acts as a Th2 adjuvant *In Vitro*. *The Journal of Immunology*, 177, 3677–3685.
- Sicherer SH and Sampson HA, 2018. Food allergy: a review and update on epidemiology, pathogenesis, diagnosis, prevention, and management. *The Journal of Allergy and Clinical Immunology*, 141, 41–58.
- Silvanovich A, Bannon GA and McClain S, 2009. The use of E-scores to determine the quality of protein alignments. *Regulatory Toxicology and Pharmacology*, 54(3 Suppl.), S26.
- Slough CL, Miday RK, Zorich NL and Jones JK, 2001. Postmarketing surveillance of new food ingredients: design and implementation of the program for the fat replacer olestra. *Regulatory Toxicology and Pharmacology*, 33, 218–223.

- Smith F, Pan X, Bellido V, Toole GA, Gates FK, Wickham MS, Shewry PR, Bakalis S, Padfield P and Mills EN, 2015. Digestibility of gluten proteins is reduced by baking and enhanced by starch digestion. *Molecular Nutrition and Food Research*, 59, 2034–2043.
- Smits M, Nooijen I, Redegeld F, de Jong A, Le TM, Knulst A, Houben G and Verhoeckx K, 2021. Digestion and transport across the intestinal epithelium affects the allergenicity of Ara h 1 and 3 but not of Ara h 2 and 6. *Molecular Nutrition and Food Research*, 65, e2000712.
- Soh WT, Aglas L, Mueller GA, Gilles S, Weiss R, Scheibhofer S, Huber S, Scheidt T, Thompson PM, Briza P, London RE, Traidl-Hoffmann C, Cabrele C, Brandstetter H and Ferreira F, 2019. Multiple roles of Bet v 1 ligands in allergen stabilization and modulation of endosomal protease activity. *Allergy*, 74, 2382–2393.
- Sollid LM, Tye-Din JA, Qiao SW, Anderson RP, Gianfrani C and Koning F, 2020. Update 2020: nomenclature and listing of celiac disease-relevant gluten epitopes recognized by CD4<sup>+</sup> T cells. *Immunogenetics*, 72, 85–88.
- Steinke K, Guertler P, Paul V, Wiedemann S, Etle T, Albrecht C, 2010. Effects of long-term feeding of genetically modified corn (event MON810) on the performance of lactating dairy cows. *Journal of Animal Physiology and Animal Nutrition*, 94, e185–e193.
- Sutton BJ, Davies AM, Bax HJ and Karagiannis SN, 2019. IgE antibodies: from structure to function and clinical translation. *Antibodies (Basel)*, 8, 19.
- Takagi K, Teshima R, Okunuki H and Sawada J-I, 2003. Comparative study of in vitro digestibility of food proteins and effect of preheating on the digestion. *Biological and Pharmaceutical Bulletin*, 26, 969–973.
- Takata S, Ohtani O and Watanabe Y, 2000. Lectin binding patterns in rat nasal-associated lymphoid tissue (NALT) and the influence of various types of lectin on particle uptake in NALT. *Archives of Histology and Cytology*, 63, 305–312.
- Taylor SL, Hefle SL, Bindslev-Jensen C, Bock SA, Burks Jr AW, Christie L, Hill DJ, Host A, Hourihane JO, Lack G, Metcalfe DD, Moneret-Vautrin DA, Vadas PA, Rance F, Skrypec DJ, Trautman TA, Yman IM and Zeiger RS, 2002. Factors affecting the determination of threshold doses for allergenic foods: how much is too much? *The Journal of Allergy and Clinical Immunology*, 109, 24–30.
- Thomas K, Aalbers M, Bannon GA, Bartels M, Dearman RJ, Esdaile DJ, Fu TJ, Glatt CM, Hadfield N, Hatzos C, Hefle SL, Heylings JR, Goodman RE, Henry B, Herouet C, Holsapple M, Ladics GS, Landry TD, MacIntosh SC, Rice EA, Privalle LS, Steiner HY, Teshima R, Van Ree R, Woolhiser M and Zawodny J, 2004. A multi-laboratory evaluation of a common in vitro pepsin digestion assay protocol used in assessing the safety of novel proteins. *Regulatory Toxicology and Pharmacology*, 39, 87–98.
- Tiller T, Meffre E, Yurasov S, Tsuiji M, Nussenzweig MC and Wardemann H, 2008. Efficient generation of monoclonal antibodies from single human B cells by single cell RT-PCR and expression vector cloning. *Journal of Immunological Methods*, 329, 112–124.
- Tordesillas L and Berin MC, 2018. Mechanisms of oral tolerance. *Clinical Reviews in Allergy and Immunology*, 55, 107–117.
- Trabalza-Marinucci M, Brandi G, Rondini C, Avellini L, Giammarini C, Costarelli S, Acuti G, Orlandi C, Filippini G, Chiaradia E, Malatesta M, Crotti S, Antonini C, Amagliani G, Manuali E, Mastrogiacomo AR, Moscati L, Naceur Haouet M, Gaiti A and Magnani M, 2008. A three-year longitudinal study on the effects of a diet containing genetically modified Bt176 maize on the health status and performance of sheep. *Livestock Science*, 113, 178–190.
- Tulinská J, Adel-Patient K, Bernard H, Lišková A, Kuricová M, Ilavská S, Horváthová M, Kebis A, Rollerová E, Babincová J, Aláčová R, Wal J-M, Schmidt K, Schmidtke J, Schmidt P, Kohl C, Wilhelm R, Schiemann J and Steinberg P, 2018. Humoral and cellular immune response in Wistar Han RCC rats fed two genetically modified maize MON810 varieties for 90 days (EU 7th Framework Programme project GRACE). *Archives of Toxicology*, 92, 2385–2399.
- Valenta R, Hochwallner H, Linhart B and Pahr S, 2015. Food allergies: the basics. *Gastroenterology*, 148, 1120–1131.
- van Bilsen JHM, Sienkiewicz-Szlapka E, Lozano-Ojalvo D, Willemsen LEM, Antunes CM, Molina E, Smit JJ, Wróblewska B, Wichers HJ, Knol EF, Ladics GS, Pieters RHH, Denery-Papini S, Vissers YM, Bavaro SL, Larré C, Verhoeckx KCM and Roggen EL, 2017. Application of the adverse outcome pathway (AOP) concept to structure the available in vivo and in vitro mechanistic data for allergic sensitization to food proteins. *Clinical and Translational Allergy*, 7, 13.
- van Boxtel EL, van den Broek LA, Koppelman SJ and Gruppen H, 2008. Legumin allergens from peanuts and soybeans: effects of denaturation and aggregation on allergenicity. *Molecular Nutrition and Food Research*, 52, 674–682.
- Vassilopoulou E, Rigby N, Moreno FJ, Zuidmeer L, Akkerdaas J, Tassios I, Papadopoulos NG, Saxoni-Papageorgiou P, van Ree R and Mills C, 2006. Effect of in vitro gastric and duodenal digestion on the allergenicity of grape lipid transfer protein. *The Journal of Allergy and Clinical Immunology*, 118, 473–480.
- Vázquez RI, Moreno-Fierros L, Neri-Bazán L, De La Riva GA and López-Revilla R, 1999. *Bacillus thuringiensis* Cry1Ac protoxin is a potent systemic and mucosal adjuvant. *Scandinavian Journal of Immunology*, 49, 578–584.
- Vázquez-Padrón RI, Moreno-Fierros L, Neri-Bazán L, de la Riva GA and López-Revilla R, 1999. Intragastric and intraperitoneal administration of Cry1Ac protoxin from *Bacillus thuringiensis* induces systemic and mucosal antibody responses in mice. *Life Sciences*, 64, 1897–1912.

- Verhoeckx K, Broekman H, Knulst A and Houben G, 2016. Allergenicity assessment strategy for novel food proteins and protein sources. *Regulatory Toxicology and Pharmacology*, 79, 118–124.
- Vivekanantham A, Belousov M, Hassan L, Nenadic G and Dixon WG, 2020. Patient discussions of glucocorticoid-related side effects within an online health community forum. *Annals of the Rheumatic Diseases*, 79, 1121–1122.
- Vriz R, Moreno FJ, Koning F and Fernandez A, 2021. Ranking of immunodominant epitopes in celiac disease: Identification of reliable parameters for the safety assessment of innovative food proteins. *Food and Chemical Toxicology*, 157, 112584.
- Walsh MC, Buzoianu SG, Gardiner GE, Rea MC, Paul Ross R, Cassidy JP and Lawlor PG, 2012. Effects of short-term feeding of Bt MON810 maize on growth performance, organ morphology and function in pigs. *British Journal of Nutrition*, 107, 364–371.
- Wang R, Edrington TC, Storrs SB, Crowley KS, Ward JM, Lee TC, Liu ZL, Li B and Glenn KC, 2017. Analyzing pepsin degradation assay conditions used for allergenicity assessments to ensure that pepsin susceptible and pepsin resistant dietary proteins are distinguishable. *PLoS One*, 12, e0171926.
- Wang R, Wang Y, Edrington TC, Liu Z, Lee TC, Silvanovich A, Moon HS, Liu ZL and Li B, 2020. Presence of small resistant peptides from new in vitro digestion assays detected by liquid chromatography tandem mass spectrometry: an implication of allergenicity prediction of novel proteins? *PLoS One*, 15, e0233745.
- Wavrin S, Bernard H, Wal JM and Adel-Patient K, 2015. Influence of the route of exposure and the matrix on the sensitisation potency of a major cows' milk allergen. *Clinical and Translational Allergy*, 5, 3.
- Westerhout J, Steeg EVD, Grossouw D, Zeijdner EE, Krul CAM, Verwei M and Wortelboer HM, 2014. A new approach to predict human intestinal absorption using porcine intestinal tissue and biorelevant matrices. *European Journal of Pharmaceutical Sciences*, 63, 167–177.
- Westerhout J, Krone T, Snippe A, Babé L, McClain S, Ladics GS, Houben GF and Verhoeckx KCM, 2019. Allergenicity prediction of novel and modified proteins: not a mission impossible! development of a random forest allergenicity prediction model. *Regulatory Toxicology and Pharmacology*, 107, 104422.
- Wickham M, Faulks R and Mills C, 2009. In vitro digestion methods for assessing the effect of food structure on allergen breakdown. *Molecular Nutrition and Food Research*, 53, 952–958.
- Worm M, Moneret-Vautrin A, Scherer K, Lang R, Fernandez-Rivas M, Cardona V, Kowalski ML, Jutel M, Poziomkowska-Gesicka I, Papadopoulos NG, Beyer K, Mustakov T, Christoff G, Bilò MB, Muraro A, Hourihane JO and Grabenhenrich LB, 2014. First European data from the network of severe allergic reactions (NORA). *Allergy*, 69, 1397–1404.
- Worm M, Reese I, Ballmer-Weber B, Beyer K, Bischoff SC, Bohle B, Brockow K, Claßen M, Fischer PJ, Hamelmann E, Jappe U, Kleine-Tebbe J, Klimek L, Koletzko B, Lange L, Lau S, Lepp U, Mahler V, Nemat K, Raithel M, Saloga J, Schäfer C, Schnadt S, Schreiber J, Szépfalusi Z, Treudler R, Wagenmann M, Werfel T and Zuberbier T, 2021. Update of the S2k guideline on the management of IgE-mediated food allergies. *Allergol Select*, 8, 195–243.
- Wurth MA, Hadadlanpour A, Horvath DJ, Daniel J, Bogdan O, Gileniewska K, Pomés A, Hamilton RG, Stockes Peebles R and Smith SA, 2018. Human IgE mAbs define variability in commercial *Aspergillus* extract allergen composition. *JCI Insight*, 3, e123387.
- Zeljenkova D, Amrusova K, Bartusova M, Kebis A, Kovriznych J, Krivosikova Z, 2014. Ninety-day oral toxicity studies on two genetically modified maize MON810 varieties in Wistar Han RCC rats (EU 7th Framework Programme project GRACE). *Archives of Toxicology*, 88, 2289–2314.

## Abbreviations

|        |  |
|--------|--|
| aAI    | Alpha-amylase inhibitor                        |
| AOP    | Adverse outcome pathway                        |
| APC    | Antigen-processing and presenting cells        |
| BAT    | Basophil activation test                       |
| BLAST  | Basic Local Alignment Search Algorithm         |
| DBPCFC | Double-blind placebo-controlled food challenge |
| EAST   | Enzyme allergosorbent assay                    |
| ED     | Eliciting dose                                 |
| ELISA  | Enzyme-linked immunosorbent assay              |
| FAO    | Food and Agriculture Organization              |
| GIT    | Gastrointestinal tract                         |
| GM     | Genetically modified                           |
| GMO    | Genetically modified organisms                 |
| IgE    | Immunoglobulin type E                          |
| IgG    | Immunoglobulin type G                          |
| HLA    | Human leucocyte antigen                        |
| hMAT   | Human-derived mast cell activation test        |
| LPS    | Lipopolysaccharide                             |

|          |   |
|----------|---|
| miBAT    | Microfluidic immunoaffinity basophil activation test      |
| MC       | Mast cells  |
| MHCII    | Class two major histocompatibility complex                |
| OECD     | Organisation for Economic Co-operation and Development    |
| PAL      | Precautionary Allergen Labelling                          |
| PTM      | Post-translational modification                           |
| RAST     | Radio allergosorbent assay                                |
| RfD      | Reference dose  |
| SDS-PAGE | Sodium dodecyl sulfate-polyacrylamide gel electrophoresis |
| WHO      | World Health Organization                                 |