

APPROVED: 10 January 2019

doi:10.2903/sp.efsa.2019.EN-1551

Literature review in support of adjuvanticity/immunogenicity assessment of proteins

Marco Daniele Parenti, Aurelia Santoro, Alberto Del Rio, Claudio Franceschi

Innovamol Srl, Modena, Italy; University of Bologna, Bologna, Italy

Abstract

Based on the risk assessment of genetically modified plants, according to Implementing Regulation (EU) No 503/201321 “In cases when known functional aspects of the newly expressed protein or structural similarity to known strong adjuvants may indicate possible adjuvant activity, the applicant shall assess the possible role of these proteins as adjuvants”. To further investigate the topic, an EFSA procurement was launched requesting a comprehensive literature review and critically appraisal on adjuvanticity and immunogenicity of proteins. A systematic literature search and critical review was performed, identifying 299 relevant publications. From the evaluation of the relevant literature emerged that: i) a clear classification of adjuvant and immunogens of proteins cannot be done; ii) structural features able to modulate adjuvanticity and immunogenicity are mainly ascribed to therapeutic proteins and in the context of allergenicity and cross-reactivity; iii) factors affecting the propensity of a protein to stimulate immune response are aggregation, thermal processing, digestion, food matrix, among others; iv) different proteins are described to have immunomodulatory effects; v) risk assessment of adjuvant and immunogenic behaviour of proteins requires specific methodologies that can be adapted from other fields; vi) adjuvanticity and immunogenicity of Cry proteins in certain experimental conditions seems plausible but due to low dosage, oral route of administration, food and feed processing and digestion, it is unlikely to emerge as a safety issue in food and feed; vii) eliciting an immune response is a very complex matter as the body responds to immune offence by inducing many processes. Based on these considerations, it is expected that the availability of new humanized animal models and the possibility to deploy artificial intelligent systems on the vastity of human data will become a general direction aiming to help answering specific questions relating to the immune systems, including the adjuvanticity and immunogenicity of food/feed proteins.

© European Food Safety Authority, 2019

Key words: adjuvanticity, immunogenicity, protein, food and feed, risk assessment, Cry proteins**Question number:** EFSA-Q-2017-00703**Correspondence:** GMO@efsa.europa.eu

Disclaimer: (For procurement:) The present document has been produced and adopted by the bodies identified above as authors. This task has been carried out exclusively by the authors in the context of a contract between the European Food Safety Authority and the authors, awarded following a tender procedure. The present document is published complying with the transparency principle to which the Authority is subject. It may not be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the authors.

Acknowledgements: The authors wish to thank EFSA for financing this project.

Suggested citation: Marco Daniele Parenti, Aurelia Santoro, Alberto Del Rio, Claudio Franceschi, 2019. Literature review in support of adjuvanticity/immunogenicity assessment of proteins. EFSA supporting publication 2019:EN-1551. 68 pp. doi:10.2903/sp.efsa.2019.EN-1551

ISSN: 2397-8325

© European Food Safety Authority, 2019

Reproduction is authorised provided the source is acknowledged.

Summary

Based on the risk assessment of genetically modified plants and according to Implementing Regulation (EU) No 503/2013 “In cases when known functional aspects of the newly expressed protein or structural similarity to known strong adjuvants may indicate possible adjuvant activity, the applicant shall assess the possible role of these proteins as adjuvants”. To further investigate the topic, an EFSA procurement was launched requesting a comprehensive literature review and critically appraisal on adjuvanticity and immunogenicity of proteins.

For this evaluation, a systematic literature search and review was performed prioritising the oral route of exposure, identifying 299 relevant publications, including those in the grey literature, describing:

- i) classification of adjuvants and immunogens;
- ii) structural and functional characteristics of adjuvants and immunogens and mechanisms underlying an adverse effect due to the inherent characteristics of a protein;
- iii) the conditions under which a protein may (de)regulate the immune response on its own or towards another bystander protein;
- iv) a list of proteins in food/feed with an adjuvant and/or an immune stimulatory capacity;
- v) available methodologies for risk assessment of protein immunogenicity/adjuvanticity;
- vi) immunogenicity and adjuvanticity of Cry proteins and
- vii) general strategies to increase or decrease the immune response through adjuvant or immunogenic behaviour of proteins.

From the evaluation of the relevant literature emerged that:

- i) a clear classification of adjuvant and immunogens cannot be done;
- ii) structural features able to modulate immunogenicity and adjuvanticity were studied mainly in the context of therapeutic proteins and the allergenicity/cross-reactivity context;
- iii) factors that affect the propensity of a protein to stimulate immune response are mainly aggregation, thermal processing, digestion, the composition of the food matrix, the presence of immunomodulators, aging and microbiota;
- iv) different proteins are described in the literature to have immunomodulatory effects, among which lectins appear to have adjuvant behaviour;
- v) there are several *in silico*, *in vitro* and *in vivo* available methodologies for risk assessment of adjuvanticity and immunogenicity of proteins but each method has strengths and weaknesses;
- vi) the adjuvanticity and immunogenicity of Cry proteins in certain experimental conditions seems plausible but due to low dosage, oral route of administration, food processing and digestion, it is unlikely to emerge as a safety issue in food and feed;
- vii) eliciting an immune response is a very complex matter as the body responds to immune offence by inducing many processes.

From the critical appraisal of the literature other important considerations emerged like the need to devise new animal models. In fact, while these are considered essential research tools genetic background and experimental conditions differences could strongly impact on the translation of the results in humans. Thus, the application of “humanized mice” is one of the promising approaches to fill this translational gap. Humanized mice can be defined simply as mice carrying human genes or tissues such as leukocytes, stem cells, organs, and tumors. However, it is anticipated that the process to create humanized models is complex and expensive as it depends on numerous interventions and many potential pitfalls still exist and impinge upon their robustness.

In addition to humanized animal model, we purport the idea that another viable and unprecedented way for a major comprehension of the immune system is to use the potential vastity of human data. Indeed, by considering the fast progress that omics and genetic technologies are experiencing as well as the impact that will likely have in the future, it is expected that a large amount of human data and information could be retrieved, even in a systematic way. This possibility opens a completely new perspective that is aimed to generate new knowledge, for instance by using artificial intelligence or sophisticated data mining builder. However, collection of human data with new technologies impinge on considerations related to privacy and ethics of massive personal data usage that should be carefully considered in the regulation framework.

In conclusion, conceptualization of the immune response triggering is a complex matter that scientifically evolved in the last decades. A wider framework for the immune response triggering that is intimately linked to the conceptualization of the immunological self is advised. Indeed, immunobiography is unique for each individual and is characterised by the combination of the type, intensity and temporal sequence of antigens (including food and feed) an individual is exposed lifelong. This feature can explain how the same adjuvant and immunogenic molecule, depending on the immunobiography of the host, can elicit strong, weak or no immune response at all. Based on these considerations, it is expected that the availability of new humanized animal models and the possibility to deploy artificial intelligent systems on the vastity of human data will become a general direction aiming to help answering specific question relating to the immune system, including the role of protein and peptides in their adjuvant and/or immunogenic behaviour towards specific subset of population.

Table of contents

Abstract.....	1
Summary.....	3
1. Introduction.....	7
1.1. Background and Terms of Reference as provided by the requestor	7
1.2. Interpretation of the Terms of Reference.....	7
1.2.1. Background as provided by EFSA	7
1.2.2. Background as provided by the tenderer	7
1.3. Additional information	8
2. Data and Methodologies	9
2.1. Overview of the methodology	9
2.2. Literature search.....	10
2.2.1. Systematic PubMed and Web of Science literature search	11
2.3. Grey literature search.....	14
2.4. Post-processing.....	14
2.5. Document filtering	14
2.5.1. Review filtering	14
2.5.2. Cry protein filtering	15
2.6. Literature relevance assessment	15
2.6.1. Inclusion criteria	15
2.6.2. Performance of the assessment	15
2.6.3. Eligibility criteria.....	16
3. Assessment/Results.....	17
3.1. Systematic and comprehensive critical evaluation of the information and methodologies and the usefulness of those for the food/feed safety assessment of proteins.....	17
3.1.1. Classification of adjuvants and immunogens	17
3.1.2. Structural and functional characteristics of adjuvants and immunogens	18
3.1.3. Conditions under which a protein may (de)regulate the immune response on its own or towards another bystander protein.....	19
3.1.4. List of proteins in food/feed with an adjuvant and an immune stimulatory capacity	23
3.1.5. An identification, classification and general description of <i>in silico</i> , <i>in vitro</i> and <i>in vivo</i> available methodologies that might be employed for the risk assessment of adjuvanticity and immunogenicity of proteins.....	24
3.1.6. Adjuvanticity and immunogenicity of Cry proteins	28
3.1.7. General strategies to increase or decrease the immune response through adjuvant and immunogenic behaviour of proteins.....	31
3.2. Possible risk assessment strategies for food and feed safety evaluation of protein adjuvanticity and immunogenicity	33
3.2.1. General information on protein/peptides in food and feed	33
3.2.2. Sequence homology	34
3.2.3. Serum Screening.....	34
3.2.4. <i>In vitro</i> digestibility test.....	35
3.2.5. Other <i>in vitro</i> tests or <i>in vivo</i> tests on animal models	35
3.3. Hypothesis and theories	36
3.4. Potential future developments: the context dimension.....	37
3.5. Implications for the food and feed safety assessment: a “personal risk assessment” scenario as future perspective	39
4. Conclusions	42
References.....	45
Glossary and Abbreviations	66



1. Introduction

1.1. Background and Terms of Reference as provided by the requestor

This contract was awarded by EFSA to a consortium with Innovamol Srl in the lead:

Contractor: Innovamol Srl

Members of the consortium are:

Innovamol Srl, Modena, Italy

University of Bologna, Bologna, Italy

Contract title: Literature review in support of adjuvanticity/immunogenicity assessment of proteins

Contract number: NP/EFSA/GMO/2017/01

1.2. Interpretation of the Terms of Reference

1.2.1. Background as provided by EFSA

The objective of this call was to outsource a comprehensive literature review, critically appraising adjuvanticity and immunogenicity of proteins that will be used as background information for further discussion within the EFSA Panel dealing with genetically modified organisms (GMO).

The tenderer shall perform a comprehensive literature review to identify and retrieve all available information on adjuvanticity and immunogenicity of proteins, including Cry proteins – state-of-the-art in science, risk assessment strategies available and future perspectives. The information obtained shall be evaluated and critically appraised.

In the context of the risk assessment of genetically modified plants and according to Implementing Regulation (EU) No 503/2013¹ it is considered that: "In cases when known functional aspects of the newly expressed protein or structural similarity to known strong adjuvants may indicate possible adjuvant activity, the applicant shall assess the possible role of these proteins as adjuvants".

Adjuvants are substances that, when co-administered with an antigen increase the immune response to the antigen and therefore might increase the allergic response. Understanding the structure and mechanisms resulting in potential adjuvanticity and immunogenicity of (novel) proteins and the conditions (e.g. environment, exposure) upon which this potential will be expressed and/or (de)regulated is an area of great development and scientific debate. The information provided by this literature review will be important for the EFSA GMO Panel to further discuss how to incorporate and streamline potential new strategies for the risk assessment of adjuvanticity and immunogenicity of (novel) proteins into the EFSA GMO Panel scientific opinions on applications.

The present Call is based on the Final work programme for grants and operational procurements 2017 as presented in Annex IX of the EFSA Programming Document 2017 – 2019, available on the EFSA's website (http://www.efsa.europa.eu/sites/default/files/corporate_publications/files/amp1719.pdf).

1.2.2. Background as provided by the tenderer

1.2.2.1. Definitions and framework for the immune response triggering

The present document is focused on adjuvanticity and immunogenicity of proteins in food/feed. Existing guidance from EFSA GMO panel addressed different components of the risk assessment mainly focusing on potential allergenicity of the novel protein(s) as well as of the whole food derived

from the GM plants². Currently, no detailed guidance on how to assess adjuvanticity and immunogenicity of proteins in food/feed is available from regulatory agencies worldwide.

Within this document, we aim to differentiate the concepts of adjuvanticity, immunogenicity, antigenicity and allergenicity by focusing on adjuvanticity and immunogenicity of proteins and peptides with a specific emphasis on Cry proteins.

We refer to antigenicity as the ability of an antigen to induce an immunological response when it is encountered by the human body. Antigenicity involves two types of immune characteristics, immunogenicity and allergenicity. Immunogenicity refers to the ability of an antigen to trigger normal and protective immune responses after being encountered by the human body. In particular, we describe the immunogenicity of an antigen using the following three aspects:

- the ability to defend the immune system (immunological defense), which is the ability to repel an exogenous antigen and to fight against infection;
- the ability to keep the immune system stable (immunological homeostasis), which is the ability of the body to recognize and eliminate damaged tissue, inflammation and/or senescent cells, and
- the ability to kill and to remove abnormally mutated cells so as to monitor and inhibit the growth of malignancies in the body (immunological surveillance).

Thus, immunogenicity reflects the strength of these three functions. On the other hand, allergenicity refers to the ability of an antigen to induce an abnormal immune response, which is an overreaction and different from a normal immune response in that it does not result in a protective/prophylaxis effect but instead causes physiological function disorder or tissue damage.

Adjuvants are substances that, when coadministered with an antigen, increase the immune response of the antigen and therefore might increase the allergic response. Therefore, while allergenicity is not the specific focus of this assignment, it was considered by the team when relevant.

In order to clarify these definitions throughout the text, a glossary of the adopted definitions is reported at the end of the document.

Conceptualization of the immune response triggering is a complex matter that scientifically evolved in the last decades at a fast pace. In order to take into account all relevant considerations for the EFSA call, this report leans on a wider framework for the immune response triggering that is intimately linked to the conceptualization of the immunological population heterogeneity further described in section 3.3.

1.3. Additional information

The objective of this EFSA call is to perform a comprehensive literature review and critically appraisal on adjuvanticity and immunogenicity of proteins. In conformity to the need of EFSA, the methodology proposed in the tender followed the general principles for systematic reviews as specified by EFSA guidance³. This document constitutes the final report (critical review) in English language addressing tasks 1, 2 and 3 as described below and in the tender specifications, including an update of the literature search.

Tasks 1 consisted in:

- A comprehensive literature search collecting state-of-the-art in science information on adjuvanticity and immunogenicity of proteins (including Cry proteins);
- The search focused on proteins (and peptides), prioritizing the oral route of exposure for food and feed, and included: i) Classification of adjuvants and immunogens; ii) the structural and functional characteristics (if any) of adjuvants and immunogens, as well as the mechanisms underlying an adverse effect due to the inherent characteristics of a protein; iii) the conditions

under which a protein may (de)regulate the immune response on its own or towards another bystander protein; and iv) a list of proteins in food/feed with an adjuvant and/or an immune stimulatory capacity;

- An identification, classification and general description of *in silico*, *in vitro* and *in vivo* available methodologies that might be employed for the risk assessment of adjuvanticity and immunogenicity of proteins (diverse safety areas to be considered, e.g. food and feed safety, pharmaceuticals, medicine, immunotherapy, etc).

Task 2 consisted in:

- Systematic and comprehensive critical evaluation of the information and methodologies described in task 1 and the usefulness of those for the food and feed safety assessment of proteins. Strengths and limitations discussed;
- Identification of possible structural features and characteristic attributes of proteins responsible for those adverse reactions as well as conditions under which a protein might (de)regulate the immune response in the context of food and feed safety;
- Taking the above into account, a proposal of possible risk assessment strategies for food and feed safety evaluation of protein adjuvanticity and immunogenicity;
- Future perspectives: a foresight study on the potential future developments (e.g. exposures scenarios, routes of administration, methodology development, etc) in the area and their possible implications for the food and feed safety assessment;

Task 3 consisted in:

- This final report (critical review) in English language addressing tasks 1 and 2, including an endnote database with an update of the literature search.
- All EFSA comments raised during the course of the contract by the EFSA Unit or the EFSA GMO Panel taken into consideration in this final report.
- The content of the report presented in the following structure: an executive summary, introduction, material and method, results, discussion and conclusion. The report submitted in electronic format (DOC format).

2. Data and Methodologies

2.1. Overview of the methodology

As shown in Figure 1 the working team completed the first three steps belonging to Task 1 that will be described in detail in the following paragraphs. The first three steps consisted in:

- Step 1: Preparing the literature search – reviewing protocol, consisting in developing the reviewing protocol, with the collaboration and agreement with EFSA, including the definition of review questions and developing the eligibility criteria for studies.
- Step 2: Searching for research studies, consisting in performing extensive literature search starting from multiple data sources that will include the requested databases (i.e. Web of Science and Pubmed)
- Step 3: Selecting the studies and collecting data from the included studies consisting in retrieving from identified documents information in order to assess relevance of studies against inclusion criteria.

For the sake of clarity, we reported in Figure 1 below the flowchart of the comprehensive literature search that led to the completion of the three steps mentioned above. We also organized the next paragraphs consistently to Figure 1 and the chronological course of the activities.

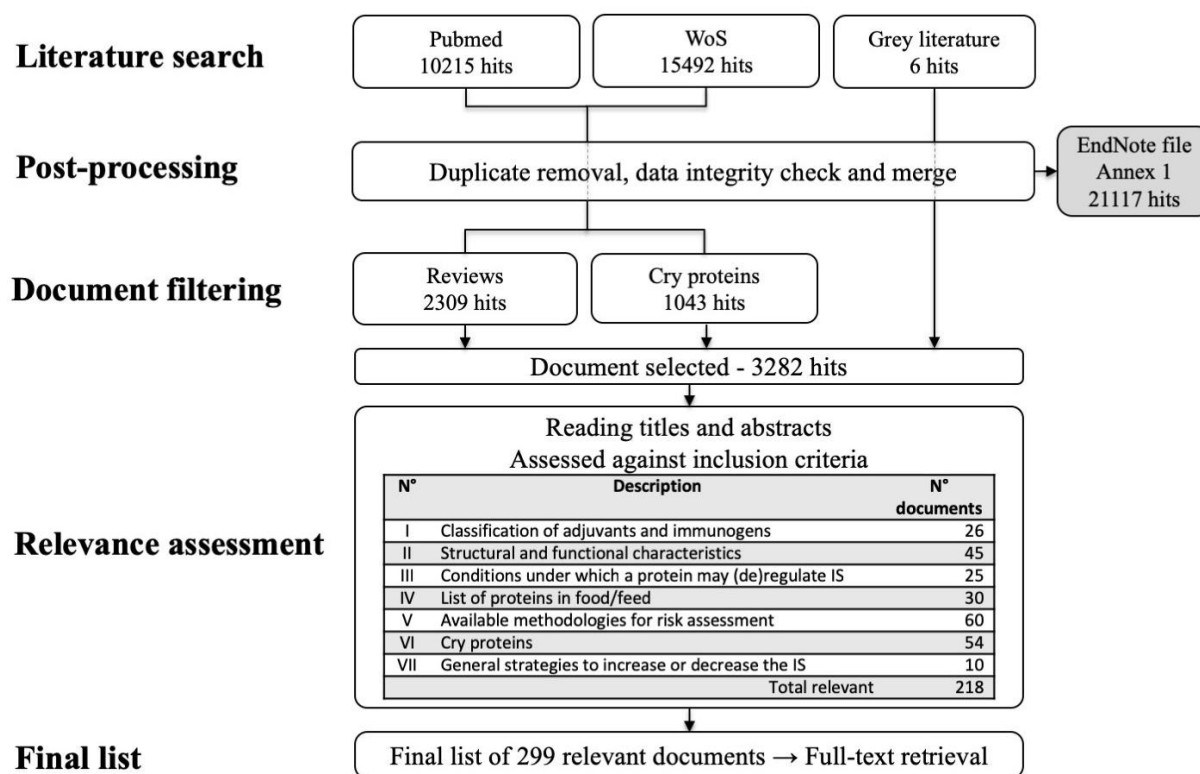


Figure 1: Overall results of the comprehensive literature review

2.2. Literature search

In this first section, the working team carried out the comprehensive literature search by collecting state-of-the-art in science information on adjuvanticity and immunogenicity of proteins. The strategy and methodology followed to perform literature searches was discussed with EFSA and took into account the inherent difficulties in collecting reliable set of data in certain conditions. Thus, the strategy and methodology were streamlined to keep into account:

- The collection of a reasonable number of documents, including those in the grey literature
- The refocus of queries by using combination of terms that could provide more relevant documents
- The importance of specific topics (e.g. Cry proteins) that were treated separately
- The need to avoid methodologies dependent from volatile definition of terms (e.g. generic Mesh terms in PubMed)

In order to keep into account the above-mentioned considerations, we devised three different searches with the objective to cover all the aspects of the call. In particular we defined three searches:

- A systematic PubMed literature search

- A systematic Web of Science *Core Collection* literature search
- A literature search specific for “grey documents” from regulatory agencies and other authorities. where different safety areas were considered, e.g. food/feed safety, pharmaceuticals, medicine, immunotherapy, etc.

The paragraphs below describe the details of these three different searches.

2.2.1. Systematic PubMed and Web of Science literature search

The systematic literature search was performed for the two requested databases (PubMed and Web of Science) by using the syntax query reported in Table 1 below. The construction of this query syntax was dictated by the necessity to include all the aspect of the call but also to avoid unnecessary and unfocused documents that appeared in more general definitions. After different test queries performed with PubMed, and in consultation with EFSA, we obtained the syntax depicted in Table 1 that returned 10215 hits and insured the best compromise between the various aspects of the call and the number of documents obtained. Consistently, the Web of Science search was devised to be specular to PubMed query. The result obtained with the *Core collection* by using the field “topic” was 15492 hits.

Table 1: List of queries performed with Pubmed and Web of Science

	Search number	Syntax ^(a)
PubMed Date performed: 14/02/2018		
Immunogenicity concepts in title/abstract	#1	(immunogen*[tiab] OR adjuvant*[tiab] OR immunopotent*[tiab] OR immunoactiv*[tiab] OR immunostimulant*[tiab] OR immunoadjuvant*[tiab] OR immuno potent*[tiab] OR immune potent*[tiab] OR immune activa*[tiab] OR immuno activa*[tiab] OR immune active*[tiab] OR immuno active*[tiab] OR immune stimulant*[tiab] OR immuno stimulant*[tiab] OR antigen*[tiab] OR epitope*[tiab] OR "B cell"[tiab] OR "B cells"[tiab] OR "T cell"[tiab] OR "T cells"[tiab] OR "B Lymphocyte"[tiab] OR "B Lymphocytes"[tiab] OR "T lymphocytes"[tiab] OR "T lymphocyte"[tiab] OR "immune response"[tiab] OR "immune responses"[tiab] OR epitope*[tiab])
Immunogenicity concepts in MeSH terms ([mesh])	#2	("Adjuvants, Immunologic"[Mesh] OR "Antigens"[Mesh:NoExp] OR "Epitopes"[Mesh:noexp] OR "Epitopes, B-Lymphocyte"[Mesh] OR "Epitopes, T-Lymphocyte"[Mesh] OR "Immunodominant Epitopes"[Mesh] OR "Immunoglobulin Idiotypes"[Mesh] OR "B-Lymphocytes"[Mesh:noexp] OR "T-Lymphocytes"[Mesh:noexp])
Immunogenicity and protein concepts combined in specific phrases	#3	("protein immunogenicity"[tiab] OR "protein immunogen"[tiab] OR "protein immunogens"[tiab] OR "protein adjuvant"[tiab] OR "protein adjuvants"[tiab] OR "peptide immunogenicity"[tiab] OR "peptide immunogen"[tiab] OR "peptide immunogens"[tiab] OR "peptide adjuvant"[tiab] OR "peptide adjuvants"[tiab] OR "toxin immunogenicity"[tiab] OR "toxin immunogen"[tiab] OR "toxin adjuvant"[tiab] OR "toxin adjuvants"[tiab] OR "immunogenic protein"[tiab] OR "immunogenic proteins"[tiab] OR "immunogenic peptide"[tiab] OR "immunogenic peptides"[tiab] OR "immunogen protein"[tiab] OR "adjuvant protein"[tiab] OR "adjuvant proteins"[tiab] OR "immunoactive peptide"[tiab] OR "immunoactive peptides"[tiab] OR "immunoactive protein"[tiab] OR "immunoactive proteins"[tiab])
Protein and food concepts	#4	((protein[tiab] OR proteins[tiab] OR peptide[tiab] OR peptides[tiab] OR toxin[tiab] OR toxins[tiab]) AND ((food[tiab] NOT "food and drug administration"[tiab]) OR food[mesh] OR feed[tiab] OR "animal feed"[mesh]))

Specific search for Cry Proteins	#5	<p>((("Adjuvants, Immunologic"[Mesh] OR adjuvan*[tiab] OR immunoadjuv*[tiab] OR immunostimul*[tiab] OR immuno stimul*[tiab] OR immunoactiv*[tiab] OR immuno activ*[tiab] OR immunopotent*[tiab] OR immuno potent*[tiab]OR immune reponse*[tiab] OR "Antigens"[Mesh:NoExp] OR immunogen*[tiab] OR immune gen*[tiab] OR antigen*[tiab] OR "Allergens"[Mesh] OR allergen*[tiab] OR "Epitopes"[Mesh:noexp] OR epitope*[tiab] OR "Epitopes, B-Lymphocyte"[Mesh] OR "Epitopes, T-Lymphocyte"[Mesh] OR "Immunodominant Epitopes"[Mesh] OR "Immunoglobulin Idiotypes"[Mesh] OR "B-Lymphocytes"[Mesh] OR "T-Lymphocytes"[Mesh] OR "B cells"[tiab] OR "T cells"[tiab] OR "B cell"[tiab] OR "T cell"[tiab] OR Immunoglobulin Idiotyp*[tiab])) AND (cry[tiab] OR cry1*[tiab] OR cry2*[tiab] OR cry3*[tiab] OR cryI*[tiab] AND (protein[tiab] OR proteins[tiab] OR toxin*[tiab])) OR BT toxin*[tiab] OR "Bacillus Thuringiensis"[tiab] OR "Bacillus thuringiensis"[Mesh] OR "B Thuringiensis"[tiab]))</p>
((#1 OR #2) AND #4) OR #3 OR #5	#6	<p>((immunogen*[tiab] OR adjuvant*[tiab] OR immunopotent*[tiab] OR immunoactiv*[tiab] OR immunostimulant*[tiab] OR immunoadjuvant*[tiab] OR immuno potent*[tiab] OR immune potent*[tiab] OR immune activa*[tiab] OR immuno activa*[tiab] OR immune active*[tiab] OR immuno active*[tiab] OR immune stimulant*[tiab] OR immuno stimulant*[tiab] OR antigen*[tiab] OR epitope*[tiab] OR "B cell"[tiab] OR "B cells"[tiab] OR "T cell"[tiab] OR "T cells"[tiab] OR "B Lymphocyte"[tiab] OR "B Lymphocytes"[tiab] OR "T lymphocytes"[tiab] OR "T lymphocyte"[tiab] OR "immune response"[tiab] OR "immune responses"[tiab] OR epitope*[tiab]) OR ("Adjuvants, Immunologic"[Mesh] OR "Antigens"[Mesh:NoExp] OR "Epitopes"[Mesh:noexp] OR "Epitopes, B-Lymphocyte"[Mesh] OR "Epitopes, T-Lymphocyte"[Mesh] OR "Immunodominant Epitopes"[Mesh] OR "Immunoglobulin Idiotypes"[Mesh] OR "B-Lymphocytes"[Mesh:noexp] OR "T-Lymphocytes"[Mesh:noexp])) AND ((protein[tiab] OR proteins[tiab] OR peptide[tiab] OR peptides[tiab] OR toxin[tiab] OR toxins[tiab]) AND ((food[tiab] NOT "food and drug administration"[tiab]) OR food[mesh] OR feed[tiab] OR "animal feed"[mesh])))) OR ("protein immunogenicity"[tiab] OR "protein immunogen"[tiab] OR "protein immunogens"[tiab] OR "protein adjuvant"[tiab] OR "protein adjuvants"[tiab] OR "peptide immunogenicity"[tiab] OR "peptide immunogen"[tiab] OR "peptide immunogens"[tiab] OR "peptide adjuvant"[tiab] OR "peptide adjuvants"[tiab] OR "toxin immunogenicity"[tiab] OR "toxin immunogen"[tiab] OR "toxin adjuvant"[tiab] OR "toxin adjuvants"[tiab] OR "immunogenic protein"[tiab] OR "immunogenic proteins"[tiab] OR "immunogenic peptide"[tiab] OR "immunogenic peptides"[tiab] OR "immunogen protein"[tiab] OR "adjuvant protein"[tiab] OR "adjuvant proteins"[tiab] OR "immunoactive peptide"[tiab] OR "immunoactive peptides"[tiab] OR "immunoactive protein"[tiab] OR "immunoactive proteins"[tiab]) OR (((("Adjuvants, Immunologic"[Mesh] OR adjuvan*[tiab] OR immunoadjuv*[tiab] OR immunostimul*[tiab] OR immuno stimul*[tiab] OR immunoactiv*[tiab] OR immuno activ*[tiab] OR immunopotent*[tiab] OR immuno potent*[tiab]OR immune reponse*[tiab] OR "Antigens"[Mesh:NoExp] OR immunogen*[tiab] OR immune gen*[tiab] OR antigen*[tiab] OR "Allergens"[Mesh] OR allergen*[tiab] OR "Epitopes"[Mesh:noexp] OR epitope*[tiab] OR "Epitopes, B-Lymphocyte"[Mesh] OR "Epitopes, T-Lymphocyte"[Mesh] OR "Immunodominant Epitopes"[Mesh] OR "Immunoglobulin Idiotypes"[Mesh] OR "B-Lymphocytes"[Mesh] OR "T-Lymphocytes"[Mesh] OR "B cells"[tiab] OR "T cells"[tiab] OR "B cell"[tiab] OR "T cell"[tiab] OR Immunoglobulin Idiotyp*[tiab])) AND (cry[tiab] OR cry1*[tiab] OR cry2*[tiab] OR cry3*[tiab] OR cryI*[tiab] AND (protein[tiab] OR proteins[tiab] OR toxin*[tiab])) OR BT toxin*[tiab] OR "Bacillus Thuringiensis"[tiab] OR "Bacillus thuringiensis"[Mesh] OR "B Thuringiensis"[tiab]))</p>
Web of Science		

Database: Core collection; Field: "topic" Date performed: 14/02/2018		
Immunogenicity concepts in title/abstract	#1	(immunogen* OR adjuvant* OR immunopotent* OR immunoactiv* OR immunostimulant* OR immunoadjuvant* OR "immuno potent*" OR "immune potent*" OR "immune activa*" OR "immuno activa*" OR "immune active*" OR "immuno active*" OR "immune stimulant*" OR "immuno stimulant*" OR antigen* OR epitope* OR "B cell" OR "B cells" OR "T cell" OR "T cells" OR "B Lymphocyte" OR "B Lymphocytes" OR "T lymphocytes" OR "T lymphocyte" OR "immune response" OR "immune responses" OR epitope*)
Immunogenicity and protein concepts combined in specific phrases	#2	("protein immunogenicity" OR "protein immunogen" OR "protein immunogens" OR "protein adjuvant" OR "protein adjuvants" OR "peptide immunogenicity" OR "peptide immunogen" OR "peptide immunogens" OR "peptide adjuvant" OR "peptide adjuvants" OR "toxin immunogenicity" OR "toxin immunogen" OR "toxin adjuvant" OR "toxin adjuvants" OR "immunogenic protein" OR "immunogenic proteins" OR "immunogenic peptide" OR "immunogenic peptides" OR "immunogen protein" OR "adjuvant protein" OR "adjuvant proteins" OR "immunoactive peptide" OR "immunoactive peptides" OR "immunoactive protein" OR "immunoactive proteins")
Protein and food concepts	#3	((protein OR proteins OR peptide OR peptides OR toxin OR toxins) AND ((food NOT "food and drug administration") OR feed))
Specific search for Cry Proteins	#4	((adjuvan* OR immunoadjuv* OR immunostimul* OR "immuno stimul*" OR immunoactiv* OR "immuno activ*" OR immunopotent* OR "immuno potent*" OR "immune response*" OR immunogen* OR "immune gen*" OR antigen* OR allergen* OR epitope* OR "B cells" OR "T cells" OR "B cell" OR "T cell" OR "Immunoglobulin Idiotype*") AND ((cry OR cry1* OR cry2* OR cry3* OR (cryI* AND (protein OR proteins OR toxin*)) OR "BT toxin*" OR "Bacillus Thuringiensis" OR "B Thuringiensis")))
((#1 AND #3) OR #2 OR #4)	#5	((((immunogen* OR adjuvant* OR immunopotent* OR immunoactiv* OR immunostimulant* OR immunoadjuvant* OR "immuno potent*" OR "immune potent*" OR "immune activa*" OR "immuno activa*" OR "immune active*" OR "immuno active*" OR "immune stimulant*" OR "immuno stimulant*" OR antigen* OR epitope* OR "B cell" OR "B cells" OR "T cell" OR "T cells" OR "B Lymphocyte" OR "B Lymphocytes" OR "T lymphocytes" OR "T lymphocyte" OR "immune response" OR "immune responses" OR epitope*) AND ((protein OR proteins OR peptide OR peptides OR toxin OR toxins) AND ((food NOT "food and drug administration") OR feed))) OR ("protein immunogenicity" OR "protein immunogen" OR "protein immunogens" OR "protein adjuvant" OR "protein adjuvants" OR "peptide immunogenicity" OR "peptide immunogen" OR "peptide immunogens" OR "peptide adjuvant" OR "peptide adjuvants" OR "toxin immunogenicity" OR "toxin immunogen" OR "toxin adjuvant" OR "toxin adjuvants" OR "immunogenic protein" OR "immunogenic proteins" OR "immunogenic peptide" OR "immunogenic peptides" OR "immunogen protein" OR "adjuvant protein" OR "adjuvant proteins" OR "immunoactive peptide" OR "immunoactive peptides" OR "immunoactive protein" OR "immunoactive proteins") OR ((adjuvan* OR immunoadjuv* OR immunostimul* OR "immuno stimul*" OR immunoactiv* OR "immuno activ*" OR immunopotent* OR "immuno potent*" OR "immune response*" OR immunogen* OR "immune gen*" OR antigen* OR allergen* OR epitope* OR "B cells" OR "T cells" OR "B cell" OR "T cell" OR "Immunoglobulin Idiotype*") AND ((cry OR cry1* OR cry2* OR cry3* OR (cryI* AND (protein OR proteins OR toxin*)) OR "BT toxin*" OR "Bacillus Thuringiensis" OR "B Thuringiensis"))))

a) as performed in the database search tool

2.3. Grey literature search

The literature search of online database was supplemented with a manual search for grey literature, to ensure no additional potential references were missed. This search was performed by accessing websites of agencies and authorities in EU and outside EU and by searching guidances, regulations and scientific opinions, including:

- Food and Drug Administration (FDA) (<https://www.fda.gov>)
- European Food Safety Authority (EFSA) (<http://www.efsa.europa.eu>)
- Canadian Food Inspection Agency (CFIA) (<http://www.inspection.gc.ca>)
- Australia Food Authority (NSW) (<http://www.foodauthority.nsw.gov.au>)
- Food Safety Commission of Japan (FSCJ) (<http://www.fsc.go.jp/english/>)
- The Norwegian Scientific Committee for Food and Environment (VKM) (<https://vkm.no>)

Because of the intrinsic differences in the organization of information in each website, a different search strategy was implemented for each source. In general, relevant documents were searched using the internal search engine of the website, and different combinations of the keywords: protein*, peptide*, adjuvant*, immunogen*. Retrieved documents were directly assessed for relevance on the topic of this call, and eventually included in the final list of relevant papers. Particular attention was dedicated to documents reporting suggested methods for risk assessment of protein adjuvanticity and immunogenicity.

2.4. Post-processing

References of documents collected from PubMed, Web of Science and the grey literature were imported in a new database by using the Mendeley software (V 1.17.11 / 2017 <https://www.mendeley.com>). The repository of data was post-processed with the software functionalities in order to

- i) merge data from the different searches,
- ii) remove duplicates
- iii) perform integrity check of each entry and,
- iv) correct reference citations.

The result of this post-processing constitutes a non-redundant and complete list of entries (19764 hits) that has been exported in EndNote format and is directly linked to documents found in the literature mentioning adjuvant and/or immunogenic proteins.

2.5. Document filtering

2.5.1. Review filtering

Because documents retrieved from PubMed and Web of Science largely exceeded the capability to perform subsequent tasks, a specific set of documents was obtained from these searches by combining non-redundant results and then by filtering reviews. Reviews were identified using though the "review" filter when performing the searches in the PubMed and WoS. The outcome of this filtering gave 2309 documents that were processed further. This filtering procedure allowed to reduce the number of papers to analyze while maintaining the capability to cover all aspects of the call, as defined in the inclusion criteria (see below).

2.5.2. Cry protein filtering

Because plant expression of *Bacillus thuringiensis* (Bt) crystal (Cry) insecticidal proteins have been the primary way to impart insect resistance in genetically modified crops, Cry proteins have an inherent importance in this assignment as described in the background of the tender specifications. Although deemed safe by regulatory agencies globally, previous studies on Cry proteins have been the basis for discussions around the potential immuno-adjuvant effects of Cry proteins. For this reason, the specific query reported in Table 1 was devised to keep into account all relevant documents on Cry proteins. It is noted that, papers reporting description of Cry proteins not related to *Bacillus thuringiensis* were not considered further. This search was included in both PubMed and Web of Science searches by keeping into account not only review but also articles.

The search on Cry proteins yielded a total of 1043 hits.

2.6. Literature relevance assessment

2.6.1. Inclusion criteria

Inclusion criteria were thoroughly consulted with EFSA during the assignment and are depicted in Table 2.

Criteria from I to V were included in the tender specifications and were considered as defined by EFSA.

Criteria VI was specifically created in order to consider the inherent importance of Cry proteins for the aim of the tender, consistently to the specific search as described in Section 2.5.2

Criteria VII was proposed by the tenderer and agreed with EFSA in order to keep into account documents that are relevant for the aim of the call by are not specifically included in the previous inclusion criteria.

Table 2: Description of inclusion criteria and numbering used in the present document

	Description of inclusion criteria
I	Classification of adjuvants and immunogens
II	Structural and functional characteristics of adjuvants and immunogens and mechanisms underlying an adverse effect due to the inherent characteristics of a protein
III	The conditions under which a protein may (de)regulate the immune response on its own or towards another bystander protein
IV	A list of proteins in food/feed with an adjuvant and an immune stimulatory capacity
V	Available methodologies for risk assessment of protein adjuvanticity and immunogenicity
VI	Adjuvanticity and immunogenicity and of Cry proteins
VII	General strategies to increase or decrease the immune response through adjuvant and immunogenic behaviour of proteins

2.6.2. Performance of the assessment

The selection of documents after filtering (Section 2.5) was analyzed for their relevance against inclusion criteria. The assessment was performed consistently to EFSA guidance³ with some exceptions described below. The relevance of documents has been evaluated by checking if the

analysed text contained relevant information on inclusion criteria (Table 2) and described a real scientific relationship between a protein/peptide and its adjuvant and immunogenic effects.

Because of the large number of selected documents, the review assessment was performed by at least one team member. Thus, for each selected document, the expert performed the following actions:

- Assessed the relevance by checking if relevant keywords (title and abstract) described a real scientific relationship between a protein/peptide and its adjuvant and immunogenic effect and/or suggested the presence of relevant information on the points included in the inclusion criteria.
- In some cases, the expert decided to parse the full-text when deemed necessary to assess the relevance of the document or to ask a second opinion to another member of the reviewing team.
- Annotate the document confirming the relevance of the article.

2.6.3. Eligibility criteria

As a general rule, papers not falling into one or more of the inclusion criteria were flagged as not relevant; some other specific exclusion criteria were also applied in order to exclude documents retrieved by search queries but not relevant to the present study, such as:

- Papers related to allergenicity only (e.g. description of allergens and allergic reactions, cross-reactivity) unless they underpinned useful information for the risk assessment of adjuvanticity and immunogenicity of proteins and peptides in food/feed, as described in section 1.2.2.1.
- Papers involving description of biopharmaceuticals only (e.g. monoclonal antibodies and vaccines) administered via parental routes which are not the focus of this call while, in the context of food/feed and as specified in the tender specs, the oral and respiratory routes of administration were considered relevant also for biopharmaceuticals.

3. Assessment/Results

3.1. Systematic and comprehensive critical evaluation of the information and methodologies and the usefulness of those for the food/feed safety assessment of proteins

3.1.1. Classification of adjuvants and immunogens

Different classes or families of immunogenic proteins appear from the literature but do not address a clear classification of adjuvant and immunogens^{4–30}. In particular, no studies reporting comprehensive classification of protein adjuvants and immunogens could be found. As required by EFSA tender specifications, the focus was on adjuvants and immunogens and documents focusing only on classification of allergens were not considered. Some classification of adjuvants and immunogens can be found in the literature but they are not specifically related to proteins. Therefore, it appears that classification of adjuvants and immunogens in food/feed proteins is a gap of information that need to be addressed by the scientific community.

Information related to classification of adjuvants and immunogens can be retrieved through databases and analysis resources of immunological interests since they allow to classify, organize and analyze large amount of data extracted from disparate sources. Databases focusing on immunological data, have become a common tool during the last years, widely and increasingly utilized by the biological and immunological research communities. Scientist utilizes them to aid the design and interpretation of experiments probing the nature of host pathogen interactions, autoimmune diseases, cancer, transplantation, and allergies. Most databases hosting primary data and experimental details relating to immune epitopes, and analysis resources that allow to analyze such data and/or predict epitopes or epitope characteristics in unknown antigenic systems.

A general overview of different resources and databases currently available was reviewed by Salimi *et al*²⁹ and Table 3 report resources that, to date, are available online. Among these, the Immune Epitope Database and Analysis Resource (IEDB)¹² seems to be by far the most complete and used resource. It contains information on immune epitopes curated from the published literature and submitted by National Institutes of Health funded epitope discovery efforts, and stores detailed experimental data regarding more than 120 000 epitopes, including 3D structural data. It also comprises prediction tools for identifying novel antibody and T cell epitopes from genome and protein sequences.

Table 3: Database and analysis resources of immunological interest

	Scope	Year	URL ^(a)
Immune Epitope Database and Analysis Resource (IEDB)	Epitope	2005	http://www.iedb.org
SYFPEITHI	Epitope	1999	http://www.syfpeithi.de
HIV Molecular Immunology Database (Los Alamos)	Epitope	1995	https://www.hiv.lanl.gov
NetMHC	Epitope	2003	http://www.cbs.dtu.dk/services/NetMHC/
Epitome	Epitope	2006	https://www.rostlab.org/services/epitome
Superficial	Epitope	2005	http://bioinformatics.charite.de/superficial/
MAPPP	Epitope	2003	http://www.mpiib-berlin.mpg.de/MAPPP/
Superhaptent	Haptens	2007	http://bioinformatics.charite.de/superhaptent/
NetChop	Human proteasomes	2002	http://www.cbs.dtu.dk/services/NetChop/
PAProc	Human proteasomes	2001	http://www.paproc.de
IPD	Immunological	2006	https://www.ebi.ac.uk/ipd/
IMGT	Immunological	1989	http://www.imgt.org

VBASE2	Immunological	2005	http://www.vbase2.org
InnateDB	Immunological	2008	http://innatedb.ca
ImmPort	Immunological	2005	http://www.immport.org/immport-open/public/home/home
Protein Data Bank	Macromolecular structures	1998	https://www.wwpdb.org
MUGEN	Vertebrate genomes	2003	http://www.mugen-noe.org

a) Database/resource have been checked for their availability online at the date of this document

3.1.2. Structural and functional characteristics of adjuvants and immunogens

The analysis of the literature^{6,9,14,15,18,22,24,27,28,31-64} revealed that structural features able to modulate adjuvanticity and immunogenicity were studied mainly in two contexts:

- the relationship between protein structure and adjuvanticity/immunogenicity in therapeutic proteins
- the impact of protein structure on allergenicity/cross-reactivity

Therapeutic proteins are commonly administered by a route different from oral, mainly intravenous or intramuscular while allergenicity/cross-reactivity is not the main topic of the present call. However, for both aspects, some studies in this context could be useful to derive general considerations on how the adjuvanticity and immunogenicity of a protein could be affected by its structural features.

The literature on the relations between the immunogenicity of the therapeutic proteins and their structural properties was reviewed by Hermeling *et al*⁴⁸. Proteins are complex molecules where a small change at a particular site of the sequence may result in a major change in their overall properties. In general, it is difficult to relate a particular change in protein structure to a change in immunogenicity. Differences in primary sequence often explain the different immunogenicity of protein from different species; a simple example is insulin, where little variations (1aa) in primary sequence among human, porcine and bovine protein will probably induce a small conformational change that leads to different immune response. The impact of a single mutation on the immunogenicity is obviously dependent on the position and the type of amino acid involved. However, drawing general principles regarding the influences of the primary structure on adjuvanticity/immunogenicity appears not to provide robust models or conclusions. Other protein modifications, such as glycosylation or physical and chemical degradation could have an impact on immunogenicity, but also in this case, no general rule can be devised. To date, aggregation of proteins however has been generally shown to increase the immunogenicity of various therapeutic proteins⁴⁸ and is discussed below.

Two categories of IgE-binding epitopes, linear and conformational, are generally considered to occur in food allergens. Conformational epitopes occur when either the secondary or the tertiary structure of the allergen is required before IgE will bind. In contrast, linear epitopes only require the primary amino acid sequence of the allergen for IgE to bind. While this is not a general rule, conformational IgE-binding epitopes are known to be important for the etiology of aeroallergen-mediated allergic reactions while linear epitopes are known to be relevant for food allergens mainly because the immune system encounters them only after they have been partially denatured and digested by the human gastrointestinal (GI) tract. Some major linear IgE-binding epitopes and its critical core amino acid residues have been identified and mapped in major food allergens, such as milk, soy, walnut, shrimps, chicken eggs and peanuts and structural database of allergenic proteins have been established. However, no obvious sequence motif shared by all peptides could be identified. Therefore, while bioinformatics tests relying on only sequence are able to filter non-allergenic proteins, the overall sequence identity is not the only determinant for allergenicity and additional quantitative descriptors are deemed to be necessary for reliable computational predictions⁴⁷. Of note,

FAO/WHO/Codex Alimentarius reports that cross-reactivity between a query protein and a known allergen has to be considered when there is (a) more than 35% identity in the amino acid sequence of the query protein using a window of 80 amino acids and a suitable gap penalty, or (b) identity contiguous amino acids of the query protein in a known allergen^{2,65}.

Other aspects of protein structure likely to be relevant for adjuvanticity and immunogenicity are solubility, stability, size, and the compactness of the overall fold. These aspects reflect dependency of allergenicity on transport over mucosal barriers and susceptibility to proteases. Even if most allergens can be grouped into a small number of structural classes, a specific fold characterizing allergenic protein was not identified, and few, if any, structural features are currently known to be common for allergens, which rather shows wide variety of secondary structure and folding. Therefore, other features than structure are likely to be more relevant for allergenicity. However, observing available 3D structure of allergenic proteins, it is becoming clear that they could be grouped into a limited number of functional or folding families, according to the classification of families and domains reported in the Pfam database. A work from Dall'antonia *et al.*⁶⁶ summarized the existing structural information on allergens and their classification in protein fold families, identifying 19 different families among more than hundred non-redundant allergen structures. The study highlights also the different amino acid composition of epitopes and diversity of the resulting physicochemical properties, such as flexibility, solvent accessibility, lipophilicity and electrostatic potential, so that no common rules for allergen-antibody binding sites could be derived.

Cross-reactivity is largely determined by structural aspects: two proteins are cross-reactive only (almost) if they share structural features⁶⁷. All IgE cross-reactions described so far have been found to reflect shared features on the level of both primary and tertiary structure of the cross-reactive proteins. Whereas all cross-reactive proteins have a similar fold, the opposite is not true: proteins with a similar fold are not necessarily cross-reactive. This is partially due to the immunological tolerance induced by autologous proteins with a similar fold. In the absence of similarity in folding with allergens, protein cross-reactivity is virtually excluded^{67,68}. If similarity in folding is observed, cross-reactivity needs to be investigated. A study from R. Aalberse⁶⁷ analyzed a series of allergenic proteins of which the protein folds are either known or can be predicted on the basis of homology, and identified four structural families: (1) antiparallel β -strands (2) antiparallel β -sheets intimately associated with one or more α -helices (3) ($\alpha+\beta$) structures, in which the α - and β -structural elements are not intimately associated and (4) α -helical. The conclusion is that allergens have no characteristic structural features other than that they need to be able to reach (and stimulate) immune cells and mast cells. Thus, the varied structures of allergens suggest that allergens may not have a specific common domain, but that any antigen can be allergenic if they have an ability to bind IgE and stimulate allergenic responses⁶⁸. The information on the atomic details of allergen structures indicates that allergens are heterogeneous, also from a structural point of view. Even if some folds are less prevalent among the currently known allergens, none of the protein folds seem to be incompatible with allergenicity. Another review describing structural features of allergen epitopes has been published⁴³; according to this study, IgE-binding epitopes would tend to be flat, whereas the known non-allergenic epitopes are more likely to be protruding/convex. However, there is not yet enough evidence that a flat surface is a common feature of IgE antibody-binding epitopes.

3.1.3. Conditions under which a protein may (de)regulate the immune response on its own or towards another bystander protein

In this category, the word "condition" is referred to a person's or animal's state of health or physical fitness and the factors or prevailing situation influencing the performance or outcome of a process. We included, for instance, conditions of host as well as conditions of administration of an immunogen such as industrial processing, heating and food matrix. The analysis of the literature revealed that various factors can affect the propensity of a protein to stimulate an immune response^{32,69–97}.

In addition, an interesting point of view to better elucidate conditions under which a protein may deregulate the immune response concerns the design and engineering of deimmunized biotherapeutics⁹⁸. Even in this field, the assessment of biotherapeutic immunogenicity is recognized to be a complex and multifaceted problem. Biotherapeutic protein engineering is achieved with a variety of different approaches that have been pursued to mitigate immunogenicity, including shielding proteins with chemical or biological blocking moieties (e.g., PEGylation, XTENylation, PASylation or reductive methylation), explicitly training the immune system to tolerate proteins, or implicitly rendering proteins tolerable by humanization, with emerging new engineering techniques for antibodies as well as non-immunoglobulin proteins. Since there are a growing number of deimmunized biologics, either moving towards or currently in human trials, it is expected that deimmunization technologies will have a profound impact on the biotherapeutics space and key opportunities will include both better versions of approved drugs with known immunogenicity issues and innovative new drugs whose therapeutic potential has yet to be tapped due to immunogenicity concerns. In the context of this call, it appears that key questions concerning protein biotherapeutics might be in the future insightful for assessing protein adjuvanticity and immunogenicity in food/feed, including what fraction of immunogenic epitopes must be deleted to deimmunize a protein, what fraction of patients exhibiting an immune response, whether a deimmunized biologic elicits a response in a significant fraction of patients, and what is the strength of the immune response of a deimmunized protein.

Because different known contributing factors that can all influence the adjuvanticity and immunogenicity of a protein, it becomes difficult to dissect the deregulation of the immune response when more than one factor occurs simultaneously. Examples of these factors include, administration-related factors such as the dosing regimen, duration, frequency, route of administration, patient-related factors like genetic variants, aging or health state (inflammation, concurrent medications, etc), or protein-related factors such as pre-processing, protein association/aggregation, glycosylation, digestion and impurities.

The literature retrieved is largely dominated by papers explaining the effects of different kind of processing (heating, digestion, etc.) on the allergenicity of food proteins. Other relevant conditions for adjuvanticity and immunogenicity, such as protein aggregation, are discussed in the context of therapeutic proteins or vaccines. While therapeutic proteins or vaccines are generally not administered via the oral route, and thus are not the main topic of this call, general considerations could be drawn on how adjuvanticity and immunogenicity of a protein could be affected by the above-mentioned external factors, in particular where allergenic behavior can be enhanced by adjuvants.

Jiskoot *et al.* reviewed some critical effect that may contribute to protein immunogenicity in the context of therapeutic proteins⁹⁴. Obviously, the route and site of administration will affect the biodistribution of a protein, its residence time, and the likelihood to encounter antigen-presenting cells, and therefore its immunogenicity. However, variations in dosing schedule or *in vivo* unexpected effects could contribute to different results in measured immunogenicity.

3.1.3.1. Aggregation and precipitation

Aggregation is a broad term, encompassing the interactions which result in the self-association of protein molecules into assemblies other than the native quaternary structure. Protein aggregates include a diverse range of protein assemblies that can differ in their biochemical and biophysical characteristics. They can range considerably in size, from dimers up to subvisible and visible particles, they can involve covalent or non-covalent linkages, be ordered or disordered in structure, soluble or insoluble, and their formation can be reversible or irreversible. Aggregation and precipitation have been speculated to increase the risk of immunogenicity response⁷⁴. However, clinical evidence in humans is missing and animal and *in vitro* data are in conflict given that experiments usually include a variety of degradants and not only protein aggregates. Transgenic mice data with purified protein particles or highly oxidized particles suggested that proteinaceous particles themselves do not lead to increased immunogenicity in mice, whereas heavily oxidized particles do⁹⁹.

3.1.3.2. Thermal Processing

Foods and feed are subjected to thermal processing mainly to preserve them by inactivating microbes (high temperature treatment), to improve their sensory qualities (e.g., flavor, texture, taste, and smell) or to obtain another food product or ingredient from a food source (e.g., protein isolates, cheese, oils). From a biochemical perspective, thermal processing promotes chemical and physical changes of food proteins, and affects protein conformation, and therefore allergenicity and immunogenicity, by promoting interactions of food proteins with other components present in the food matrix. Changes in secondary structure start to occur at temperature above 55°C, and tertiary structure are lost above 70°C. Protein denaturation may result in protein aggregation and cross-linking between amino acids. Moreover, if sugars are present during the heat treatment, the free amino groups of side chains of amino acids can be blocked due to the Maillard reaction. These conformational changes may also affect digestion and absorption of protein and the recognition by the immune system. The effects of this reaction on immunogenicity and allergenicity of food proteins were reviewed extensively by Teodorowicz *et al.*⁸⁰ and Gupta *et al.*⁶⁹; in general, many studies have revealed a clear effect on digestibility, bioavailability, immunogenicity and consequently allergenicity of food proteins. Biochemical and conformational changes of proteins caused by Maillard reaction may result in masking of existing antibody binding epitopes, but also in creating new structures that are more immunogenic and are thus able to promote the initiation of IgE-mediated allergies. The effects of several food processing on allergenicity was also extensively reviewed^{70,76–78,83,88,92,97}, highlighting that, depending on intensity of treatment and molecular characteristics of a food protein, allergenicity can be increased, decreased or remain unaltered by the processing method. Microbial fermentation and enzymatic or acid hydrolysis seems to have the potential to reduce allergenic integrity and allergenicity to such an extent that reactions will not be elicited but, in general, food processing may influence but not abolish completely the allergenic potential of proteins. In conclusion, thermal processing provides many beneficial effects, but also brings major changes in allergenicity that are highly complex and not easily predictable as there are different chemical pathways leading to distinct patterns of modifications¹⁰⁰. For the risk assessment point of view, it is therefore clear that a deeper understanding of thermally induced chemical changes would be essential, for instance, to achieve more effective quality control protocols.

3.1.3.3. Digestion

Protein digestion has been mainly studied for allergens which data are of inspiration for assessing risk assessment of adjuvant and immunogenic proteins in food and feed. The allergenicity risk assessment usually includes an evaluation of protein stability to digestion, under the premise that intestinal exposure would be directly linked with this property. Nevertheless, stability to digestion is not a necessity for a dietary protein to act as an allergen. The data presented on susceptibility of food allergens to gastric (pepsin) as well as gastro-duodenal digestion (pepsin, followed by trypsin and chymotrypsin) clearly illustrates that even major allergens may be extremely labile as intact proteins, being digested to small peptide fragments within few minutes^{101,102}. No clear correlation exists between the profile of digestion products and associated risk of allergenicity, therefore caution should be taken when extrapolating allergenicity of proteins from digestibility studies alone, which should be complemented by an evaluation of the allergenicity of the generated peptide fragments^{102,103}. It has been hypothesized that the poor correlation between purified protein digestion and the allergenic status of proteins might be due to the exclusion of plant matrix components or alterations in protein structure during the purification process, but a study from Schafer *et al.* showed that digestion results with genetically engineered proteins in seed/grain extracts, and with purified proteins from microbes, were comparable⁹¹.

3.1.3.4. Food matrix

Foods are complex multicomponent mixtures that can contain, in addition to proteins, polysaccharides, in many cases interacting as mixed biopolymers. The food matrix has been suggested to affect the allergenic properties of orally administered proteins by providing adjuvant stimuli to the specialized gut mucosal immune system or by protecting them from digestion. For

example, the IgE-binding of egg allergens is considerably increased in the presence of pectin, gum arabic and xylan, functional biopolymers commonly used in the food industry, and their susceptibility to digestion is diminished as compared with the isolated proteins, since it is known that the presence of soluble polysaccharides commonly used in the preparation of a wide range of foods, as stabilizers, thickeners and emulsifiers, reduces protein digestibility⁷¹. In the same way, purified peanut allergens possess little intrinsic immune-stimulating capacity in contrast to a whole peanut extract, and the presence of a food matrix enhanced the immune response to the individual allergens. Peanut consists of proteins, carbohydrates and fatty acids, and all these different components, and their interactions, may be responsible for an intrinsic adjuvant effect⁸⁶. These studies underline the importance of the food matrix in the digestibility of food allergens and in their potential to trigger an immune response.

3.1.3.5. Immunomodulators

As for other sections, immunomodulators have been mainly studied for allergens but risk assessment considerations of adjuvant and immunogenic proteins in food and feed can be done. Associated molecules, such as other proteins, carbohydrates and lipids, may influence the host response to the allergenic proteins as they are in contact with the allergen or co-liberated from the allergen carrier. There are many examples of such immune-regulators contributing to the enhancement of allergic responses or exerting a preventative role, thus dampening allergic reactions. Relevant examples of intrinsic allergen-associated immunomodulators are lipocalins, lectins, caseins, iron chelators, plant lipid transfer protein or glycoproteins; extrinsic immunomodulators are toll-like receptor ligands, lipopolysaccharides, beta-glucan and chitin³². Interestingly, proteins and peptides have been shown to enhance the interaction of an immunomodulator with the IS via increased immunostimulation and this behavior, in the context of drug delivery, is used to enhance oral delivery and achieve depot effects¹⁰⁴. Immunomodulators as adjuvants are mainly studied in the context of vaccines since the effectiveness of many of them currently in use is due in part to adjuvants, i.e. molecules that have little immunogenicity by themselves but which help enhance and appropriately skew the immune response to an antigen¹⁰⁵. Valuable example of proteins with immunomodulating with adjuvant capability are cytokines as endogenous immunomodulators and host defense peptides (HDPs) as exogenous immunomodulators¹⁰⁵. Other examples of protein/peptide molecules with immunomodulatory capability are reported in Section 3.1.7.

3.1.3.6. Aging

Aging results in a phenomenon known as immunosenescence. While immunosenescence has protean effects on the health of the organism, it can particularly impair the host's ability to defend itself against microbial invasion by pathogens such as bacteria and viruses. This results from defects in innate and adaptive humoral and cellular immunity. Specific factors that have been shown to be associated with this response are chronological age, body mass index (BMI), CD28 expression on CD8 T-cells, telomerase reverse transcriptase (TERT) and T-cell receptor excisions circles (TREC)⁷². The changes of the innate and adaptive immune system during aging was reviewed by Hasler *et al*⁸⁷. Several changes related to aging have a bearing on defence against infection diseases and autoimmunity, such as defective clearance, inflammation, age-related post-translational modification of proteins, infections and neoplasia.

3.1.3.7. Microbiota

The intestinal microbiota is likely to have multiple complex roles in initiating, regulating, and promoting allergic sensitization. In the healthy state, the microbiome stimulates the intestinal mucosa to produce a protective mucous layer that reduces the ability of food allergens to cross the epithelial barrier and gain access to the systemic circulation. Defects in innate or adaptive bacteria-induced barrier protective responses might exacerbate genetic pre-dispositions that render the host susceptible to allergen contact and entry and elicit direct or indirect stress in the epithelial barrier, particularly in the skin or intestinal mucosa. Microbiome-modulating strategies might be efficacious either as a preventive therapy (to restore functionality in the context of environmentally induced dysbiosis) or as an adjunctive treatment co-administered with orally administered allergens⁹³.

3.1.4. List of proteins in food/feed with an adjuvant and an immune stimulatory capacity

Vast information on the literature concern proteins and peptides with allergenic properties ^{6,8,10,14,28,35,38,49–52,58,59,64,71,78,85,97,106–126}. As mentioned, allergenicity is not the main topic of this call and subtle differences exist between adjuvanticity, immunogenicity and allergenicity definitions. Therefore we discussed immunomodulators in section 3.1.3.5 and, more in general, strategies to increase or decrease the immune response in section 3.1.7.

When considering the immunogenicity of proteins injected directly into the body (i.v., intravenously; s.c., subcutaneously; or i.p., intraperitoneally), all proteins except for self-proteins are basically immunogenic, and some could be also allergenic. Conversely, the assessment of immunogenicity and allergenicity of proteins which are ingested as food or feed is more complicated because several factors could affect oral immunogenicity and allergenicity, such as enzymatic degradation and oral tolerance. In the context of immunogenicity/allergenicity assessment of protein in food, these two terms are often used as synonyms, where the word “immunogenic” is often used to specify a molecule able to stimulate the immune system but also to induce an overreaction, or namely an allergic reaction. A common hypothesis behind this is that commonly allergenic foods are intrinsically more immunogenic than rarely allergenic or nonallergenic foods in allergy-susceptible hosts. This hypothesis is not always true, as shown by the study of Birmingham *et al*²³ where groups of mice were injected intraperitoneally with the protein extracts (plus alum as an adjuvant) from chicken eggs, peanuts, almonds, filberts-hazelnuts, walnuts, soybeans, and wheat (commonly allergenic foods) and coffee, sweet potatoes, carrots, white potatoes, cherries, lettuce, and spinach (rarely allergenic and nonallergenic foods). The study demonstrates that: (i) foods vary widely with regard to their relative immunogenicity in allergy-susceptible hosts and (ii) intrinsic immunogenicity in mice does not distinguish commonly allergenic foods from rarely allergenic or nonallergenic foods. As a consequence, the results of the literature search to find “proteins in food/feed with an adjuvant and/or an immune stimulatory capacity” is largely dominated by studies related to allergenicity, which on its own is not the focus of the present tender. As requested by the EFSA tender specifications, we focused our attention on studies reporting adjuvanticity and immunogenicity in food and feed but, since allergenicity is an interconnected concept, allergen literature has been considered to retrieve relevant information for this assignment.

Adjuvants may be selected in food immunotherapy for their ability to suppress the acute allergic reaction and/or modulate the underlying allergic immune response, respectively¹²⁷. Of note, adjuvants and other co-formulants used in therapeutic agents, feed additives and pesticide formulations have long been considered inactive ingredients and legal regulations of the approval and marketing of these additives specified significantly less stringent risk assessment requirements than those specified for the active ingredients¹²⁷. Nevertheless, a growing number of studies have shown additive, synergistic, or antagonistic side effects between active ingredients and their additives in formulated products purporting the idea that adjuvant behavior in food and feed need to be considered more carefully. For instance, a thorough toxicological evaluation of surfactants and other additives is essential for proper risk assessment of formulations, not only those used in agriculture, animal husbandry and plant protection, but also formulation of food and feed¹²⁷. In food, we identified essentially caseins and lectins proteins with immunomodulatory effect but, for lectins, it is also identified their adjuvant potential. Lectins are a complex group of proteins and/or glycoproteins of non-immune origin, possessing at least one non-catalytic domain which binds reversibly and specifically to monosaccharides, oligosaccharides and glycoconjugates. These proteins have also been named agglutinins or hemagglutinins and are found as monomers, homo- and heterodimers, as well as homo- and heterotetramer molecules, and they are widely distributed in nature. Lectins are ubiquitous proteins and have been isolated from viruses, fungi, bacteria, invertebrates, unicellular organisms, animals and plants. Several plant lectins exert immunomodulatory activities that are initiated by their interaction with glycan's moieties present over the surface of immune cells. Such interaction may

trigger signal transduction, to produce certain cytokines and induce efficient immune responses against tumors or microbial infections. A study from Lavelle *et al.*¹²⁶ reported that certain plant lectins, in particular lectins extracted from European mistletoe (*Viscum Album*), are strong mucosal immunogens, and are able to stimulate systemic and mucosal antibody responses after oral or intranasal delivery. A strong mucosal adjuvanticity of mistletoe lectins was also demonstrated using intranasal and oral administration and compared with known adjuvant such as cholera toxin^{121,122,124}. A recent review was published by Souza *et al.*¹¹³ on the immunomodulatory effect of plant lectins. The review discusses plant lectins that exert immunomodulatory effects, in particular ArtinM (Artocarpus heterophyllus lectin manose binding), and the mechanisms accounting for these activities concluding that some plant lectins exerting immunomodulatory activity are able to positively modify the immune response to certain pathological conditions, such as cancer and infections.

3.1.5. An identification, classification and general description of *in silico*, *in vitro* and *in vivo* available methodologies that might be employed for the risk assessment of adjuvanticity and immunogenicity of proteins

In this category the reviewing team considered documents concerning identification, classification and general description of *in silico*, *in vitro* and *in vivo* available methodologies that might be employed for the risk assessment of adjuvanticity and immunogenicity of proteins (diverse safety areas considered, e.g. food and feed safety, pharmaceuticals, medicine, immunotherapy, etc), as specified in tender specifications. A vast literature has been collected^{30,94,115,118,120,125,128–183} and is discussed below.

3.1.5.1. *In silico* models

Predictive immunogenicity screening often involves more than one approach, as each method has strengths and weaknesses. A first step in the process may be to screen a protein for the presence of T cell epitopes by sequence analysis *in silico*. The core residues of the T cell epitope sequence that mainly define the affinity and stability of binding to HLA pockets are limited in length to 9–10 amino acids, thus prediction of T cell epitopes based on the amino acid sequence of a peptide for risk assessment is computationally feasible when sufficient information on a set of peptides that are known to bind to a particular MHC is available. For instance, considering the context of IgE-mediated allergy where the knowledge on HLA phenotyping is still limited, *in silico* prediction methods may have limited robustness because of the limited set of peptides that would constitute a reliable data training set. Nevertheless, useful databases such as the Immune Epitope Database Analysis Resource are expected provide the raw material for developing T cell epitope prediction tools. A brief overview of most popular T cell epitope mapping tools is reported in the table below. A common denominator among these tools is the ability to quickly screen large datasets, including whole genomes or proteomes, for putative T cell epitopes. While methods are reviewed in strength and limitations by different studies^{136,143,148,151}, their application in risk assessment of adjuvanticity and immunogenicity of proteins in food and feed remains an open challenge.

Table 4: Overview of most popular T cell epitope mapping tools

Name of the service	Mapping tool URL
Bimas	http://bimas.dcrn.nih.gov/molbio/hla_bind
SYFPEITHI	www.syfpeithi.de
EpiDirect	http://epipredict.de/index.html
NetMHCpan	www.cbs.dtu.dk/services/NetMHCpan
HIV	http://hiv.lanl.gov/content/hiv-db/ALABAMA/epitope_analyzer.html
TEPITOPE	www.vaccinome.com
RANKPEP	http://bio.dfci.harvard.edu/Tools/rankpep.html

MHC-BPS	http://bidd.cz3.nus.edu.sg/mhc
MHCpred	www.jenner.ac.uk/MHCPred
EpiJen	www.jenner.ac.uk/EpiJen
SVMHC	www-bs.informatik.uni-tuebingen.de/SVMHC
ProPred	www.imtech.res.in/raghava/propred
ProPred-I	www.imtech.res.in/raghava/propred1
nHLAPRED w	ww.imtech.res.in/raghava/nhlapred/neural.html
IEDB	www.immuneepitope.org
AntiJen	www.jenner.ac.uk/antijen/aj_mhc.htm

3.1.5.2. *In vitro* methods

Several review have been published regarding the development and validation of different assay formats to measure antibodies specifically generated against a specific protein, mainly in the context of the development and safety evaluation of biotherapeutics proteins^{132,156,169}. Current methods are summarized in the table below. Recent progress in technological approaches that are useful for the clinical and non-clinical risk assessment of immunogenicity was also described in literature¹⁷⁸.

Table 5: Overview of *in vitro* methods

Type of Assay	General Description	Advantages	Disadvantages
Direct/Indirect ELISA	Serum or plasma samples are incubated with the immobilized antigen and the bound antibody detected using an enzyme-labelled anti-immunoglobulin reagent of appropriate specificity conjugated to an enzyme (for example, alkaline phosphatase, horseradish peroxidase), or a small molecule, (for example, biotin), which acts to amplify the signal following binding of an enzyme conjugate, (for example, streptavidine-alkaline phosphatase). The final colour due to enzyme substrate addition is directly proportional to the antibody concentration in test samples and is measured spectrophotometrically. Direct/indirect methods refer to the immobilization of antigen or the capturing agent respectively.	<ul style="list-style-type: none"> • Easy to use and automate • High throughput • High therapeutic tolerance • Inexpensive • Generic reagents and instrument 	<ul style="list-style-type: none"> • May bind non-specifically • High background • May fail to detect low-affinity antibodies • Requires species specific secondary reagent
Bridging ELISA	As above but uses the antigen both for capturing the antibody	<ul style="list-style-type: none"> • Easy to use and automate • High through- 	<ul style="list-style-type: none"> • Antigen labelling required

	and for detection. The antigen is appropriately conjugated or tagged such that a colorimetric signal is developed.	<ul style="list-style-type: none"> • Low background, High therapeutic tolerance in solution phase • High specificity (dual-arm binding) • Generic reagents and instrument 	<ul style="list-style-type: none"> • May fail to detect low-affinity antibodies • Highly susceptible to interference by therapeutic, serum components e.g., anti-human Ig molecules, multivalent targets • May not detect IgG4
Electrochemiluminescence	Employs a ruthenium-conjugated protein instead of the enzyme conjugate. An oxidation/reduction reaction of ruthenium ions in the presence of tripropylamine generates an ECL reaction under appropriate voltage stimulation. Since the ECL instrument (MSD) uses carbon electrode plates, available as standard, high bind or precoated with streptavidin or avidin, ECL based assays may be developed in either conventional formats or bridging assay protocols as used for ELISA	<ul style="list-style-type: none"> • High through-put, large dynamic range • Minimally affected by matrix • High tolerance to therapeutic in solution phase • Detection signal consistent during life of TAG conjugate 	<ul style="list-style-type: none"> • May require two antigen conjugates • Antigen labelling required • Susceptible to interference by therapeutic, serum components e.g., anti-human Ig molecules, multivalent targets • May not detect IgG4 • Vendor-specific equipment & reagents
Radioimmunoprecipitation	Serum is incubated with a radio-labelled antigen and the resulting antigen-antibody complex precipitated using polyethylene glycol or immobilized protein A/G or antiglobulin and the precipitated radioactivity assessed.	<ul style="list-style-type: none"> • Moderate through-put • High sensitivity • Can be specific • Inexpensive 	<ul style="list-style-type: none"> • Can be isotype specific • May not detect low-affinity antibodies. • Requires radiolabelled antigen. • Decay of radio-label may affect antigen stability
Surface Plasmon Resonance	The sample flows over an antigen immobilized sensor chip and generates a signal due to a change in the refractive index caused by a difference in mass as the analyte binds to the ligand. This change	<ul style="list-style-type: none"> • Automated • Determines specificity, isotype, relative binding affinity • Enables detection of both 'low-affinity' and 	<ul style="list-style-type: none"> • Antigen immobilization may alter therapeutic. Regeneration step may degrade antigen. • Sensitivity

	in refractive index is directly proportional to the amount (mass) of binding antibody in the sample being tested	<ul style="list-style-type: none"> high affinity antibodies. Detection reagent not required 	<ul style="list-style-type: none"> often less than binding assay. Expensive vendor-specific equipment & reagents
--	--	---	--

In addition to the methods reported above, proteomic methods were also applied to characterization of allergens. Also, immune epitopes derived from such allergens can be defined using technologies such as X-ray diffraction or hydrogen deuterium exchange (HDX) mass spectrometry (MS). Moreover, proteomics can provide semi-quantitative or quantitative information regarding those molecules, for example, within food or environmental samples¹³⁰. The main aspects of current and perspective applications of proteomic technologies to the study of food protein allergens (allergenomics) and derived peptides was critically reviewed^{137,139}. Interestingly, *in vitro* safety measures/evaluations of raw material/products involving plant cell, tissue, and organ culture systems as sources of food ingredients have been already proposed and described in the literature¹⁸⁴.

3.1.5.3. *In vivo* models

The usefulness and drawbacks of different mouse models that have been used for evaluation of immune response was extensively reviewed⁹⁴; an overview of main mouse model is reported in the table below.

Table 6: Overview of *in vivo* methods

Mouse Model	Description	Advantages	Disadvantages
Wild-type mice	Evaluate murine variants of human proteins	<ul style="list-style-type: none"> Relevant model with murine immune system evaluating murine variant-induced tolerance 	<ul style="list-style-type: none"> Murine proteins are not necessarily predictive of human proteins and their binding to relevant target
Transgenic mice	The HLA transgenic lines are generated by incorporation of specific human HLA genes into murine MHC class II-deficient mice, producing a mouse strain that expresses human class II HLA in the absence of mouse class II MHC	<ul style="list-style-type: none"> Mimic human subjects that are born with a specific tolerance to an endogenous protein 	<ul style="list-style-type: none"> Labor intensive Each protein that needs evaluation requires a new transgenic mouse to be bred Need additional characterization for confirming the presence of transgene
Xenomouse	"Humanized" mice engrafted with a functional human immune system. Immunocompromised mice, utilized as recipients to facilitate acceptance of human	<ul style="list-style-type: none"> Can respond to neo-antigens Tolerant to human IgG 	<ul style="list-style-type: none"> Weak B cell signaling Unable to mount robust immune response to molecules with low

	<p>tissue, are engrafted with functional human hematopoietic stem cells (CD34+), liver, and thymus. The result is a cohort of mice in which human myeloid and lymphoid lineages are reconstituted from a single human donor, and the interactions of these cells in a complex biological environment can be studied.</p>		<p>immunogenic potential</p> <ul style="list-style-type: none"> • May not be sufficiently robust and sensitive to detect small changes in attributes
--	--	--	---

The detailed influence of several key factors in the establishment of a mouse model protocol for allergy, such as mouse genders, genetic background of mouse strains, routes of sensitization, nature of food allergens and usage of adjuvants was discussed by Liu *et al.*²⁹. The use of other animal models in the context of risk assessment of food allergic potential, such as rat, dogs and swine was also reported^{141,142,144,159}. The available data suggest that the sensible use of an accurate animal model, along with other needed approaches, could be considerably useful in protein adjuvanticity and immunogenicity assessment; however, it is worth to note that currently none of the approaches have been validated in this context. Probably, the best approach would be using a combination of various models in order to replicate the genetic background as well as different environmental factors and conditions of exposure to risk groups.

3.1.6. Adjuvanticity and immunogenicity of Cry proteins

Microbial products derived from various strains of the common soil bacterium *Bacillus thuringiensis* (Bt) have been used as insecticides since the 1930s, and have been widely used in genetically modified (GM) crops to confer insect protection. Several relevant articles and reviews have been identified in the literature, including grey literature^{36,91,185–243}.

Safety studies on extensive uses have provided robust evidence of vertebrate safety of Cry protein-containing products, but also prompted some preliminary investigation of the potential for Cry proteins, like Cry1Ac, to act as medically safe vaccine adjuvants in animals. Therefore, adjuvanticity of Cry proteins has been investigated to explore their potential as vaccine adjuvants.

The adjuvanticity of Cry1Ac protoxin was tested by exposing mice to microgram amount of crystallized or soluble form of the protein either intragastrically (ig), intranasally (in) or intraperitoneally (ip)^{194,210,213,215,220,223,225,234,235,237,238}. Several studies demonstrated that Cry1Ac is a mucosal and systemic adjuvant as potent as other known toxins, such cholera toxin²³⁸, and it enhances mostly serum and intestinal IgG antibody responses, especially at the large intestine. Stronger adjuvant effects have been shown with recombinant soluble Cry1Ac protoxin administered to mice by the intraperitoneal (IP) or the intragastric (IG) route of exposure²³⁷. These features make Cry1Ac of potential use as carrier and/or adjuvant in mucosal and parenteral vaccines. The mechanism of action through which the Cry1Ac protein exerts its immunomodulatory effects on antigen presenting cells, such as macrophages, is unknown; two studies from Moreno-Fierros *et al.* showed that Cry1Ac activates macrophages by inducing the upregulation of CD86, CD80 MHCII and the production of IL-6, TNF- α and MCP-1, and these effects are mediated by MAPK pathways and the transcription factor NF- κ B^{202,244}. A direct interaction of Cry1Ac protein with HSP70 was also demonstrated by the same group in a more recent publication where purified Cry1Ac was used alone for MAPK or antibody-based co-immunoprecipitation assays, while for binding and endocytic assays, it was covalently conjugated with FITC or Cy5 or with biotin and tested macrophage culture RAW264.7 cells cultivated in DMEM¹⁹⁵.

Despite these clear results, other studies highlight different results on toxicity and adjuvanticity of this protein. For instance, the *in silico* analysis of the amino acid full-length sequences of Cry8Ka5 and Cry1Ac revealed no relevant similarity to sequences of known toxic, antinutritional and/or allergenic proteins deposited in the NCBI database. Search for similarity in primary amino acid sequences of Cry8Ka5 and Cry1Ac proteins in different databases of proteins showed no identity (as per Codex Alimentarius and EFSA >35%) with any allergens¹⁹². The fusion protein Cry1ab/Ac was also tested revealing that this protein does not possess the characteristics associated with food toxins or allergens, i.e., it has no sequence homology with any known allergens or toxins, and no N-glycosylation sites, can be rapidly degraded in gastric and intestinal fluids, and is devoid of adverse effects in mice by gavage at a high dose level of 5 g (Cry1Ab/Ac protein)/kg body weight²¹⁴. Therefore, the analysis of sequence similarity is not expected to provide information able to predict potential adjuvant or immunogenic effects of Cry proteins. Finally, an *in vivo* study examined the potential influences of GM Bt rice on the immune system of cynomolgus monkeys, stating that monkeys fed on a diet of GM rice containing the Cry1Ab/Ac gene for 6 months did not exhibit adverse immunotoxicological effects¹⁸⁶.

The potential adjuvanticity of Cry1Ab (a protein with 86% amino acid homology to Cry1Ac) has been investigated in several studies. In a mouse model of peanut allergy Cry1Ab did not demonstrate adjuvant effects on oral sensitisation to peanut when compared to the effects of cholera toxin in similar conditions²¹⁸. The same authors also demonstrated the high resistance to digestion of this protein under specific experimental conditions²¹¹, a property shared by some of the dietary proteins known to sensitize atopic patients by the gastrointestinal route. However, while the resistance of Cry1Ab and Cry1Ac protein to pancreatin degradation is reported, when these proteins are subjected to gastro-duodenal, the peptides in the simulated intestinal fluid are completely digested (US EPA, 2010). In addition, Cry8Ka5 and Cry1Ac proteins were demonstrated highly susceptible to simulated gastric fluid (SGF) digestion but were resistant to simulated intestinal fluid (SIF), i.e. resistant to pancreatin digestion but rapidly degraded by pepsin^{245,246}.

Other relevant studies examined the potential immune-related effects of Cry proteins, where oral exposure to the GM grain in the animals' diet was employed. This represent a key difference from previous studies in that purified proteins were tested instead of the whole food. It is important to note that, compared to treatment with purified protein, mice in this kind of studies consumed similar or even higher amounts of Cry1Ab protein. Reiner *et al.* showed that there is no adjuvant effect on an allergic response to a non-crossreactive protein upon Cry1Ab consumption²⁰⁰. Similarly, Adel-Patient *et al.* demonstrated that Cry1Ab is immunogenic when it is administered as a purified protein by the i.p. route, while this effect was not observed after administration of a protein extract from MON810 which resulted in an immune response against maize proteins but not against Cry1Ab²⁰⁷. Kroghsbo *et al.* reported no adverse immunotoxicological effects of Bt toxin for either the transgenic rice or the corresponding recombinant protein after 28 or 90 days dosing to Wistar rats, using lectin from kidney bean as a control²¹⁶.

A study from Finamore *et al.*²⁴⁷ report that the MON810 maize, which include a DNA sequence that encodes a bioactive form of Cry1Ab protein, when given to both weaning and old mice for 30 and 90 days, induced several changes to the immunophenotype of the gut, spleen, and circulating lymphocytes and to the level of serum cytokines. These data suggest that age was an important factor in the immune response to this protein. This fact is not surprising, considering that the immune system during weaning and aging can less efficiently or inappropriately respond to external stimuli than during adult age. Conversely, Walsh *et al.*²⁰⁴ reported that long-term feeding of Bt maize to pigs did not elicit an allergic or inflammatory-type peripheral immune response. This was evidenced by the lack of antigen-specific antibody production and the absence of alterations in T cell populations and inflammatory cytokine production. Peripheral immune response to Bt maize did not appear to be age-related, as there were no differences in cytokines, antigen-specific Ig production or T cell populations between pigs fed Bt maize from 40 or 70 days of age.

Several reviews tried to summarize these controversial results about safety and immunological effects of Cry proteins in mammals. Rubio-Infante and Moreno-Fierros¹⁸⁸ summarized some findings

regarding the Cry toxins and their effect on the immune system, reporting that these proteins may have inherent adjuvanticity, immunogenicity and allergenicity effects. The researcher stated that although the effects of these proteins on mammals could not be classified as “toxic”, they cannot be considered innocuous, and more investigation is required¹⁸⁸.

Joshi *et al.* also discussed literature concerning potential adjuvanticity of the Cry proteins¹⁸⁷. In summary, this review reports that studies done to assess the immunomodulatory potential of Cry1Ac have major flaws in the design that preclude valid interpretation. For Cry1Ab, studies were better executed and controlled, but evidence of reproducibility is lacking as there are no studies employing the same model with the same protocol. Moreover, even in the unlikely event of there being an identified adjuvant property of Cry proteins, the authors suggest that exposure would be negligible for these types of proteins since the low expression levels in seed and grains, the presence of chemical and thermal food processing and enzymatic digestion in the gut. The authors conclude that “it is highly unlikely that Cry proteins, as expressed in GM crops, have any potential to act as an adjuvant”.

Then *et al.*¹⁷ discussed the health risks associated with Bt toxins present in genetically engineered soybean and the residues left from spraying with the complementary herbicide. The article highlights some possible regulatory issues with the risk assessment of Bt soybean plants, in particular: “it is known that Bt toxins have immunogenic properties; since many allergens occur naturally in soybeans, these immunogenic properties raise specific questions regarding health effects that so far have not been taken into account during risk assessment”.

Adjuvanticity and immunogenicity and of Cry proteins is a complex issue requiring a deep and focused analysis. Nevertheless, some general considerations can be made.

- Protein immunogenicity depends on multiple factors that are related not only to the product, but also to the patient (e.g., disease state) and the administration regimen; since putative adjuvant/immunogenic effects in foods occurs mainly through the oral route, for the purpose of discriminating whether Cry proteins possess adjuvanticity under the expected conditions of dietary exposure, the oral route of administration is considered more relevant. Nevertheless, other route of exposure such as dermal routes or respiratory are considered and summarised in the following sections.
- Unlike *in silico* and *in vitro* models, animal models provide an intact immune system; however, they are different from humans and the extrapolation of results from animal to human could be very delicate and the level of risk to human patients cannot be completely defined from results with animal studies. A recent study from Abolins *et al.*²⁴⁸ compared the immune systems of 460 wild mice with mice bred in captivity. The study found that these two groups of mice have major differences in their immune make-up where wild mice had highly-activated immune systems, most likely because they are more exposed to infections, and are also characterized by a tight control of their responses to new infections, probably to prevent immune-mediated disease. These results indicate that more caution on the interpretation of results and in extrapolating data from the lab to the wild is necessary. Nevertheless, laboratory mouse models will continue to be hugely important in biological and biomedical research.

Based on literature retrieved, the adjuvanticity of Cry proteins in certain experimental conditions seems plausible. However, due to low dosage, oral route of administration, food processing and digestion, it is unlikely that this property could emerge as a safety issue in food. This is consistent with the assessment by the EFSA Genetically Modified Organisms (GMO) panel whereby they concluded that there is not a safety concern for the health of humans or animals that consume food/feed derived from GM plants containing Cry proteins. Equally, following review of relevant data in submitted dossiers from registrants^{248–251}, the U.S. FDA and EPA have not, to date, considered additional animal toxicology studies with whole foods as necessary to confirm safety (https://www3.epa.gov/pesticides/chem_search/reg_actions/pip/bt_brad2/2-id_health.pdf).

3.1.7. General strategies to increase or decrease the immune response through adjuvant and immunogenic behaviour of proteins

Eliciting an immune response is a very complex matter as the body responds to immune offence by inducing many processes. While different studies are valuable to understand how immunity is controlled, it is still apparent and a major problem in the field of immunology which of the many processes dominates in different circumstances and even what is the final effect of the body in terms of increase or decrease of the immune response^{85,94,125,163,178,252–264}. With all this in mind, some general considerations can be summarized as follows:

- Proteins that increase immune response and whereby this effect does not represent an overreaction and does not result in physiological function disorder or tissue damage are mainly ascribed to vaccines and therapeutic proteins where, usually, the route of administration is different than oral
- Proteins that increase immune response and whereby this effect represents an overreaction resulting in physiological function disorder or tissue damage are mainly ascribed to allergens
- Oral tolerance, route of exposure and digestion of food proteins strongly influence the capacity to define increase or decrease capacity of proteins to modulate immune response

Owing that the scientific fields is moving at a fast pace (see Chapter 4) and the topic of immune response in the food/feed safety context in the European regulatory framework is more and more important, the following list has to be considered non-exhaustive and prone to be updated as soon as new findings and evidence appear in the scientific literature or other regulatory agencies.

Table 7: General strategies to increase or decrease immune response by means of exogenous proteins or peptides

	Description	Behaviour/Classification	References
Cytokines	Cytokines like IFN γ or GM-CSF have been popular for over a decade as effective adjuvant molecules	Increase response, Adjuvant	265,266
Interleukins	Induction of local delayed hypersensitivity (DTH) is commonly observed after the use of Pro-inflammatory cytokines IL-1, IL-2, TNF, IFN, IL-6, IL-8	Increase response, Adjuvant	265,267
Bacterial flagellin	Bacterial flagellin is an effective adjuvant for CD4+ T cells <i>in vivo</i>	Increase response, Adjuvant	265,268
mHSP70	Mycobacterial heat shock protein 70 (mHSP70)	Increase response, Adjuvant	265,269
Amino acids	Various tissues and alternatively activated macrophages and monocytes may consume essential amino acids (arginine, tryptophan, phenylalanine, cysteine glutamine, and histidine), thus denying important nutrients to growing T cells	Increase response	270,271
Immunoglobulin	The injection of immunoglobulin (Ig) molecules intravenously has been found to reduce inflammation in autoimmune patients and in mouse models of autoimmunity	Decrease response	271,272
Protein allergens	EFSA "Scientific Opinion on the evaluation of allergenic foods and food ingredients for labelling	Increase response/Allergens	255,273

	purposes" includes information on the prevalence of food allergy in unselected populations, proteins identified as food allergens, cross-reactivities, the effects of food processing on the allergenicity of foods and ingredients, methods for the detection of allergens and allergenic foods, doses observed to trigger adverse reactions in sensitive individuals and risk assessment methodologies that have been used to derive individual and population thresholds for selected allergenic foods		
	EFSA "Scientific Opinion on the assessment of allergenicity of GM plants and microorganisms and derived food and feed" conclusions and recommendations are provided to update and complement current risk assessment strategies for the allergenicity assessment of newly expressed protein(s) and whole GM food and feed	Increase response/Allergens	125,163,258
	The COMprehensive Protein Allergen Resource, developed by the Health and Environmental Sciences Institute is a new comprehensive repository of protein sequences of known or putative allergens	Increase response/Allergens	http://comparedatabase.org/
	Dietary proteins usually induce immune tolerance, but may trigger life-threatening immune responses in the case of food allergy. Several key stages of life (e.g. early dietary allergen exposures) present mechanistic points that might participate in tipping the balance between food protein tolerance and allergy	Tolerance/Allergens	274
Therapeutic protein products	FDA outlines and recommends adoption of a risk-based approach to evaluating and mitigating immune responses to or adverse immunologically related responses associated with therapeutic protein products that affect their safety and efficacy, including patient-specific and product specific factors that affect immunogenicity	Increase response	163,275
Lectins and agglutinins	Lectins are carbohydrate-binding proteins present throughout nature that act as agglutinins. Approximately 30% of our food contains lectins, some of which may be resistant enough to digestion to enter the circulation. Shared amino acid motifs between dietary lectins,	Increase response	264

	exogenous peptides, and various body tissues may lead to immunostimulation, resulting in the production of antibodies against lectin and bacterial antigens, followed by autoimmunity		
Short immunomodulating peptides	Higher levels of protection for efficacious vaccination regimens can be achieved by the addition of immunostimulating adjuvants. Many adjuvants elicit strong, undefined inflammation, which produces increased immunogenicity but may also lead to undesirable effects	Increase response/Adjuvant	255
Altered peptide ligands	The basis of T cell immune responses is the specific recognition of an immunogenic peptide epitope by a T cell receptor. Peptide alterations of such T cell epitopes with single or few amino acid variations can have drastic effects on the outcome of this recognition. These altered peptide ligands can act as modulators of immune responses as they are capable of downregulating or upregulating responses	Increase response/Decrease response	259

3.2. Possible risk assessment strategies for food and feed safety evaluation of protein adjuvanticity and immunogenicity

As mentioned above, there is no universal predictive and validated method to assess the adjuvanticity and immunogenicity of proteins or protein-containing products. As specified in 1.2.2.1, allergenicity was not the specific focus of this call. Nevertheless the principles coming from the guideline for Genetically Modified Organisms published by EFSA^{2,260} can be used as starting point to define an effective strategy to the adjuvanticity and immunogenicity risk assessment. The guideline suggests a weight-of-evidence approach involving an integrated case-by-case approach to be used in the allergenicity risk assessment of newly expressed proteins in genetically modified feed and foods. For adjuvanticity, it should be recognized that there is no definite test yet available. In addition, because the substance properties and the mechanisms causing adjuvant activity are not well known, experimental work to reveal adjuvant activity of a substance must first of all consist of immune function studies in the intact host. As already reported by EFSA, it is possible that in a near future adjuvant activity of newly expressed proteins or any product derived from GMOs may be assessed also using *in vitro* test such as cultures of APC in which a few of the multiple parameters that characterise activation, both at genomic and phenotypic levels can be determined. To date, such tests may not detect all mechanisms for adjuvanticity/ types of adjuvants nor allow to distinguish IgE and cytotoxic adjuvanticity from IgA/IgG/IgM but they would provide useful information particularly when used in association with animal models²⁵⁸.

The application of the above-mentioned strategy to the adjuvanticity and immunogenicity risk assessment might require additional adjustments, as proposed also by Verhoeckx *et al*²⁷⁶. Based on these findings, we suggest a general framework strategy to keep into account different aspects of risk assessment strategies for food and feed safety.

3.2.1. General information on protein/peptides in food and feed

- A thorough investigation of food composition, including the source of transgene, can provide useful information such as:
 - o History of exposure (environmental and geographic factors, previous adverse effects, history of safe use)
 - o Taxonomy (adjuvanticity and immunogenicity of related species using phylogenetic or evolutionary tree)
 - o Protein identification linked to the source of transgene (typically performed by LC-MS/MS analysis and database searches after digestion of the proteins with trypsin)
 - o Protein usage (how the protein is intended to be used in a food product, food processing and components of food matrix)
 - o Level of use (the expected amount of proteins that will be present in the food product and how often will this product be consumed)
- The above information should be used to determine the need of experimental testing on adjuvanticity and immunogenicity, and therefore to select the appropriate sample (whole food, protein extract, hydrolyzed proteins, purified/recombinant protein, etc).

3.2.2. Sequence homology

- Most of the sequence homology comparison routines were mainly studies in the field of allergenicity. At present, no tool to specifically perform risk assessment on the adjuvanticity and immunogenicity of proteins has been designed yet. In the context of allergenicity, it should be noted that the mere sequence homology comparison between protein and known allergens could lead to misleading or unreliable conclusions. Nevertheless, the increasing availability of data (see 3.1.1) together with the capability to create knowledge from data pool, i.e. by means of data mining and artificial intelligent systems, promise to help in the future. However, inconsistencies may arise when considering that it is not always true that known allergenic foods are more immunogenic than nonallergenic foods. Indeed, a protein could exert an immunogenic effect without inducing allergenic reaction and/or with poor similarity with known allergenic sequences, as suggested for instance by Cry proteins under defined condition of exposure (see above).
- Moreover, small changes in primary sequences could lead to large variations in protein structure, which are often difficult to predict, thus it is challenging to estimate the influences of these changes on the immunogenicity behavior.
- Despite the T-cell repertoire is highly variable and very large, the use of complete tools such as the Immune Epitope Database and other updated epitope prediction tools in a consensus approach allows to overcome most of the possible limitations of each prediction software and may lead to more accurate results, which could be used to roughly estimate the presence of a putative immunogenic sequence and therefore highlight the need of supplementary experimental testing.
- The use of immunological databases and analysis resources could also compensate the literature gaps in classification of adjuvants and immunogens, which are largely underrepresented in the available databases.

3.2.3. Serum Screening

- Testing the immunogenicity of a protein involves measuring antibodies specifically generated against the protein. Therefore, detection and characterization of antibodies are important to

assess food safety, and a well devised bioanalytical strategy involving a panel of assays is required. A typical strategy involves a screening assay for detection based on the ability of the antibodies to bind to the protein complemented with a confirmatory step (e.g., adsorption with excess antigen).

- All assays should be optimized and validated for their intended purpose using samples from a similar patient population. Validation of antibody assays is an essential pre-requisite for obtaining results that are reproducible, accurate and meaningful.

3.2.4. *In vitro* digestibility test

- It has been established that no absolute correlation exists between resistance of proteins to pepsin digestion and allergenicity, and the same consideration could be done for adjuvanticity and immunogenicity. This test is still proposed as an additional criterion to be considered in an overall risk assessment; however, there is a need to develop more physiologically relevant *in vitro* gastrointestinal digestion models reflecting the conditions of human digestion in the general population or even at-risk groups with modified or impaired digestive function.
- For example, digestibility test should take into consideration the interactions with the food matrix that may alter the digestion and should be conducted on both purified protein in solution and in the whole complex food.

3.2.5. Other *in vitro* tests or *in vivo* tests on animal models

- *In vitro* cell based assays or *in vivo* tests on animal models have not been validated so far for regulatory purposes, but they can provide useful information on the immunogenic potential of proteins.
- Although studies on mice have strongly contributed to the understanding of immune system biology, often results obtained from animal models are not transferable to humans because of several factors. Mouse genders, genetic background of mouse strains, routes of sensitization, nature of food, usage of adjuvants could be implicated in the divergent results. Moreover, the immune system has rapidly evolved as host species coevolve with their pathogens and commensal microbiota. Since mice and humans experienced different sets of microbiome and pathogens, it is easy to understand as this coevolution led to different immune systems.
- Alternative methods to investigate important clinical questions including the mechanisms of the immune system response are required. In this direction, the application of "humanized mice" is one of the promising approaches to fill this translational gap. Humanized mice can be defined simply as mice carrying human genes or tissues such as leukocytes, stem cells, organs, and tumors²⁷⁷.
- Skin sensitisation validated regulatory tests (i.e. OECD 442 and OECD 406) can be used for potential immunoreactive effects in terms of skin sensitisation. Both methods involve the use of animal models (mouse and guinea pig, respectively), therefore their application on protein adjuvanticity and immunogenicity undergo the same limitation of animal models discussed above. In addition, skin sensitization tests do not incorporate considerations about oral tolerance which is extremely relevant in the context of adjuvanticity and immunogenicity of food/feed proteins.
- A suggested approach would be using a combination of various models in order to replicate the genetic background as well as different environmental factors and conditions of exposure to risk groups.

3.3. Hypothesis and theories

In the early 1980s, several experimental data confirmed that the immune system (IS) may not only sometimes fail to mount the expected immune response against foreign material (non-self elements), including proteins and peptides, but, as in the case of autoimmune diseases, it also directs its activity against autologous materials (self elements). In this scenario, self-non-self (SNS) discrimination began to be seen as a useless or erroneous theory. In fact, according to the classic SNS theory, only non-self entities trigger an immune response while the danger theory of Matzinger^{278,279} offers different predictions by saying that what triggers an immune response is not “foreignness”, but the release of “alarm signals” by damaged tissues.

However, even the Matzinger’s most modern conceptualization about immune response triggering, shows problematic issues in several key points that may finally prove this theory not to be completely satisfying²⁸⁰. For instance, such a theory offers no insight on geographical and population contexts that should be considered inevitable and intrinsic in the IS dynamics as well as in the output foreseen by EFSA in this assignment (i.e. mechanisms underlying an adverse effect; conditions under which a protein may deregulate the immune response on its own or towards bystander protein; risk assessment strategies for food and feed safety). Furthermore, danger theory is insufficient to explain innate immunity and response to symbiotic bacteria that are known to help breaking down foods containing fiber as well as molecular mechanisms that the IS uses to detect “strangers” vis-à-vis to endogenous signs of cellular distress playing a role in provoking immune responses (e.g. adjuvant properties of heat shock proteins, another relevant issue for this EFSA assignment). Therefore, the concept of “danger” is a theoretical suggestion, while, conversely, the idea of molecular “damage” signals has led to a number of experimental studies especially focused on endogenous damage, which is where the innovation of the danger theory lies²⁸¹.

On top of this, it is to be emphasized that the IS, which is constituted by innate immunity (the most ancestral) and adaptive immunity (the most recent and sophisticated), has memory and large plasticity. Indeed, recent findings in the field of immune memory have demonstrated that B and T cell mediated immunity following infections are enhanced by the so called *trained immunity*^{282–284}, mostly studied in relation to vaccination. Owing to its memory and plasticity, the IS is capable of recording all immunological experiences and stimuli it was exposed to: type, intensity and timing of antigenic stimuli induce a lifelong continuous adaptation, eventually responsible for the capability to mount strong, weak or tolerogenic immune response²⁸⁵.

In order to overcome all the aforementioned limitations and to take into account all relevant considerations for this EFSA tender, this document proposes a wider framework for the immune response triggering.

Indeed, the conceptualization of the immune response triggering is a complex matter that scientifically evolved in the last decades at a fast pace. In order to take into account all relevant considerations for the EFSA call, this report lean on a wider framework for the immune response triggering that is intimately linked to the conceptualization of the immunological self.

In this context, it is suggested the adoption of the notion of *liquid self*²⁸⁶ by hypothesizing that:

- The definition of the self is a process, an evolving configuration of states, and should not be strictly referred to the only physical entities (proteins and peptides);
- As a process, the self is dynamic because it varies depending on the internal (inflammatory, mostly) and external (ecological) contexts;
- The self is mainly defined within a continuum of states by the immunological history at the species (evolutionary) and the individual (ontogenetic) level, i.e., by the quantitative, qualitative, and temporal aspects of the immunological stimuli that each of us is exposed to in our lifelong history. In particular, besides structure, dose, time, and localization of antigen²⁸¹,

the host's temporal dimension in terms of antigen exposure in utero²⁸⁷, during birth²⁸⁸, and in senescence²⁸⁹.

The self is mainly defined within a continuum of states by the immunological history at the species (evolutionary) and the individual (ontogenetic) level, i.e., by the quantitative, qualitative, and temporal aspects of the immunological stimuli that each of us is exposed to in our lifelong history. In particular, besides structure, dose, time, and localization of antigen²⁸⁰, the host's temporal dimension in terms of antigen exposure in utero²⁸⁷, during birth²⁸⁸, and in senescence²⁸⁹

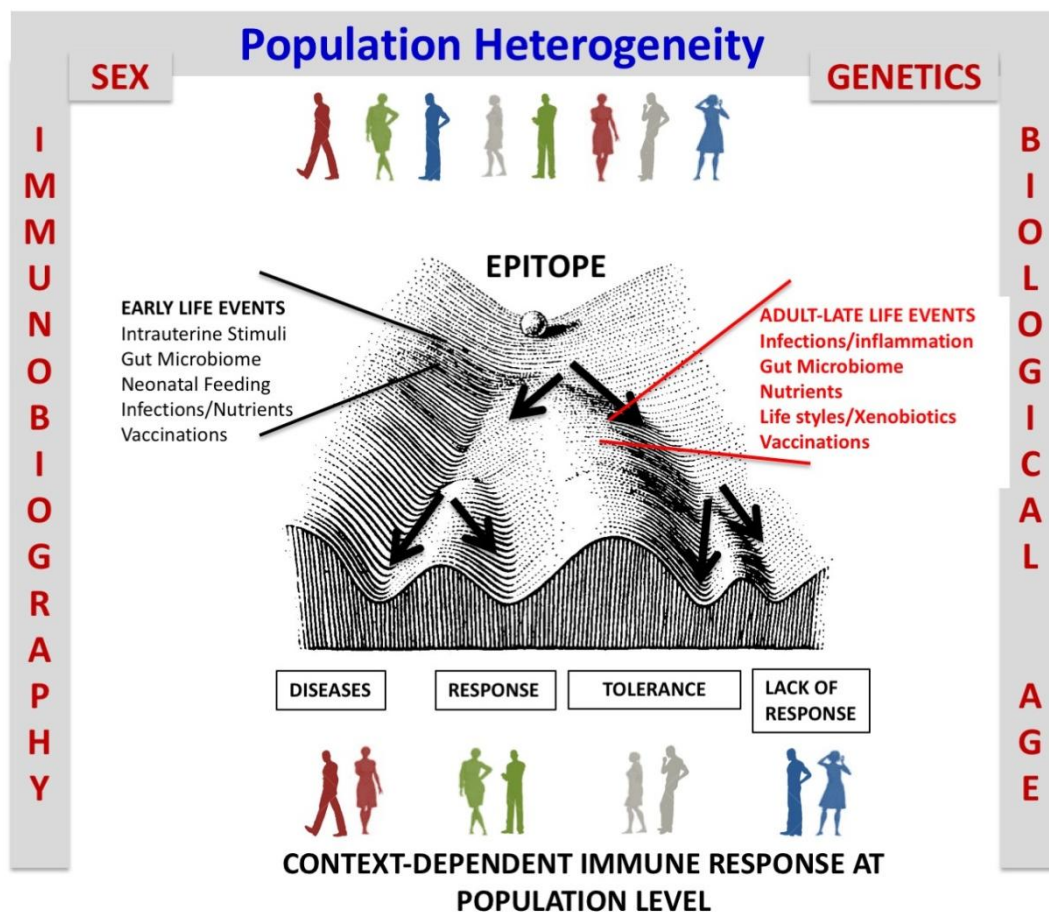
As a consequence, the question whether a given molecule (adjuvant or immunogen) belongs to self or non-self loses its significance as it largely depends on the context²⁸⁷, which lead to the wider perspective of the *immunological biography*²⁹⁰.

Immunobiography is indeed unique for each individual and is characterized by the combination of the type, intensity and temporal sequence of antigens (including food) we are exposed lifelong. This feature can explain how the same antigenic molecule, depending on the immunobiography of the host, can become either a strong or weak antigen or can induce tolerance²⁸⁵.

3.4. Potential future developments: the context dimension

Adjuvanticity and immunogenicity, which are the focus of this call, are not only intrinsically associated with a given epitope or structure, but rather are highly context-dependent. Accordingly, lack of information regarding the context (such as species of the host, assay utilized to measure response, dose and route of administration) limit the usefulness of data relating to the epitope's antigenicity or immunogenicity because of the missing link with the population heterogeneity.

The cells of the IS are not only able to recognize external and internal stressors but also to adapt and modify according to the variety of stimuli they are exposed to, this basic characteristic of the IS as a whole is known as plasticity²⁸⁶. For this reason, it has been suggested that together with the type of molecular stimuli and their doses also the temporal sequence is critical. As a consequence, the combination and subsequent integration of these factors is able to produce a variety of immunological outputs (strong response, weak response, anergy, tolerance, memory, etc.). At every contact with an antigen/stressor an integration occurs. It is easy to understand how the food which we are exposed daily can impact on the remodeling of the IS. The whole history of antigenic encounters, i.e. the immunobiography, can be represented as a Waddington Landscape²⁸⁶.



Adapted from Franceschi *et al.*, 2017, *Frontiers in Immunology*

Figure 2: The immune response to specific antigens is modulated by the lifelong (early and late life events) personal history of antigenic exposure (immunobiography), represented as a Waddington Landscape. The response to every single antigenic molecule depends on the conditions of immune system (IS) when it meets the antigen. A variety of conditions, including population heterogeneity, age, gender, nutrition, clinical history and the socioeconomical status, impinge upon the IS. The antigens can be met during life under different environmental conditions that can shape the immune response (i.e., what slope the ball will follow in its path). As a whole, this process can lead to the creation at population level of a large heterogeneity of immune responsiveness to specific antigens of the figure.

Immunobiography is strongly dependent by the individual's lifelong events as exemplified in Fig.2. It starts *in utero* and continues during the whole life since the very first day of life. Hence, the immunological "history," i.e., the summation and interaction of all the immunological experiences/stimuli will create a unique set of immune responses in each single individual.

One of the most important drivers of immune response is the gut microbiota and other microbial constituent of the human body which are able to regulate host-pathogen balance and to produce systemic pro-inflammatory stimuli. The lifelong antigenic load represented by foods and bacteria/bacterial products leads to a profound remodeling of the gut microbiota and these changes are emerging as a driving force of the functional homeostasis of the immune system. As a matter of fact, a perturbation of the gut microbiota homeostasis due to irregular lifestyles, stress and age may lead to gut microbiota dysbiosis. This condition may predispose the host to metabolic disorders and inflammation²⁹¹. The IS changes during decades of life, however these changes are very

heterogeneous. An emerging concept is that chronological age is not enough to discriminate the rate of aging and that markers of biological age should be used instead.

On top of this, it should be noted that CD8+T cell specificity depends on the recognition of MHC class I–epitope complexes and these epitopes are mainly produced via degradation of proteins by the proteasome, generating fragments of the original sequence. Interestingly, proteasomes can produce a significant portion of epitopes by reshuffling the antigen sequence, thus expanding enormously the potential antigenic repertoire. Indeed, the proteasome not only cuts proteins into fragments through canonical peptide bond hydrolysis but also ligates them through proteasome-catalyzed peptide splicing (PCPS). Through PCPS, the proteasome can significantly shape antigen presentation and this process is estimated to produce about one fourth of all antigenic peptide molecules and to enlarge the antigenic landscape – in term of antigenic peptide diversity and antigens presented at the cell surface – by around 30%. Thus, PCPS have potential implications for our understanding of CD8+T cells and the mechanisms generating non-canonical antigenic epitopes targeted by the T cell response²⁹².

The continuous reshaping of the IS²⁸⁵ that impinge upon the context of risk assessment of proteins in food and feed is due to several components of the immunobiography. Temporal and geographical dimensions, as well socio-economic and psychological status, nutrition, gut microbiota and new potential source of unexpected epitopes are indeed key players in the individual's IS response lifelong.

3.5. Implications for the food and feed safety assessment: a “personal risk assessment” scenario as future perspective

According to the previous chapters, the immune responses to potentials antigens, including pathogens, food, and vaccines, will be quantitatively and qualitatively different according to the overall immune-biographical background of the host, including age, sex, lifestyle, socioeconomic and psychological status, and geography/genetics. Given these context-dependent factors it is needed to re-think and work towards the concept of risk assessment in a personalized scenario as exemplified in the figure below.

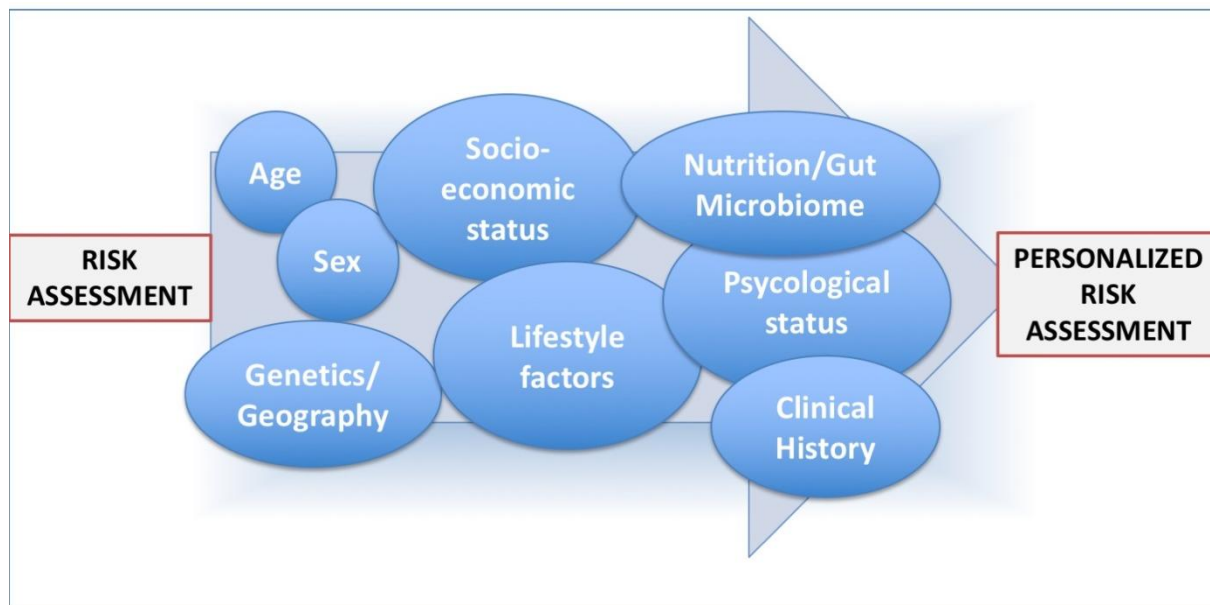


Figure 3: Context-dependent factors imply to re-think the concept of risk assessment towards a “personalized risk assessment” scenario where, for instance, a given protein or peptide can elicit immunogenic and adjuvant effects in a context-dependent manner.

Many studies on animal models and also humans have shown that the capability to react to different stimuli lifelong is the result of an intriguing mixture of gene-environment (G×E) interactions. Humans are characterized by a higher level of complexity because of their biological and cultural capabilities of adapting to all areas of the planet and changing their environments, by developing an extraordinary variety of cultural adaptive strategies including different food habits. This specific characteristic had and still have a strong impact on the molecular and cellular mechanisms involved in the immune system function. Accordingly, the immune response is a highly context-dependent phenomenon, within a new integrated, ecological, and evolutionary perspective, and it is presented as a dynamic process, both historically and individually. During the entire lifespan, new G×E interactions emerge as a consequence of the continuous remodeling process that the body set up to adapt to the changes occurring over time, which on the other hand occur concomitantly with the changes of the environment²⁹³. In this complex scenario, many factors (population genetics, demography, sex, family, immunobiography, physical/geographical and cultural/ anthropological environment, social networks, socioeconomic status and education) need to be carefully considered and integrated to understand the contribution of IS in food and feed risk assessment.

Recently, biomarkers of biological versus chronological age have been identified²⁹⁴, and an increasing number of investigations showed that such tools are effectively capable to catch the aging rate at the individual level, which in turn correlates with relevant health parameters in the elderly. These studies show that people who are or will be affected in coming years by age-related diseases tend to present a DNA methylation signature of accelerated aging, as well as in human model of accelerated aging, such as Down syndrome people²⁹⁵. Conversely, when these markers were applied to centenarians and their offspring, a signature of decelerated aging emerged²⁹⁶. Besides DNA methylation, among the most promising markers of biological age, it is important to mention N-glycans profiling²⁹⁷ as well as markers derived from biochemical and anthropometric measurements, such as hand grip, chair stand, and lung capacity²⁹⁸.

As a future perspective for the assessment of individual's biological age, it would result particularly promising the combination of the above-mentioned innovative biomarkers with the integration of classical biochemical and hematological markers (high-density lipoprotein, low-density lipoprotein, tryglicerides, glucose, insulin, albumin, urea, number of leukocytes, presence of anemia, among others) and longitudinal medical data (drugs assumption, hospitalizations). Moreover, of particular interest would be the idea to generate an inflammatory biological age marker capable to assess the inflammaging status. Thus, biological age represents an innovative trait that should be integrated in the study of the individual's immune response. In particular, biological age should complement chronological age data in the control cohorts, in which, with age, increase the health status heterogeneity.

By accepting the challenge of personalized risk assessment, we can hypothesize to create a score to measure the individual risk factors in their context-dependency above discussed (see table below) and use this score to discern people into groups with different food and feed risk. A mathematical model could then assess the specific weight of each contributing factor taking into account the heterogeneity of the population and define a specific risk assessment score for clustered group of people.

Table 8: Exemplificative score table of factors that can influence the personalized risk assessment.

Testable predictors	Range	Risk ^(a)	Personalized score ^(b)	Personalized risk ^(c)
Age (biological)	0-100+
Gender	Man/woman
Country of origin	Continent/Country/State/Region, etc
Population/Genetic background	Caucasian/Africans/Americans,

	etc			
Nutrition	Mediterranean diet/ Western diet, etc
Gut microbiota	GM family abundance and diversity
Clinical history	Diseases, drugs, etc
Exposure	Pollutants, environment etc

a) Risk defined as hazard + exposure

b) Calculated so as to consider heterogeneity of the population

c) Personalized risk defined as hazard + exposure + context-dependency

However, in order to have a comprehensive picture of the context at different levels, the risk assessment should be further investigated and developed according to two apparently opposite but in reality, complementing directions represented by:

- i) the in-depth analysis of individuals, i.e. single persons, in order to catch the personalized characteristics of the immunological response and risk assessment, but also
- ii) the exploitation of large datasets at population level regarding a large number of subjects of different ages, health status and exposure and the use of ad hoc mathematical models (machine learning, artificial intelligence).

Indeed, the use of big data and omics in a multidisciplinary approach analysis in population studies aimed at identify biomarkers of risk assessment should have as a final goal the definition of a diagnostic tool able to set tailored risk assessment actions. For instance, recently machine learning techniques have been applied to multi-omics in the field of clinical predictions in cancer therapy to personalised dietary interventions based on prediction of postprandial glucose responses from gene and gut microbiota sequences²⁹⁹.

Strategies to implement personalized risk assessment into guidelines should be then developed to set out new regulation frameworks for security food/feed agencies and policy makers. This will help to better understand patterns that might be relevant for the pre-risk assessment and the post-marketing monitoring of food/feed.

4. Conclusions

For this call, a systematic literature search and critical review was performed, identifying 299 relevant publications, including those in the grey literature. From the evaluation of the relevant literature, it emerged that:

- i) A clear classification of adjuvant and immunogens of proteins cannot be done. No study describes a comprehensive classification of protein adjuvants and immunogens. Information can be retrieved through immunological databases of which the Immune Epitope Database and Analysis Resource (IEDB) seems to be the most complete and used resource. However, these tools are mainly used to aid the design and interpretation of experiments probing the nature of host pathogen interactions, autoimmune diseases, cancer, transplantation, and allergies.
- ii) Structural features able to modulate immunogenicity and adjuvanticity were studied mainly in the context of therapeutic proteins and the allergenicity/cross-reactivity context which are outside the main scope of the present call. The primary structure of a protein do not provide information for robust models on adjuvanticity/immunogenicity and no obvious sequence motif shared by all peptides could be identified, also for food allergens. Three-dimensional structures of allergenic proteins could be grouped into a limited number of functional or folding families and cross-reactivity is largely determined by structural aspects.
- iii) Factors that affect the propensity of a protein to stimulate immune response are mainly aggregation, thermal processing, digestion, the composition of the food matrix, the presence of immunomodulators, aging and microbiota. Also in this context the literature is dominated by allergenicity. Other relevant conditions are discussed in the context of therapeutic proteins or vaccines which are generally not administered with oral route, thus not the main topic of this call. It is noted the particular relevance of food matrix (e.g. presence of pectin, gum arabic and xylan, functional biopolymers used in the food industry) and the fact that immunomodulators as adjuvants are mainly studied in the context of vaccines.
- iv) Different proteins are described in the literature to have immunomodulatory effects, among which lectins appear to have adjuvant behaviour. It is to be noted that, when considering proteins injected directly into the body, all proteins are basically immunogenic and some could be also allergenic. Assessment of adjuvanticity and immunogenicity and of proteins ingested as food/feed is complicated by enzymatic degradation, oral tolerance, etc; and it is not always true that allergenic foods are intrinsically more immunogenic than rarely allergenic (or nonallergenic) foods in allergy-susceptible host. Finally, adjuvants and other co-formulants used in therapeutic agents, feed additives and pesticide formulations have long been considered inactive ingredients while it is not clear if they should be considered more carefully.
- v) There are several *in silico*, *in vitro* and *in vivo* available methodologies for risk assessment of adjuvanticity and immunogenicity of proteins but each method has strengths and weaknesses. Application of *in silico* methods in risk assessment of adjuvanticity and immunogenicity of proteins in food and feed remains an open challenge. The application in risk assessment of *in vitro* methods for the assessment of protein adjuvanticity in food and feed have not been yet described in the literature (mainly developed for biotherapeutics proteins). Accurate *in vivo* animal model, along with other needed approaches, could be considerably useful in protein adjuvanticity and immunogenicity assessment but none of the approaches have been validated in this context. Probably, a valuable approach is the combination of various models in order to replicate genetic background, different environmental factors and conditions of exposure to risk groups.
- vi) The adjuvanticity and immunogenicity of Cry proteins in certain experimental conditions seems plausible but due to low dosage, oral route of administration, food and feed processing and digestion, it is unlikely to emerge as a safety issue in food and feed. This assessment is

consistent with the assessment by the EFSA Genetically Modified Organisms (GMO) panel whereby they concluded that there is not a safety concern for the health of humans or animals that consume food/feed derived from GM plants containing Cry proteins. In addition, following review of relevant data in submitted dossiers from registrants, the U.S. FDA and EPA have not, to date, considered additional animal toxicology studies with whole foods as necessary to confirm safety.

- vii) Stimulating an immune response is a very complex matter as the body responds to immune offence by inducing many processes. Proteins that increase immune response are mainly ascribed to vaccines and therapeutic proteins where, usually, the route of administration is different than oral. Other proteins that increase immune response are allergens. It is important to note that oral tolerance, route of exposure and digestion of food proteins strongly influence the capacity to define increase or decrease capacity of proteins to modulate immune response. Proteins with known role in increasing/decreasing immune response: cytokines, interleukins, bacterial flagellin, mHSP70, amino acids, immunoglobulin, protein allergens, therapeutic protein products, lectins and agglutinins, short immunomodulating peptides, altered peptide ligands.

In addition to the above-mentioned conclusions, with this assignment we discussed also the implications for the food and feed safety assessment of adjuvant and immunogenic proteins and possible future scenarios.

A particular emphasis should be dedicated to *in vivo* models. Although animal models are considered essential research tools and have been employed as precursor to clinical trials, there is a well-funded and accepted appreciation of their limitations. The application of "humanized mice" is one of the promising approaches to fill translational gaps from animals to humans. Humanized mice can be defined simply as mice carrying human genes or tissues such as leukocytes, stem cells, organs, and tumors. The process to create humanized models is complex and depend on numerous interventions and many potential pitfalls still exist and impinge upon their robustness. Often, the graft is incomplete or inefficient and the mice native immune system is preserved instead of the recreation of the supportive human cytokine environment. Sometimes the restoration of the immune system is incomplete with a poor recovery of certain leukocyte population with the inability to mimic the humoral responses and thus driving an inappropriate immune system (IS) response difficult to be translated. Furthermore, this model is significant expensive. Even if humanized mice are a useful tool, the study of the IS demands more sophisticated models where several complex systems may interact with each other.

Another viable way for a major comprehension of the IS is to use the potential vastity of human data. By considering the fast progress that omics and genetic technologies are experiencing as well as the impact that will likely have in the future, it is expected that lot of human data and information could be retrieved, even in a systematic way. Indeed, genomics, transcriptomics, proteomics and metabolomics are able to reveal both the static sequences of genes and proteins as well as the biological function of the gene product. Over the past two decades, advances in genomic technology have allowed laboratories to generate vast amounts of biological data including gene sequences, protein structures, information on gene expression and metabolic pathways. Large volumes of data have been generated by automated instrumentation and automatically stored in computer databases and *ad hoc* platforms (GEO, ArrayExpress, and other resources) and this data are very heterogeneous as different formats are present based on the instruments/methods used. In addition to the new information gathered from genomic technologies, pharmaceutical and biotechnology companies have large amounts of data inherited from their own and other sources on chemical structures and properties of compounds, and clinical, phenotypic and toxicological information. Most of this is stored in older types of databases designed for the particular type of data, and a major computational challenge is to integrate the new genomic information with current database systems in order to facilitate decision-making. In the field of bioinformatics, for example, major advances were made in the field of systems biology *i.e.* from the integration of computational and data acquisition technologies, rather than from faster statistical analysis of data after the acquisition. It is now possible

to consider how to evaluate the vast amounts of information generated by "omic" technologies using data-mining tools made possible by rapid advances in computational storage capacity and speed. This possibility opens up a completely new perspective that is aimed to generate new knowledge, for instance by using artificial intelligence (AI) or sophisticated data mining builder. AI based machine learning approaches allow learning of complex functional relationships from data in an unbiased fashion without the need of *a priori* assumptions. AI is indeed particularly appealing for building predictive models on the basis of biological networks when underlying molecular mechanisms are unknown.

Recent advances in nutrigenomics studies are owed to the completion of human genome project and the new biomics technologies that provide means for the simultaneous determination of the expression of many thousands of genes. These new disciplines and their attendant technologies are changing the paradigms of health research. A number of genetic variations have been shown to increase the susceptibility to diet-related diseases. These include variants that have been associated with Type 2 diabetes mellitus, obesity, cardiovascular diseases, some autoimmune diseases and cancers. Nutrigenetics aims to study these susceptible genes and provide dietary interventions for individuals at risk of such diseases. This could be translated also into the field of personalized risk assessment. However, collection of human data with new technologies impinge on considerations related to privacy and ethics of massive personal data usage that should be carefully considered in the regulation framework. It is relevant that all the data will be totally anonymous and efforts to improve security of database are required.

Based on these considerations, it is expected that the availability of new humanized animal models and the possibility to deploy artificial intelligent systems on the vastity of human data will become a general direction aiming to help answering specific question relating to the IS, including the role of protein and peptides in their adjuvant and immunogenic behaviour towards specific subset of population.

References

- (1) European Commission. 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 Commis. *Off. J. Eur. Union* **2013**, 1–48.
- (2) EFSA GMO Panel. European Food Safety Authority. Guidance for Risk Assessment of Food and Feed from Genetically Modified Plants. *EFSA J.* **2011**, 9 (5), 1–37 DOI: 10.2903/j.efsa.2011.2150.
- (3) Application of Systematic Review Methodology to Food and Feed Safety Assessments to Support Decision Making. EFSA Guidance for Those Carrying out Systematic Reviews. *EFSA J.* **2010**, 8 (6), 1637 DOI: 10.2903/j.efsa.2010.1637.
- (4) Bucholska, J.; Minkiewicz, P.; Darewicz, M.; Iwaniak, A. Databases and Associated Bioinformatic Tools in Studies of Food Allergens, Epitopes and Haptens – a Review. *Polish J. Food Nutr. Sci.* **2018**, 68 (2), 103–113 DOI: 10.1515/pjfn-2017-0022.
- (5) Radauer, C. Navigating through the Jungle of Allergens: Features and Applications of Allergen Databases. *Int. Arch. Allergy Immunol.* **2017**, 173 (1), 1–11 DOI: 10.1159/000471806.
- (6) Pele, M. Peanut Allergens. *Rom. Biotechnol. Lett.* **2010**, 15 (2), 5204–5212.
- (7) Molenda, J.; Bania, J.; Bystron, J.; Twardon, J. Importance of Newly Detected Staphylococcal Enterotoxins for Food Safety. *Pol. J. Vet. Sci.* **2008**, 11 (1), 79–82.
- (8) Battais, F.; Richard, C.; Jacquenet, S.; Denery-Papini, S.; Moneret-Vautrin, D. A. Wheat Grain Allergies: An Update on Wheat Allergens. *Eur. Ann. Allergy Clin. Immunol.* **2008**, 40 (3), 67–76.
- (9) Hofmann, A.; Burks, A. W. Pollen Food Syndrome: Update on the Allergens. *Curr. Allergy Asthma Rep.* **2008**, 8 (5), 413–417.
- (10) Nowak-Wegrzyn, A. Food Allergy to Proteins. *Nestle Nutr. Workshop Ser. Pediatr. Program.* **2007**, 59, 17–31; discussion 31–6 DOI: 10.1159/000098510.
- (11) Schein, C. H.; Ivanciuc, O.; Braun, W. Bioinformatics Approaches to Classifying Allergens and Predicting Cross-Reactivity. *Immunol. Allergy Clin. North Am.* **2007**, 27 (1), 1–27 DOI: 10.1016/j.iac.2006.11.005.
- (12) Peters, B.; Sidney, J.; Bourne, P.; Bui, H. H.; Buus, S.; Doh, G.; Fleri, W.; Kronenberg, M.; Kubo, R.; Lund, O.; Nemazee, D.; Ponomarenko, J. V.; Sathiamurthy, M.; Schoenberger, S. P.; Stewart, S.; Surko, P.; Way, S.; Wilson, S.; Sette, A. The Design and Implementation of the Immune Epitope Database and Analysis Resource. *Immunogenetics* **2005**, 57 (5), 326–336 DOI: 10.1007/s00251-005-0803-5.
- (13) Peters, B.; Sidney, J.; Bourne, P.; Bui, H.-H.; Buus, S.; Doh, G.; Fleri, W.; Kronenberg, M.; Kubo, R.; Lund, O.; Nemazee, D.; Ponomarenko, J. V.; Sathiamurthy, M.; Schoenberger, S.; Stewart, S.; Surko, P.; Way, S.; Wilson, S.; Sette, A. The Immune Epitope Database and Analysis Resource: From Vision to Blueprint. *PLoS Biol.* **2005**, 3 (3), e91 DOI: 10.1371/journal.pbio.0030091.
- (14) Breiteneder, H.; Mills, E. N. C. Plant Food Allergens—Structural and Functional Aspects of Allergenicity. *Biotechnol. Adv.* **2005**, 23 (6), 395–399 DOI: 10.1016/j.biotechadv.2005.05.004.
- (15) MILLS, E. N. C.; JENKINS, J. A.; ALCOCKER, M. J. C.; SHEWRY, P. R. Structural, Biological, and

- Evolutionary Relationships of Plant Food Allergens Sensitizing via the Gastrointestinal Tract. *Crit. Rev. Food Sci. Nutr.* **2004**, *44* (5), 379–407 DOI: 10.1080/10408690490489224.
- (16) C. Kucuksezer, U.; Ozdemir, C. Role of Galectins in Allergic Disorders. *Recent Pat. Inflamm. Allergy Drug Discov.* **2016**, *10* (1), 2–12 DOI: 10.2174/1872213X10666160426152917.
- (17) Mothes, N.; Horak, F.; Valenta, R. Transition from a Botanical to a Molecular Classification in Tree Pollen Allergy: Implications for Diagnosis and Therapy. *Int. Arch. Allergy Immunol.* **2004**, *135* (4), 357–373 DOI: 10.1159/000082332.
- (18) Baker, M. D.; Acharya, K. R. Superantigens: Structure-Function Relationships. *Int. J. Med. Microbiol.* **2004**, *293* (7–8), 529–537 DOI: 10.1078/1438-4221-00298.
- (19) Sabra, A.; Bellanti, J. A.; Rais, J. M.; Castro, H. J.; Mendez de Inocencio, J.; Sabra, S. IgE and Non-IgE Food Allergy. *Ann. Allergy, Asthma Immunol.* **2003**, *90* (6), 71–76 DOI: 10.1016/S1081-1206(10)61664-X.
- (20) Andersson, K.; Lidholm, J. Characteristics and Immunobiology of Grass Pollen Allergens. *Int. Arch. Allergy Immunol.* **2003**, *130* (2), 87–107 DOI: 10.1159/000069013.
- (21) Hoffmann-Sommergruber, K. Pathogenesis-Related (PR)-Proteins Identified as Allergens. *Biochem. Soc. Trans.* **2002**, *30* (6), 930–935 DOI: 10.1042/bst0300930.
- (22) Breiteneder, H.; Ebner, C. Atopic Allergens of Plant Foods. *Curr. Opin. Allergy Clin. Immunol.* **2001**, *1* (3), 261–267.
- (23) Johnson, H. M.; Torres, B. A.; Soos, J. M. Superantigens: Structure and Relevance to Human Disease. *Proc. Soc. Exp. Biol. Med.* **1996**, *212* (2), 99–109.
- (24) Sinha, M.; Singh, R. P.; Kushwaha, G. S.; Iqbal, N.; Singh, A.; Kaushik, S.; Kaur, P.; Sharma, S.; Singh, T. P. Current Overview of Allergens of Plant Pathogenesis Related Protein Families. *Sci. World J.* **2014**, *2014*, 1–19 DOI: 10.1155/2014/543195.
- (25) Costa, J.; Carrapatoso, I.; Oliveira, M. B. P. P.; Mafra, I. Walnut Allergens: Molecular Characterization, Detection and Clinical Relevance. *Clin. Exp. Allergy* **2014**, *44* (3), 319–341 DOI: 10.1111/cea.12267.
- (26) Flower, D. R. Systematic Identification of Small Molecule Adjuvants. *Expert Opin. Drug Discov.* **2012**, *7* (9), 807–817 DOI: 10.1517/17460441.2012.699958.
- (27) Kumar, S.; Verma, A. K.; Das, M.; Dwivedi, P. D. Allergenic Diversity among Plant and Animal Food Proteins. *Food Rev. Int.* **2012**, *28* (3), 277–298 DOI: 10.1080/87559129.2011.635391.
- (28) Afrache, H.; Gouret, P.; Ainouche, S.; Pontarotti, P.; Olive, D. The Butyrophilin (BTN) Gene Family: From Milk Fat to the Regulation of the Immune Response. *Immunogenetics* **2012**, *64* (11), 781–794 DOI: 10.1007/s00251-012-0619-z.
- (29) Salimi, N.; Fleri, W.; Peters, B.; Sette, A. Design and Utilization of Epitope-Based Databases and Predictive Tools. *Immunogenetics* **2010**, *62* (4), 185–196 DOI: 10.1007/s00251-010-0435-2.
- (30) Harrer, A.; Egger, M.; Gadermaier, G.; Erler, A.; Hauser, M.; Ferreira, F.; Himly, M. Characterization of Plant Food Allergens: An Overview on Physicochemical and Immunological Techniques. *Mol. Nutr. Food Res.* **2010**, *54* (1), 93–112 DOI: 10.1002/mnfr.200900096.
- (31) Balakireva, A.; Zamyatnin, A. Properties of Gluten Intolerance: Gluten Structure, Evolution, Pathogenicity and Detoxification Capabilities. *Nutrients* **2016**, *8* (10), 644 DOI:

- 10.3390/nu8100644.
- (32) Gómez-Casado, C.; Díaz-Perales, A. Allergen-Associated Immunomodulators: Modifying Allergy Outcome. *Arch. Immunol. Ther. Exp. (Warsz)*. **2016**, *64* (5), 339–347 DOI: 10.1007/s00005-016-0401-2.
- (33) Vasta, G. R.; Feng, C.; Bianchet, M. A.; Bachvaroff, T. R.; Tasumi, S. Structural, Functional, and Evolutionary Aspects of Galectins in Aquatic Mollusks: From a Sweet Tooth to the Trojan Horse. *Fish Shellfish Immunol*. **2015**, *46* (1), 94–106 DOI: 10.1016/j.fsi.2015.05.012.
- (34) Minkiewicz, P.; Darewicz, M.; Iwaniak, A.; Sokołowska, J.; Starowicz, P.; Bucholska, J.; Hryniewicz, M. Common Amino Acid Subsequences in a Universal Proteome—Relevance for Food Science. *Int. J. Mol. Sci*. **2015**, *16* (9), 20748–20773 DOI: 10.3390/ijms160920748.
- (35) He, L.; Han, M.; Qiao, S.; He, P.; Li, D.; Li, N.; Ma, X. Soybean Antigen Proteins and Their Intestinal Sensitization Activities. *Curr. Protein Pept. Sci*. **2015**, *16* (7), 613–621 DOI: 10.2174/1389203716666150630134602.
- (36) Xu, C.; Wang, B.-C.; Yu, Z.; Sun, M. Structural Insights into Bacillus Thuringiensis Cry, Cyt and Parasporin Toxins. *Toxins (Basel)*. **2014**, *6* (9), 2732–2770 DOI: 10.3390/toxins6092732.
- (37) Salunke, D. M.; Gill, J.; Dwevedi, A. Comparative Structural Proteomics of Allergenic Proteins from Plant Pollen. *J. INDIAN Inst. Sci*. **2014**, *94* (1, SI), 119–126.
- (38) Mueller, G. A.; Maleki, S. J.; Pedersen, L. C. The Molecular Basis of Peanut Allergy. *Curr. Allergy Asthma Rep*. **2014**, *14* (5), 429 DOI: 10.1007/s11882-014-0429-5.
- (39) Chruszcz, M.; Mikolajczak, K.; Mank, N.; Majorek, K. A.; Porebski, P. J.; Minor, W. Serum Albumins—Unusual Allergens. *Biochim. Biophys. Acta - Gen. Subj*. **2013**, *1830* (12), 5375–5381 DOI: 10.1016/j.bbagen.2013.06.016.
- (40) Juhász, A.; Gell, G.; Békés, F.; Balázs, E. The Epitopes in Wheat Proteins for Defining Toxic Units Relevant to Human Health. *Funct. Integr. Genomics* **2012**, *12* (4), 585–598 DOI: 10.1007/s10142-012-0302-3.
- (41) Aalberse, R. C.; Cramer, R. IgE-Binding Epitopes: A Reappraisal. *Allergy* **2011**, *66* (10), 1261–1274 DOI: 10.1111/j.1398-9995.2011.02656.x.
- (42) Agostino, M.; Sandrin, M. S.; Thompson, P. E.; Farrugia, W.; Ramsland, P. A.; Yuriev, E. Carbohydrate-Mimetic Peptides: Structural Aspects of Mimicry and Therapeutic Implications. *Expert Opin. Biol. Ther*. **2011**, *11* (2), 211–224 DOI: 10.1517/14712598.2011.542140.
- (43) Pomés, A. Relevant B Cell Epitopes in Allergic Disease. *Int. Arch. Allergy Immunol*. **2010**, *152* (1), 1–11 DOI: 10.1159/000260078.
- (44) Silano, M.; Vincentini, O.; De Vincenzi, M. Toxic, Immunostimulatory and Antagonist Gluten Peptides in Celiac Disease. *Curr. Med. Chem*. **2009**, *16* (12), 1489–1498 DOI: 10.2174/092986709787909613.
- (45) TANABE, S. Analysis of Food Allergen Structures and Development of Foods for Allergic Patients. *Biosci. Biotechnol. Biochem*. **2008**, *72* (3), 649–659 DOI: 10.1271/bbb.70708.
- (46) Tanabe, S. Epitope Peptides and Immunotherapy. *Curr. Protein Pept. Sci*. **2007**, *8* (1), 109–118 DOI: 10.2174/138920307779941569.
- (47) Bannon, G. A. What Makes a Food Protein an Allergen? *Curr. Allergy Asthma Rep*. **2004**, *4* (1), 43–46.

- (48) Hermeling, S.; Crommelin, D. J. A.; Schellekens, H.; Jiskoot, W. Structure-Immunogenicity Relationships of Therapeutic Proteins. *Pharm. Res.* **2004**, *21* (6), 897–903 DOI: 10.1023/B:PHAM.0000029275.41323.a6.
- (49) Maleki, S. J.; Hurlburt, B. K. Structural and Functional Alterations in Major Peanut Allergens Caused by Thermal Processing. *J. AOAC Int.* **2004**, *87* (6), 1475–1479.
- (50) Wal, J.-M. Cow's Milk Proteins/Allergens. *Ann. Allergy, Asthma Immunol.* **2002**, *89* (6), 3–10 DOI: 10.1016/S1081-1206(10)62115-1.
- (51) Lehrer, S. B.; Ayuso, R.; Reese, G. Current Understanding of Food Allergens. *Ann. N. Y. Acad. Sci.* **2002**, *964*, 69–85.
- (52) Lorenz, A.-R.; Scheurer, S.; Haustein, D.; Vieths, S. Recombinant Food Allergens. *J. Chromatogr. B Biomed. Sci. Appl.* **2001**, *756* (1–2), 255–279 DOI: 10.1016/S0378-4347(01)00086-X.
- (53) Wal, J. M. Structure and Function of Milk Allergens. *Allergy* **2001**, *56* (s67), 35–38 DOI: 10.1034/j.1398-9995.2001.00911.x.
- (54) Bredehorst, R.; David, K. What Establishes a Protein as an Allergen? *J. Chromatogr. B Biomed. Sci. Appl.* **2001**, *756* (1–2), 33–40 DOI: 10.1016/S0378-4347(01)00069-X.
- (55) Showalter, A. M. Arabinogalactan-Proteins: Structure, Expression and Function. *Cell. Mol. Life Sci.* **2001**, *58* (10), 1399–1417 DOI: 10.1007/PL00000784.
- (56) Aalberse, R. C. Structural Biology of Allergens. *J. Allergy Clin. Immunol.* **2000**, *106* (2), 228–238 DOI: 10.1067/mai.2000.108434.
- (57) Ueda, H.; Ogawa, H. Glycobiology of the Plant Glycoprotein Epitope: Structure, Immunogenicity and Allergenicity of Plant Glycotopes. *Trends Glycosci. Glycotechnol.* **1999**, *11* (62), 413–428 DOI: 10.4052/tigg.11.413.
- (58) Reese, G.; Ayuso, R.; Lehrer, S. B. Tropomyosin: An Invertebrate Pan-Allergen. *Int. Arch. Allergy Immunol.* **1999**, *119* (4), 247–258 DOI: 10.1159/000024201.
- (59) Rao, P. V. S.; Rajagopal, D.; Ganesh, K. A. B- and T-Cell Epitopes of Tropomyosin, the Major Shrimp Allergen. *Allergy* **1998**, *53* (46 Suppl), 44–47 DOI: 10.1111/j.1398-9995.1998.tb04959.x.
- (60) Elsayed, S.; Apold, J.; Holen, E.; Vik, H.; Florvaag, E.; Dybendal, T. The Structural Requirements of Epitopes with Ige Binding Capacity Demonstrated by Three Major Allergens from Fish, Egg and Tree Pollen. *Scand. J. Clin. Lab. Invest.* **1991**, *51* (sup204), 17–31 DOI: 10.3109/00365519109104592.
- (61) Rothbard, J. B.; Geftter, M. L. Interactions between Immunogenic Peptides and MHC Proteins. *Annu. Rev. Immunol.* **1991**, *9* (1), 527–565 DOI: 10.1146/annurev.iy.09.040191.002523.
- (62) van der Drift, A. C.; van Noort, J. M.; Krüse, J. Catheptic Processing of Protein Antigens: Enzymic and Molecular Aspects. *Semin. Immunol.* **1990**, *2* (4), 255–271.
- (63) Werner, G. H. Natural and Synthetic Peptides (Other than Neuropeptides) Endowed with Immunomodulating Activities. *Immunol. Lett.* **1987**, *16* (3–4), 363–370.
- (64) Cunsolo, V.; Saletti, R.; Muccilli, V.; Gallina, S.; Di Francesco, A.; Foti, S. Proteins and Bioactive Peptides from Donkey Milk: The Molecular Basis for Its Reduced Allergenic Properties. *Food Res. Int.* **2017**, *99* (1), 41–57 DOI: 10.1016/j.foodres.2017.07.002.

- (65) Ivanciuc, O.; Schein, C. H.; Garcia, T.; Oezguen, N.; Negi, S. S.; Braun, W. Structural Analysis of Linear and Conformational Epitopes of Allergens. *Regul. Toxicol. Pharmacol.* **2009**, *54* (3 SUPPL.), S11–S19 DOI: 10.1016/j.yrtph.2008.11.007.
- (66) Dall'Antonia, F.; Pavkov-Keller, T.; Zangger, K.; Keller, W. Structure of Allergens and Structure Based Epitope Predictions. *Methods* **2014**, *66* (1), 3–21 DOI: 10.1016/j.ymeth.2013.07.024.
- (67) Aalberse, R. C. Structural Biology of Allergens. *J. Allergy Clin. Immunol.* **2000**, *106* (2), 228–238 DOI: 10.1067/mai.2000.108434.
- (68) Devis, D. L.; Davies, J. M.; Zhang, D. Molecular Features of Grass Allergens and Development of Biotechnological Approaches for Allergy Prevention. *Biotechnol. Adv.* **2017**, *35* (5), 545–556 DOI: 10.1016/j.biotechadv.2017.05.005.
- (69) Gupta, R. K.; Gupta, K.; Sharma, A.; Das, M.; Ansari, I. A.; Dwivedi, P. D. Maillard Reaction in Food Allergy: Pros and Cons. *Crit. Rev. Food Sci. Nutr.* **2018**, *58* (2), 208–226 DOI: 10.1080/10408398.2016.1152949.
- (70) Jiménez-Saiz, R.; Benedé, S.; Molina, E.; López-Expósito, I. Effect of Processing Technologies on the Allergenicity of Food Products. *Crit. Rev. Food Sci. Nutr.* **2015**, *55* (13), 1902–1917 DOI: 10.1080/10408398.2012.736435.
- (71) Benedé, S.; López-Expósito, I.; Molina, E.; López-Fandiño, R. Egg Proteins as Allergens and the Effects of the Food Matrix and Processing. *Food Funct.* **2015**, *6* (3), 694–713 DOI: 10.1039/C4FO01104J.
- (72) Poland, G. A.; Ovsyannikova, I. G.; Kennedy, R. B.; Lambert, N. D.; Kirkland, J. L. A Systems Biology Approach to the Effect of Aging, Immunosenescence and Vaccine Response. *Curr. Opin. Immunol.* **2014**, *29* (3), 62–68 DOI: 10.1016/j.coi.2014.04.005.
- (73) Toda, M.; Heilmann, M.; Ilchmann, A.; Vieths, S. The Maillard Reaction and Food Allergies: Is There a Link? *Clin. Chem. Lab. Med.* **2014**, *52* (1), 61–67 DOI: 10.1515/cclm-2012-0830.
- (74) Ratanji, K. D.; Derrick, J. P.; Dearman, R. J.; Kimber, I. Immunogenicity of Therapeutic Proteins: Influence of Aggregation. *J. Immunotoxicol.* **2014**, *11* (2), 99–109 DOI: 10.3109/1547691X.2013.821564.
- (75) Bublin, M.; Eiwegger, T.; Breiteneder, H. Do Lipids Influence the Allergic Sensitization Process? *J. Allergy Clin. Immunol.* **2014**, *134* (3), 521–529 DOI: 10.1016/j.jaci.2014.04.015.
- (76) Masthoff, L. J.; Hoff, R.; Verhoeckx, K. C. M.; van Os-Medendorp, H.; Michelsen-Huisman, A.; Baumert, J. L.; Pasmans, S. G.; Meijer, Y.; Knulst, A. C. A Systematic Review of the Effect of Thermal Processing on the Allergenicity of Tree Nuts. *Allergy* **2013**, *68* (8), 983–993 DOI: 10.1111/all.12185.
- (77) Kostı, R. I.; Triga, M.; Tsabouri, S.; Priftis, K. N. Food Allergen Selective Thermal Processing Regimens May Change Oral Tolerance in Infancy. *Allergol. Immunopathol. (Madr.)* **2013**, *41* (6), 407–417 DOI: 10.1016/j.aller.2012.08.011.
- (78) Verma, A. K.; Kumar, S.; Das, M.; Dwivedi, P. D. Impact of Thermal Processing on Legume Allergens. *Plant Foods Hum. Nutr.* **2012**, *67* (4), 430–441 DOI: 10.1007/s11130-012-0328-7.
- (79) Hall, E. H.; Crowe, S. E. Environmental and Lifestyle Influences on Disorders of the Large and Small Intestine: Implications for Treatment. *Dig. Dis.* **2011**, *29* (2), 249–254 DOI: 10.1159/000323930.
- (80) Teodorowicz, M.; van Neerven, J.; Savelkoul, H. Food Processing: The Influence of the Maillard

- Reaction on Immunogenicity and Allergenicity of Food Proteins. *Nutrients* **2017**, *9* (8), 835 DOI: 10.3390/nu9080835.
- (81) Perrier, C.; Corthésy, B. Gut Permeability and Food Allergies. *Clin. Exp. Allergy* **2011**, *41* (1), 20–28 DOI: 10.1111/j.1365-2222.2010.03639.x.
- (82) Pali-Schöll, I.; Jensen-Jarolim, E. Anti-Acid Medication as a Risk Factor for Food Allergy. *Allergy* **2011**, *66* (4), 469–477 DOI: 10.1111/j.1398-9995.2010.02511.x.
- (83) Nowak-Wegrzyn, A.; Fiocchi, A. Rare, Medium, or Well Done? The Effect of Heating and Food Matrix on Food Protein Allergenicity. *Curr. Opin. Allergy Clin. Immunol.* **2009**, *9* (3), 234–237 DOI: 10.1097/ACI.0b013e32832b88e7.
- (84) Moreno, F. J. Gastrointestinal Digestion of Food Allergens: Effect on Their Allergenicity. *Biomed. Pharmacother.* **2007**, *61* (1), 50–60 DOI: 10.1016/j.biopha.2006.10.005.
- (85) Strobel, S.; Mowat, A. M. Oral Tolerance and Allergic Responses to Food Proteins. *Curr. Opin. Allergy Clin. Immunol.* **2006**, *6* (3), 207–213 DOI: 10.1097/01.all.0000225162.98391.81.
- (86) van Wijk, F.; Nierkens, S.; Hassing, I.; Feijen, M.; Koppelman, S. J.; de Jong, G. A. H.; Pieters, R.; Knippels, L. M. J. The Effect of the Food Matrix on in Vivo Immune Responses to Purified Peanut Allergens. *Toxicol. Sci.* **2005**, *86* (2), 333–341 DOI: 10.1093/toxsci/kfi187.
- (87) Hasler, P.; Zouali, M. Immune Receptor Signaling, Aging, and Autoimmunity. *Cell. Immunol.* **2005**, *233* (2), 102–108 DOI: 10.1016/j.cellimm.2005.04.012.
- (88) Poms, R. E.; Anklam, E. Effects of Chemical, Physical, and Technological Processes on the Nature of Food Allergens. *J. AOAC Int.* **2004**, *87* (6), 1466–1474.
- (89) Davis, P. J.; Williams, S. C. Protein Modification by Thermal Processing. *Allergy* **1998**, *53* (46), 102–105 DOI: 10.1111/j.1398-9995.1998.tb04975.x.
- (90) Reinhardt, M. C. Macromolecular Absorption of Food Antigens in Health and Disease. *Ann. Allergy* **1984**, *53* (6 Pt 2), 597–601.
- (91) Schafer, B. W.; Embrey, S. K.; Herman, R. A. Rapid Simulated Gastric Fluid Digestion of In-Seed/Grain Proteins Expressed in Genetically Engineered Crops. *Regul. Toxicol. Pharmacol.* **2016**, *81*, 106–112 DOI: 10.1016/j.yrtph.2016.08.004.
- (92) Rahaman, T.; Vasiljevic, T.; Ramchandran, L. Effect of Processing on Conformational Changes of Food Proteins Related to Allergenicity. *Trends Food Sci. Technol.* **2016**, *49*, 24–34 DOI: 10.1016/j.tifs.2016.01.001.
- (93) Wesemann, D. R.; Nagler, C. R. The Microbiome, Timing, and Barrier Function in the Context of Allergic Disease. *Immunity* **2016**, *44* (4), 728–738 DOI: 10.1016/j.immuni.2016.02.002.
- (94) Jiskoot, W.; Kijanka, G.; Randolph, T. W.; Carpenter, J. F.; Koulov, A. V.; Mahler, H.-C.; Joubert, M. K.; Jawa, V.; Narhi, L. O. Mouse Models for Assessing Protein Immunogenicity: Lessons and Challenges. *J. Pharm. Sci.* **2016**, *105* (5), 1567–1575 DOI: 10.1016/j.xphs.2016.02.031.
- (95) Bøgh, K. L.; Madsen, C. B. Food Allergens: Is There a Correlation between Stability to Digestion and Allergenicity? *Crit. Rev. Food Sci. Nutr.* **2016**, *56* (9), 1545–1567 DOI: 10.1080/10408398.2013.779569.
- (96) Carrard, A.; Rizzuti, D.; Sokollik, C. Update on Food Allergy. *Allergy* **2015**, *70* (12), 1511–1520 DOI: 10.1111/all.12780.

- (97) Verhoeckx, K. C. M.; Vissers, Y. M.; Baumert, J. L.; Faludi, R.; Feys, M.; Flanagan, S.; Herouet-Guicheney, C.; Holzhauser, T.; Shimojo, R.; van der Bolt, N.; Wichers, H.; Kimber, I. Food Processing and Allergenicity. *Food Chem. Toxicol.* **2015**, *80*, 223–240 DOI: 10.1016/j.fct.2015.03.005.
- (98) Griswold, K. E.; Bailey-Kellogg, C. Design and Engineering of Deimmunized Biotherapeutics. *Curr. Opin. Struct. Biol.* **2016**, *39*, 79–88 DOI: 10.1016/j.sbi.2016.06.003.
- (99) Mahler, H.; Allmendinger, A. Stability , Formulation , and Delivery of Biopharmaceuticals. *Protein Ther.* **2017**, 469–491.
- (100) Davis, P. J.; Smales, C. M.; James, D. C. How Can Thermal Processing Modify the Antigenicity of Proteins? *Allergy* **2001**, *56* (s67), 56–60 DOI: 10.1034/j.1398-9995.2001.00918.x.
- (101) Verhoeckx, K. C. M.; Vissers, Y. M.; Baumert, J. L.; Faludi, R.; Feys, M.; Flanagan, S.; Herouet-Guicheney, C.; Holzhauser, T.; Shimojo, R.; van der Bolt, N.; Wichers, H.; Kimber, I. Food Processing and Allergenicity. *Food Chem. Toxicol.* **2015**, *80*, 223–240 DOI: 10.1016/j.fct.2015.03.005.
- (102) Bøgh, K. L.; Madsen, C. B. Food Allergens: Is There a Correlation between Stability to Digestion and Allergenicity? *Crit. Rev. Food Sci. Nutr.* **2016**, *56* (9), 1545–1567 DOI: 10.1080/10408398.2013.779569.
- (103) Vanga, S. K.; Singh, A.; Raghavan, V. Review of Conventional and Novel Food Processing Methods on Food Allergens. *Crit. Rev. Food Sci. Nutr.* **2017**, *57* (10), 2077–2094 DOI: 10.1080/10408398.2015.1045965.
- (104) Anderson, P. M. Delivery Systems for Immunomodulatory Proteins and Peptides. *BioDrugs* **1997**, *7*(1), 51–65 DOI: 10.2165/00063030-199707010-00007.
- (105) Nicholls, E. F.; Madera, L.; Hancock, R. E. W. Immunomodulators as Adjuvants for Vaccines and Antimicrobial Therapy. *Ann. N. Y. Acad. Sci.* **2010**, *1213* (1), 46–61 DOI: 10.1111/j.1749-6632.2010.05787.x.
- (106) Matsuo, H.; Yokooji, T.; Taogoshi, T. Common Food Allergens and Their IgE-Binding Epitopes. *Allergol. Int.* **2015**, *64* (4), 332–343 DOI: 10.1016/j.alit.2015.06.009.
- (107) Bøgh, K. L.; Barkholt, V.; Madsen, C. B. Characterization of the Immunogenicity and Allergenicity of Two Cow's Milk Hydrolysates--A Study in Brown Norway Rats. *Scand. J. Immunol.* **2015**, *81* (5), 274–283 DOI: 10.1111/sji.12271.
- (108) Urisu, A.; Kondo, Y.; Tsuge, I. Hen's Egg Allergy. *Chem. Immunol. Allergy* **2015**, *101*, 124–130 DOI: 10.1159/000375416.
- (109) Kuehn, A.; Swoboda, I.; Arumugam, K.; Hilger, C.; Hentges, F. Fish Allergens at a Glance: Variable Allergenicity of Parvalbumins, the Major Fish Allergens. *Front. Immunol.* **2014**, *5* DOI: 10.3389/fimmu.2014.00179.
- (110) Leung, N. Y. H.; Wai, C. Y. Y.; Shu, S.; Wang, J.; Kenny, T. P.; Chu, K. H.; Leung, P. S. C. Current Immunological and Molecular Biological Perspectives on Seafood Allergy: A Comprehensive Review. *Clin. Rev. Allergy Immunol.* **2014**, *46* (3), 180–197 DOI: 10.1007/s12016-012-8336-9.
- (111) Nieuwenhuizen, N. E.; Lopata, A. L. Anisakis – A Food-Borne Parasite That Triggers Allergic Host Defences. *Int. J. Parasitol.* **2013**, *43* (12–13), 1047–1057 DOI: 10.1016/j.ijpara.2013.08.001.

- (112) Barros, A.; Cosme, F. Allergenic Proteins in Foods and Beverages. *FOOD Technol. Biotechnol.* **2013**, *51* (2, SI), 153–158.
- (113) Souza, M. A.; Carvalho, F. C.; Ruas, L. P.; Ricci-Azevedo, R.; Roque-Barreira, M. C. The Immunomodulatory Effect of Plant Lectins: A Review with Emphasis on ArtinM Properties. *Glycoconj. J.* **2013**, *30* (7), 641–657 DOI: 10.1007/s10719-012-9464-4.
- (114) Minkiewicz, P.; Miciński, J.; Darewicz, M.; Bucholska, J. Biological and Chemical Databases for Research into the Composition of Animal Source Foods. *Food Rev. Int.* **2013**, *29* (4), 321–351 DOI: 10.1080/87559129.2013.818011.
- (115) Zhuang, Y.; Dreskin, S. C. Redefining the Major Peanut Allergens. *Immunol. Res.* **2013**, *55* (1–3), 125–134 DOI: 10.1007/s12026-012-8355-x.
- (116) Pabst, O.; Mowat, A. M. Oral Tolerance to Food Protein. *MUCOSAL Immunol.* **2012**, *5* (3), 232–239 DOI: 10.1038/mi.2012.4.
- (117) Clement, F.; Venkatesh, Y. P. Dietary Garlic (*Allium Sativum*) Lectins, ASA I and ASA II, Are Highly Stable and Immunogenic. *Int. Immunopharmacol.* **2010**, *10* (10), 1161–1169 DOI: 10.1016/j.intimp.2010.06.022.
- (118) Hoffmann-Sommergruber, K.; Mills, E. N. C.; Vieths, S. Coordinated and Standardized Production, Purification and Characterization of Natural and Recombinant Food Allergens to Establish a Food Allergen Library. *Mol. Nutr. Food Res.* **2008**, *52* (S2), S159–S165 DOI: 10.1002/mnfr.200800466.
- (119) Bittencourt, A. L.; Soares, M. F. de M.; Pires, R. R.; Honmoto, C. S.; Tanaka, M. K.; Jacob, C. M. A.; Abdalla, D. S. P. Immunogenicity and Allergenicity of 2S, 7S and 11S Soy Protein Fractions. *Rev. Bras. Ciências Farm.* **2007**, *43* (4), 597–606 DOI: 10.1590/S1516-93322007000400013.
- (120) Monaci, L.; Tregoeat, V.; van Hengel, A. J.; Anklam, E. Milk Allergens, Their Characteristics and Their Detection in Food: A Review. *Eur. Food Res. Technol.* **2006**, *223* (2), 149–179 DOI: 10.1007/s00217-005-0178-8.
- (121) Lavelle, E. C.; Grant, G.; Pfuller, U.; O'Hagan, D. T. Immunological Implications of the Use of Plant Lectins for Drug and Vaccine Targeting to the Gastrointestinal Tract. *J. Drug Target.* **2004**, *12* (2), 89–95 DOI: 10.1080/10611860410001693733.
- (122) Lavelle, E. C.; Grant, G.; Pusztai, A.; Pfüller, U.; Leavy, O.; McNeela, E.; Mills, K. H. G.; O'Hagan, D. T. Mistletoe Lectins Enhance Immune Responses to Intranasally Co-Administered Herpes Simplex Virus Glycoprotein D2. *Immunology* **2002**, *107* (2), 268–274.
- (123) Birmingham, N.; Thanavorakul, S.; Gangur, V. Relative Immunogenicity of Commonly Allergenic Foods versus Rarely Allergenic and Nonallergenic Foods in Mice. *J. Food Prot.* **2002**, *65* (12), 1988–1991.
- (124) Lavelle, E. C.; Grant, G.; Pusztai, A.; Pfüller, U.; O'Hagan, D. T. The Identification of Plant Lectins with Mucosal Adjuvant Activity. *Immunology* **2001**, *102* (1), 77–86 DOI: 10.1046/j.1365-2567.2001.01157.x.
- (125) Taylor, S. L.; Hefle, S. L. Will Genetically Modified Foods Be Allergenic? *J. Allergy Clin. Immunol.* **2001**, *107* (5), 765–771 DOI: 10.1067/mai.2001.114241.
- (126) Lavelle, E. C.; Grant, G.; Pusztai, A.; Pfüller, U.; O'Hagan, D. T. Mucosal Immunogenicity of Plant Lectins in Mice. *Immunology* **2000**, *99* (1), 30–37 DOI: 10.1046/j.1365-2567.2000.00932.x.

- (127) Loh, W.; Tang, M. Adjuvant Therapies in Food Immunotherapy. *Immunol. Allergy Clin. North Am.* **2018**, *38* (1), 89–101 DOI: 10.1016/j.iac.2017.09.008.
- (128) de Moraes, A.; Kubota, L. Recent Trends in Field-Effect Transistors-Based Immunosensors. *Chemosensors* **2016**, *4* (4), 20 DOI: 10.3390/chemosensors4040020.
- (129) Liu, T.; Navarro, S.; Lopata, A. L. Current Advances of Murine Models for Food Allergy. *Mol. Immunol.* **2016**, *70*, 104–117 DOI: 10.1016/j.molimm.2015.11.011.
- (130) Nony, E.; Le Mignon, M.; Brier, S.; Martelet, A.; Moingeon, P. Proteomics for Allergy: From Proteins to the Patients. *Curr. Allergy Asthma Rep.* **2016**, *16* (9), 64 DOI: 10.1007/s11882-016-0642-5.
- (131) Snyder, A.; Chan, T. A. Immunogenic Peptide Discovery in Cancer Genomes. *Curr. Opin. Genet. Dev.* **2015**, *30*, 7–16 DOI: 10.1016/j.gde.2014.12.003.
- (132) Wadhwa, M.; Knezevic, I.; Kang, H.-N.; Thorpe, R. Immunogenicity Assessment of Biotherapeutic Products: An Overview of Assays and Their Utility. *Biologicals* **2015**, *43* (5), 298–306 DOI: 10.1016/j.biologicals.2015.06.004.
- (133) Ladics, G. S.; Fry, J.; Goodman, R.; Herouet-Guicheney, C.; Hoffmann-Sommergruber, K.; Madsen, C. B.; Penninks, A.; Pomés, A.; Roggen, E. L.; Smit, J.; Wal, J.-M. Allergic Sensitization: Screening Methods. *Clin. Transl. Allergy* **2014**, *4* (1), 13 DOI: 10.1186/2045-7022-4-13.
- (134) McClain, S.; Bowman, C.; Fernández-Rivas, M.; Ladics, G. S.; Ree, R. van. Allergic Sensitization: Food- and Protein-Related Factors. *Clin. Transl. Allergy* **2014**, *4* (1), 11 DOI: 10.1186/2045-7022-4-11.
- (135) Tekin, H. C.; Gijs, M. A. M. Ultrasensitive Protein Detection: A Case for Microfluidic Magnetic Bead-Based Assays. *Lab Chip* **2013**, *13* (24), 4711 DOI: 10.1039/c3lc50477h.
- (136) Jawa, V.; Cousens, L. P.; Awwad, M.; Wakshull, E.; Kropshofer, H.; De Groot, A. S. T-Cell Dependent Immunogenicity of Protein Therapeutics: Preclinical Assessment and Mitigation. *Clin. Immunol.* **2013**, *149* (3), 534–555 DOI: 10.1016/j.clim.2013.09.006.
- (137) Kanduc, D. Homology, Similarity, and Identity in Peptide Epitope Immunodefinition. *J. Pept. Sci.* **2012**, *18* (8), 487–494 DOI: 10.1002/psc.2419.
- (138) Picariello, G.; Mamone, G.; Addeo, F.; Ferranti, P. The Frontiers of Mass Spectrometry-Based Techniques in Food Allergenomics. *J. Chromatogr. A* **2011**, *1218* (42), 7386–7398 DOI: 10.1016/j.chroma.2011.06.033.
- (139) Mamone, G.; Picariello, G.; Addeo, F.; Ferranti, P. Proteomic Analysis in Allergy and Intolerance to Wheat Products. *Expert Rev. Proteomics* **2011**, *8* (1), 95–115 DOI: 10.1586/epr.10.98.
- (140) Sanz, M. L.; Blázquez, A. B.; Garcia, B. E. Microarray of Allergenic Component-Based Diagnosis in Food Allergy. *Curr. Opin. Allergy Clin. Immunol.* **2011**, *11* (3), 204–209 DOI: 10.1097/ACI.0b013e3283466fe4.
- (141) Ladics, G. S.; Knippels, L. M. J.; Penninks, A. H.; Bannon, G. A.; Goodman, R. E.; Herouet-Guicheney, C. Review of Animal Models Designed to Predict the Potential Allergenicity of Novel Proteins in Genetically Modified Crops. *Regul. Toxicol. Pharmacol.* **2010**, *56* (2), 212–224 DOI: 10.1016/j.yrtph.2009.09.018.
- (142) Ahuja, V.; Quatchadze, M.; Ahuja, V.; Stelter, D.; Albrecht, A.; Stahlmann, R. Evaluation of Biotechnology-Derived Novel Proteins for the Risk of Food-Allergic Potential: Advances in the

- Development of Animal Models and Future Challenges. *Arch. Toxicol.* **2010**, *84* (12), 909–917 DOI: 10.1007/s00204-010-0582-0.
- (143) Lundegaard, C.; Lund, O.; Buus, S.; Nielsen, M. Major Histocompatibility Complex Class I Binding Predictions as a Tool in Epitope Discovery. *Immunology* **2010**, *130* (3), 309–318 DOI: 10.1111/j.1365-2567.2010.03300.x.
- (144) Dearman, R. J.; Kimber, I. Animal Models of Protein Allergenicity: Potential Benefits, Pitfalls and Challenges. *Clin. Exp. Allergy* **2009**, *39* (4), 458–468 DOI: 10.1111/j.1365-2222.2008.03194.x.
- (145) Bowman, C. C.; Selgrade, M. K. Utility of Rodent Models for Evaluating Protein Allergenicity. *Regul. Toxicol. Pharmacol.* **2009**, *54* (3), S58–S61 DOI: 10.1016/j.yrtph.2008.10.002.
- (146) Taylor, S. L.; Nordlee, J. A.; Niemann, L. M.; Lambrecht, D. M. Allergen Immunoassays—Considerations for Use of Naturally Incurred Standards. *Anal. Bioanal. Chem.* **2009**, *395* (1), 83–92 DOI: 10.1007/s00216-009-2944-0.
- (147) Flower, D. R. Advances in Predicting and Manipulating the Immunogenicity of Biotherapeutics and Vaccines. *BioDrugs* **2009**, *23* (4), 231–240 DOI: 10.2165/11317530-000000000-00000.
- (148) Gowthaman, U.; Agrewala, J. N. In Silico Methods for Predicting T-Cell Epitopes: Dr Jekyll or Mr Hyde? *Expert Rev. Proteomics* **2009**, *6* (5), 527–537 DOI: 10.1586/epr.09.71.
- (149) Selgrade, M. K.; Bowman, C. C.; Ladics, G. S.; Privalle, L.; Laessig, S. A. Safety Assessment of Biotechnology Products for Potential Risk of Food Allergy: Implications of New Research. *Toxicol. Sci.* **2009**, *110* (1), 31–39 DOI: 10.1093/toxsci/kfp075.
- (150) Nagata, S.; Pastan, I. Removal of B Cell Epitopes as a Practical Approach for Reducing the Immunogenicity of Foreign Protein-Based Therapeutics☆. *Adv. Drug Deliv. Rev.* **2009**, *61* (11), 977–985 DOI: 10.1016/j.addr.2009.07.014.
- (151) Toussaint, N. C.; Kohlbacher, O. Towards in Silico Design of Epitope-Based Vaccines. *Expert Opin. Drug Discov.* **2009**, *4* (10), 1047–1060 DOI: 10.1517/17460440903242283.
- (152) Zhou, P.; Tian, F.; Wu, Y.; Li, Z.; Shang, Z. Quantitative Sequence-Activity Model (QSAM): Applying QSAR Strategy to Model and Predict Bioactivity and Function of Peptides, Proteins and Nucleic Acids. *Curr. Comput. Aided-Drug Des.* **2008**, *4* (4), 311–321 DOI: 10.2174/157340908786785994.
- (153) Marzban, G.; Herndl, A.; Maghuly, F.; Katinger, H.; Laimer, M. Mapping of Fruit Allergens by 2D Electrophoresis and Immunodetection. *Expert Rev. Proteomics* **2008**, *5* (1), 61–75 DOI: 10.1586/14789450.5.1.61.
- (154) Smith, D. S.; Eremin, S. A. Fluorescence Polarization Immunoassays and Related Methods for Simple, High-Throughput Screening of Small Molecules. *Anal. Bioanal. Chem.* **2008**, *391* (5), 1499–1507 DOI: 10.1007/s00216-008-1897-z.
- (155) Hartmann, R.; Wal, J.-M.; Bernard, H.; Pentzien, A.-K. Cytotoxic and Allergenic Potential of Bioactive Proteins and Peptides. *Curr. Pharm. Des.* **2007**, *13* (9), 897–920.
- (156) De Groot, A. S.; Scott, D. W. Immunogenicity of Protein Therapeutics. *Trends Immunol.* **2007**, *28* (11), 482–490 DOI: 10.1016/j.it.2007.07.011.
- (157) Gupta, S.; Indelicato, S. R.; Jethwa, V.; Kawabata, T.; Kelley, M.; Mire-Sluis, A. R.; Richards, S. M.; Rup, B.; Shores, E.; Swanson, S. J.; Wakshull, E. Recommendations for the Design, Optimization, and Qualification of Cell-Based Assays Used for the Detection of Neutralizing

- Antibody Responses Elicited to Biological Therapeutics. *J. Immunol. Methods* **2007**, *321* (1–2), 1–18 DOI: 10.1016/j.jim.2006.12.004.
- (158) McClain, S.; Bannon, G. A. Animal Models of Food Allergy: Opportunities and Barriers. *Curr. Allergy Asthma Rep.* **2006**, *6* (2), 141–144 DOI: 10.1007/s11882-006-0052-1.
- (159) Prescott, V. E.; Hogan, S. P. Genetically Modified Plants and Food Hypersensitivity Diseases: Usage and Implications of Experimental Models for Risk Assessment. *Pharmacol. Ther.* **2006**, *111* (2), 374–383 DOI: 10.1016/j.pharmthera.2005.10.005.
- (160) Lee, L. J.; Yang, S.-T.; Lai, S.; Bai, Y.; Huang, W.-C.; Juang, Y.-J. Microfluidic Enzyme-Linked Immunosorbent Assay Technology. In *ADVANCES IN CLINICAL CHEMISTRY, VOL 42*; Makowski, G., Ed.; Advances in Clinical Chemistry; ELSEVIER ACADEMIC PRESS INC: 525 B STREET, SUITE 1900, SAN DIEGO, CA 92101-4495 USA, 2006; Vol. 42, pp 255–295.
- (161) Viatte, S.; Alves, P. M.; Romero, P. Reverse Immunology Approach for the Identification of CD8 T-Cell-Defined Antigens: Advantages and Hurdles. *Immunol. Cell Biol.* **2006**, *84* (3), 318–330 DOI: 10.1111/j.1440-1711.2006.01447.x.
- (162) Taylor, S. L. Review of the Development of Methodology for Evaluating the Human Allergenic Potential of Novel Proteins. *Mol. Nutr. Food Res.* **2006**, *50* (7), 604–609 DOI: 10.1002/mnfr.200500275.
- (163) Matsuda, T.; Matsubara, T.; Hino, S. Immunogenic and Allergenic Potentials of Natural and Recombinant Innocuous Proteins. *J. Biosci. Bioeng.* **2006**, *101* (3), 203–211 DOI: 10.1263/jbb.101.203.
- (164) Alexopoulos, C.; Krikorian, D.; Panou-Pomonis, E.; Sakarellos-Daitsiotis, M.; Sakarellos, C. Innovative, Multifunctional Sequential Oligopeptide Carriers Socn-I and SOCn-II: Functions-Technology-Perspectives. *Protein Pept. Lett.* **2005**, *12* (6), 601–607 DOI: 10.2174/0929866054395914.
- (165) Torreri, P.; Ceccarini, M.; Macioce, P.; Petrucci, T. C. Biomolecular Interactions by Surface Plasmon Resonance Technology. *Ann. Ist. Super. Sanita* **2005**, *41* (4), 437–441.
- (166) Dearman, R. J.; Kimber, I. Characterisation of Immune Responses to Food Allergens in Mice. *Proc. Nutr. Soc.* **2005**, *64* (04), 426–433 DOI: 10.1079/PNS2005456.
- (167) Knippels, L. M. J.; van Wijk, F.; Penninks, A. H. Food Allergy: What Do We Learn from Animal Models? *Curr. Opin. Allergy Clin. Immunol.* **2004**, *4* (3), 205–209.
- (168) Westphal, C. D.; Raybourne, R. B. Potential Allergenicity of Novel Proteins in Murine Models. *J. AOAC Int.* **2004**, *87* (6), 1433–1440.
- (169) Mire-Sluis, A. R.; Barrett, Y. C.; Devanarayan, V.; Koren, E.; Liu, H.; Maia, M.; Parish, T.; Scott, G.; Shankar, G.; Shores, E.; Swanson, S. J.; Taniguchi, G.; Wierda, D.; Zuckerman, L. A. Recommendations for the Design and Optimization of Immunoassays Used in the Detection of Host Antibodies against Biotechnology Products. *J. Immunol. Methods* **2004**, *289* (1–2), 1–16 DOI: 10.1016/j.jim.2004.06.002.
- (170) Kimber, I.; Betts, C. J.; Dearman, R. J. Assessment of the Allergenic Potential of Proteins. *Toxicol. Lett.* **2003**, *140–141* (SI), 297–302 DOI: 10.1016/S0378-4274(03)00025-0.
- (171) Kimber, I.; Stone, S.; Dearman, R. J. Assessment of the Inherent Allergenic Potential of Proteins in Mice. *Environ. Health Perspect.* **2003**, *111* (2), 227–231 DOI: 10.1289/ehp.5703.
- (172) Atherton, K. T.; Dearman, R. J.; Kimber, I. Protein Allergenicity in Mice - A Potential Approach

- for Hazard Identification. In *GENETICALLY ENGINEERED FOODS ASSESSING POTENTIAL ALLERGENICITY*; Fu, TJ and Gendel, S., Ed.; ANNALS OF THE NEW YORK ACADEMY OF SCIENCES; NEW YORK ACAD SCIENCES: 2 EAST 63RD ST, NEW YORK, NY 10021 USA, 2002; Vol. 964, pp 163–171.
- (173) Helm, R. M.; Burks, A. W. Animal Models of Food Allergy. *Curr. Opin. Allergy Clin. Immunol.* **2002**, *2* (6), 541–546 DOI: 10.1097/01.all.0000044541.45448.bb.
- (174) Knippels, L. M. J.; Penninks, A. H. Assessment of Protein Allergenicity - Studies in Brown Norway Rats. In *GENETICALLY ENGINEERED FOODS ASSESSING POTENTIAL ALLERGENICITY*; Fu, TJ and Gendel, S., Ed.; ANNALS OF THE NEW YORK ACADEMY OF SCIENCES; NEW YORK ACAD SCIENCES: 2 EAST 63RD ST, NEW YORK, NY 10021 USA, 2002; Vol. 964, pp 151–161.
- (175) Taylor, S. L. Protein Allergenicity Assessment of Foods Produced through Agricultural Biotechnology. *Annu. Rev. Pharmacol. Toxicol.* **2002**, *42* (1), 99–112 DOI: 10.1146/annurev.pharmtox.42.082401.130208.
- (176) Gendel, S. M. Sequence Analysis for Assessing Potential Allergenicity. *Ann. N. Y. Acad. Sci.* **2002**, *964*, 87–98.
- (177) Bayard, C.; Lottspeich, F. Bioanalytical Characterization of Proteins. *J. Chromatogr. B Biomed. Sci. Appl.* **2001**, *756* (1–2), 113–122 DOI: 10.1016/S0378-4347(01)00096-2.
- (178) Sauna, Z. E.; Lagassé, D.; Pedras-Vasconcelos, J.; Golding, B.; Rosenberg, A. S. Evaluating and Mitigating the Immunogenicity of Therapeutic Proteins. *Trends Biotechnol.* **2018**, *0* (0), 1–17 DOI: 10.1016/j.tibtech.2018.05.008.
- (179) Becker, W.-M.; Reese, G. Immunological Identification and Characterization of Individual Food Allergens. *J. Chromatogr. B Biomed. Sci. Appl.* **2001**, *756* (1–2), 131–140 DOI: 10.1016/S0378-4347(01)00098-6.
- (180) Lehrer, S. B.; Horner, W. E.; Reese, G.; Taylor, S. Why Are Some Proteins Allergenic? Implications for Biotechnology. *Crit. Rev. Food Sci. Nutr.* **1996**, *36* (6), 553–564 DOI: 10.1080/10408399609527739.
- (181) van Vliet, E.; Kühnl, J.; Goebel, C.; Martinozzi-Teissier, S.; Alépée, N.; Ashikaga, T.; Blömeke, B.; Del Bufalo, A.; Cluzel, M.; Corsini, E.; Delrue, N.; Desprez, B.; Gellatly, N.; Giese, C.; Gribaldo, L.; Hoffmann, S.; Klaric, M.; Maillere, B.; Naisbitt, D.; Pallardy, M.; Vocanson, M.; Petersohn, D. State-of-the-Art and New Options to Assess T Cell Activation by Skin Sensitizers: Cosmetics Europe Workshop. *ALTEX* **2018**, *35* (2), 179–192 DOI: 10.14573/altex.1709011.
- (182) Cloete, W. J.; Klumperman, B.; Cloete, T. E. Increased Functionality Through Surface Grafting: Polymeric Surfaces. *Curr. Org. Chem.* **2017**, *21* (24), 2606–2611 DOI: 10.2174/1385272821666170620112440.
- (183) Arena, S.; Renzone, G.; D'Ambrosio, C.; Salzano, A. M.; Scaloni, A. Dairy Products and the Maillard Reaction: A Promising Future for Extensive Food Characterization by Integrated Proteomics Studies. *Food Chem.* **2017**, *219*, 477–489 DOI: 10.1016/j.foodchem.2016.09.165.
- (184) Murthy, H.; Paek, K.; Park, S.; Dandin, V. Safety Issues of Food Ingredients from Plant Cell, Tissue, and Organ Cultures: An Explication. In *Food Toxicology*; CRC Press: Taylor & Francis Group, 6000 Broken Sound Parkway NW, Suite 300, Boca Raton, FL 33487-2742, 2016; pp 151–168.
- (185) Zhao, K.; Ren, F.; Han, F.; Liu, Q.; Wu, G.; Xu, Y.; Zhang, J.; Wu, X.; Wang, J.; Li, P.; Shi, W.; Zhu, H.; Lv, J.; Zhao, X.; Tang, X. Edible Safety Assessment of Genetically Modified Rice T1C-1

- for Sprague Dawley Rats through Horizontal Gene Transfer, Allergenicity and Intestinal Microbiota. *PLoS One* **2016**, *11* (10), e0163352 DOI: 10.1371/journal.pone.0163352.
- (186) Tan, X.; Zhou, X.; Tang, Y.; Lv, J.; Zhang, L.; Sun, L.; Yang, Y.; Miao, Y.; Jiang, H.; Chen, G.; Huang, Z.; Wang, X. Immunotoxicological Evaluation of Genetically Modified Rice Expressing Cry1Ab/Ac Protein (TT51-1) by a 6-Month Feeding Study on Cynomolgus Monkeys. *PLoS One* **2016**, *11* (9), e0163879 DOI: 10.1371/journal.pone.0163879.
- (187) Joshi, S. S.; Barnett, B.; Doerrer, N. G.; Glenn, K.; Herman, R. A.; Herouet-Guichenev, C.; Hunst, P.; Kough, J.; Ladics, G. S.; McClain, S.; Papineni, S.; Poulsen, L. K.; Rasclé, J.-B.; Tao, A.-L.; van Ree, R.; Ward, J.; Bowman, C. C. Assessment of Potential Adjuvanticity of Cry Proteins. *Regul. Toxicol. Pharmacol.* **2016**, *79*, 149–155 DOI: 10.1016/j.yrtph.2016.04.005.
- (188) Rubio-Infante, N.; Moreno-Fierros, L. An Overview of the Safety and Biological Effects of *Bacillus Thuringiensis* Cry Toxins in Mammals. *J. Appl. Toxicol.* **2016**, *36* (5), 630–648 DOI: 10.1002/jat.3252.
- (189) Naegeli, H.; Birch, A. N.; Casacuberta, J.; De Schrijver, A.; Gralak, M. A.; Guerche, P.; Jones, H.; Manachini, B.; Messéan, A.; Nielsen, E. E.; Nogué, F.; Robaglia, C.; Rostoks, N.; Sweet, J.; Tebbe, C.; Visioli, F.; Wal, J.-M.; Divéki, Z.; Fernández-Dumont, A.; Gennaro, A.; Lanzoni, A.; Maria Neri, F.; Paraskevopoulos, K. Scientific Opinion on an Application by Dow AgroSciences (EFSA-GMO-NL-2013-116) for Placing on the Market of Genetically Modified Insect-resistant Soybean DAS-81419-2 for Food and Feed Uses, Import and Processing under Regulation (EC) No 1829/2003. *EFSA J.* **2016**, *14* (12) DOI: 10.2903/j.efsa.2016.4642.
- (190) Andreassen, M.; Bohn, T.; Wikmark, O.-G.; Bodin, J.; Traavik, T.; Lovik, M.; Nygaard, U. C. Investigations of Immunogenic, Allergenic and Adjuvant Properties of Cry1Ab Protein after Intra-gastric Exposure in a Food Allergy Model in Mice. *BMC Immunol.* **2016**, *17* (1), 10 DOI: 10.1186/s12865-016-0148-x.
- (191) Fard, N.; Minuchehr, Z.; Rahgozar, M. Novel Genetically Modified Foods and Allergenicity Assessment of Them, Case Study: Tarom GM Rice. *Curr. Nutr. Food Sci.* **2015**, *11* (1), 11–15 DOI: 10.2174/1573401311666141231192227.
- (192) Farias, D. F.; Viana, M. P.; Oliveira, G. R.; Santos, V. O.; Pinto, C. E. M.; Viana, D. A.; Vasconcelos, I. M.; Grossi-de-Sa, M. F.; Carvalho, A. F. U. Food Safety Assessment of Cry8Ka5 Mutant Protein Using Cry1Ac as a Control Bt Protein. *Food Chem. Toxicol.* **2015**, *81*, 81–91 DOI: 10.1016/j.fct.2015.04.008.
- (193) Baktavachalam, G. B.; Delaney, B.; Fisher, T. L.; Ladics, G. S.; Layton, R. J.; Locke, M. E.; Schmidt, J.; Anderson, J. A.; Weber, N. N.; Herman, R. A.; Evans, S. L. Transgenic Maize Event TC1507: Global Status of Food, Feed, and Environmental Safety. *GM Crops Food* **2015**, *6* (2), 80–102 DOI: 10.1080/21645698.2015.1054093.
- (194) Mezzomo, B. P.; Miranda-Vilela, A. L.; Grisolia, C. K. Toxicological Evaluation of a Potential Immunosensitizer for Use as a Mucosal Adjuvant--*Bacillus Thuringiensis* Cry1Ac Spore-Crystals: A Possible Inverse Agonist That Deserves Further Investigation. *Toxins (Basel)*. **2015**, *7* (12), 5348–5358 DOI: 10.3390/toxins7124881.
- (195) Rubio-Infante, N.; Ilhucatzí-Alvarado, D.; Torres-Martínez, M.; Reyes-Grajeda, J. P.; Nava-Acosta, R.; González-González, E.; Moreno-Fierros, L. The Macrophage Activation Induced by *Bacillus Thuringiensis* Cry1Ac Protoxin Involves ERK1/2 and P38 Pathways and the Interaction with Cell-Surface-HSP70. *J. Cell. Biochem.* **2018**, *119* (1), 580–598 DOI: 10.1002/jcb.26216.
- (196) Mathur, C.; Kathuria, P. C.; Dahiya, P.; Singh, A. B. Lack of Detectable Allergenicity in Genetically Modified Maize Containing “Cry” Proteins as Compared to Native Maize Based on In

- Silico & In Vitro Analysis. *PLoS One* **2015**, *10* (2), e0117340 DOI: 10.1371/journal.pone.0117340.
- (197) Andreassen, M.; Bøhn, T.; Wikmark, O.-G.; Van den Berg, J.; Løvik, M.; Traavik, T.; Nygaard, U. C. Cry1Ab Protein from *Bacillus Thuringiensis* and MON810 Cry1Ab -Transgenic Maize Exerts No Adjuvant Effect After Airway Exposure. *Scand. J. Immunol.* **2015**, *81* (3), 192–200 DOI: 10.1111/sji.12269.
- (198) Song, Y.; Liang, C.; Wang, W.; Fang, J.; Sun, N.; Jia, X.; Li, N. Immunotoxicological Evaluation of Corn Genetically Modified with *Bacillus Thuringiensis* Cry1Ah Gene by a 30-Day Feeding Study in BALB/c Mice. *PLoS One* **2014**, *9* (2), e78566 DOI: 10.1371/journal.pone.0078566.
- (199) Andreassen, M.; Bøhn, T.; Wikmark, O.-G.; Van den Berg, J.; Løvik, M.; Traavik, T.; Nygaard, U. C. CRY1AB Protein from MON810 Transgenic Maize and *Bacillus Thuringiensis* Has No Clear Adjuvanticity after Intranasal Exposure. *Toxicol. Lett.* **2014**, *229* (S), S207 DOI: 10.1016/j.toxlet.2014.06.696.
- (200) Reiner, D.; Lee, R.-Y.; Dekan, G.; Epstein, M. M. No Adjuvant Effect of *Bacillus Thuringiensis*-Maize on Allergic Responses in Mice. *PLoS One* **2014**, *9* (8), e103979 DOI: 10.1371/journal.pone.0103979.
- (201) Wang, J.; Sun, N.; Zhou, C.; Zhou, X.; Lu, J.; Wang, C.; Che, H. Food Proteins from Different Allergen Families Sensitize Balb/c Mice to Family-Specific Immune Responses. *J. Immunotoxicol.* **2014**, *11* (2), 172–179 DOI: 10.3109/1547691X.2013.819053.
- (202) Moreno-Fierros, L.; García-Hernández, A. L.; Ilhucatz-Alvarado, D.; Rivera-Santiago, L.; Torres-Martínez, M.; Rubio-Infante, N.; Legorreta-Herrera, M. Cry1Ac Protoxin from *Bacillus Thuringiensis* Promotes Macrophage Activation by Upregulating CD80 and CD86 and by Inducing IL-6, MCP-1 and TNF- α Cytokines. *Int. Immunopharmacol.* **2013**, *17* (4), 1051–1066 DOI: 10.1016/j.intimp.2013.10.005.
- (203) Cao, S.; He, X.; Xu, W.; Luo, Y.; Ran, W.; Liang, L.; Dai, Y.; Huang, K. Potential Allergenicity Research of Cry1C Protein from Genetically Modified Rice. *Regul. Toxicol. Pharmacol.* **2012**, *63* (2), 181–187 DOI: 10.1016/j.yrtph.2012.03.017.
- (204) Walsh, M. C.; Buzoianu, S. G.; Rea, M. C.; O'Donovan, O.; Gelencsér, E.; Ujhelyi, G.; Ross, R. P.; Gardiner, G. E.; Lawlor, P. G. Effects of Feeding Bt MON810 Maize to Pigs for 110 Days on Peripheral Immune Response and Digestive Fate of the Cry1Ab Gene and Truncated Bt Toxin. *PLoS One* **2012**, *7* (5), e36141 DOI: 10.1371/journal.pone.0036141.
- (205) Randhawa, G. J.; Singh, M.; Grover, M. Bioinformatic Analysis for Allergenicity Assessment of *Bacillus Thuringiensis* Cry Proteins Expressed in Insect-Resistant Food Crops. *Food Chem. Toxicol.* **2011**, *49* (2), 356–362 DOI: 10.1016/j.fct.2010.11.008.
- (206) Razavi, A.; Malhotra, I.; Ghosh, A.; Pusztai-Carey, M.; Marks, J.; King, C. Antibodies as Epidemiological Markers of Genetically Modified Crop Exposure: Detection of Cry1Ab-Specific IgG. *Food Agric. Immunol.* **2017**, *28* (5), 779–788 DOI: 10.1080/09540105.2017.1313200.
- (207) Adel-Patient, K.; Guimaraes, V. D.; Paris, A.; Drumare, M.-F.; Ah-Leung, S.; Lamourette, P.; Nevers, M.-C.; Canlet, C.; Molina, J.; Bernard, H.; Créminon, C.; Wal, J.-M. Immunological and Metabolomic Impacts of Administration of Cry1Ab Protein and MON 810 Maize in Mouse. *PLoS One* **2011**, *6* (1), e16346 DOI: 10.1371/journal.pone.0016346.
- (208) Quemada, H.; Zarka, K.; Pett, W.; Bothma, G.; Felcher, K.; Mirendil, H.; Koch, M.; Brink, J.; Douches, D. Safety Evaluations of the Cry1Ia1 Protein Found in the Transgenic Potato 'SpuntaG2'. *J. Am. Soc. Hortic. Sci.* **2010**, *135* (4), 325–332.

- (209) Cao, S.; He, X.; Xu, W.; Ran, W.; Liang, L.; Luo, Y.; Yuan, Y.; Zhang, N.; Zhou, X.; Huang, K. Safety Assessment of Cry1C Protein from Genetically Modified Rice According to the National Standards of PR China for a New Food Resource. *Regul. Toxicol. Pharmacol.* **2010**, *58* (3), 474–481 DOI: 10.1016/j.yrtph.2010.08.018.
- (210) Legorreta-Herrera, M.; Meza, R. O.; Moreno-Fierros, L. Pretreatment with Cry1Ac Protoxin Modulates the Immune Response, and Increases the Survival of Plasmodium-Infected CBA/Ca Mice. *J. Biomed. Biotechnol.* **2010**, *2010*, 198921 DOI: 10.1155/2010/198921.
- (211) Guimaraes, V.; Drumare, M.-F.; Lereclus, D.; Gohar, M.; Lamourette, P.; Nevers, M.-C.; Vaisanen-Tunkelrott, M.-L.; Bernard, H.; Guillon, B.; Créminon, C.; Wal, J.-M.; Adel-Patient, K. In Vitro Digestion of Cry1Ab Proteins and Analysis of the Impact on Their Immunoreactivity. *J. Agric. Food Chem.* **2010**, *58* (5), 3222–3231 DOI: 10.1021/jf903189j.
- (212) Kim, J.-H.; Seo, Y.-J.; Kim, J.-Y.; Han, Y.-S.; Lee, K.-S.; Kim, S.-A.; Kim, H.-N.; Ahn, K.; Lee, S.-I.; Kim, H.-Y. Allergenicity Assessment of Cry Proteins in Insect-Resistant Genetically Modified Maize Bt11, MON810, and MON863. *FOOD Sci. Biotechnol.* **2009**, *18* (5), 1273–1278.
- (213) Verdin-Teran, S. L.; Vilches-Flores, A.; Moreno-Fierros, L. Immunization with Cry1Ac from *Bacillus Thuringiensis* Increases Intestinal IgG Response and Induces the Expression of FcRn in the Intestinal Epithelium of Adult Mice. *Scand. J. Immunol.* **2009**, *70* (6), 596–607 DOI: 10.1111/j.1365-3083.2009.02332.x.
- (214) Xu, W.; Cao, S.; He, X.; Luo, Y.; Guo, X.; Yuan, Y.; Huang, K. Safety Assessment of Cry1Ab/Ac Fusion Protein. *Food Chem. Toxicol.* **2009**, *47* (7), 1459–1465 DOI: 10.1016/j.fct.2009.03.029.
- (215) Jarillo-Luna, A.; Moreno-Fierros, L.; Campos-Rodriguez, R.; Rodriguez-Monroy, M. A.; Lara-Padilla, E.; Rojas-Hernandez, S. Intranasal Immunization with *Naegleria Fowleri* Lysates and Cry1Ac Induces Metaplasia in the Olfactory Epithelium and Increases IgA Secretion. *Parasite Immunol.* **2008**, *30* (1), 31–38 DOI: 10.1111/j.1365-3024.2007.00999.x.
- (216) Kroghsbo, S.; Madsen, C.; Poulsen, M.; Schrøder, M.; Kvist, P. H.; Taylor, M.; Gatehouse, A.; Shu, Q.; Knudsen, I. Immunotoxicological Studies of Genetically Modified Rice Expressing PHA-E Lectin or Bt Toxin in Wistar Rats. *Toxicology* **2008**, *245* (1–2), 24–34 DOI: 10.1016/j.tox.2007.12.005.
- (217) Then, C.; Bauer-Panskus, A. Possible Health Impacts of Bt Toxins and Residues from Spraying with Complementary Herbicides in Genetically Engineered Soybeans and Risk Assessment as Performed by the European Food Safety Authority EFSA. *Environ. Sci. Eur.* **2017**, *29* (1), 1 DOI: 10.1186/s12302-016-0099-0.
- (218) Guimaraes, V. D.; Drumare, M.-F.; Ah-Leung, S.; Lereclus, D.; Bernard, H.; Créminon, C.; Wal, J.-M.; Adel-Patient, K. Comparative Study of the Adjuvant/immunogenicity of *Bacillus Thuringiensis* Cry1Ab Protein and Cholera Toxin on Allergic Sensitisation and Elicitation to Peanut. *Food Agric. Immunol.* **2008**, *19* (4), 325–337 DOI: 10.1080/09540100802495651.
- (219) Delaney, B.; Astwood, J. D.; Cunney, H.; Conn, R. E.; Herouet-Guicheney, C.; MacIntosh, S.; Meyer, L. S.; Privalle, L.; Gao, Y.; Mattsson, J.; Levine, M. Evaluation of Protein Safety in the Context of Agricultural Biotechnology. *Food Chem. Toxicol.* **2008**, *46* (2), S71–S97 DOI: 10.1016/j.fct.2008.01.045.
- (220) Román Calderón, M. E.; Alcocer González, J. M.; Franco Molina, M. A.; Tamez Guerra, R. S.; Padilla, C. R. Adjuvant Effects of Crystal Proteins from a Mexican Strain of *Bacillus Thuringiensis* on the Mouse Humoral Response. *Biologicals* **2007**, *35* (4), 271–276 DOI: 10.1016/j.biologicals.2007.01.006.

- (221) Guerrero, G. G.; Russell, W. M.; Moreno-Fierros, L. Analysis of the Cellular Immune Response Induced by Bacillus Thuringiensis Cry1A Toxins in Mice: Effect of the Hydrophobic Motif from Diphtheria Toxin. *Mol. Immunol.* **2007**, *44* (6), 1209–1217 DOI: 10.1016/j.molimm.2006.06.007.
- (222) Son, D.-Y. Research on the Allergic Potential of Insecticidal Cry1Ac Proteins of Genetically Modified Rice. *FOOD Sci. Biotechnol.* **2006**, *15* (3), 385–391.
- (223) Esquivel-Perez, R.; Moreno-Fierros, L. Mucosal and Systemic Adjuvant Effects of Cholera Toxin and Cry1Ac Protoxin on the Specific Antibody Response to HIV-1 C4/V3 Peptides Are Different and Depend on the Antigen Co-Administered. *Viral Immunol.* **2005**, *18* (4), 695–708 DOI: 10.1089/vim.2005.18.695.
- (224) Guerrero, G. G.; Dean, D. H.; Moreno-Fierros, L. Structural Implication of the Induced Immune Response by Bacillus Thuringiensis Cry Proteins: Role of the N-Terminal Region. *Mol. Immunol.* **2004**, *41* (12), 1177–1183 DOI: 10.1016/j.molimm.2004.06.026.
- (225) Rojas-Hernandez, S.; Rodriguez-Monroy, M. A.; Lopez-Revilla, R.; Resendiz-Albor, A. A.; Moreno-Fierros, L. Intranasal Coadministration of the Cry1Ac Protoxin with Amoebal Lysates Increases Protection against Naegleria Fowleri Meningoencephalitis. *Infect. Immun.* **2004**, *72* (8), 4368–4375 DOI: 10.1128/IAI.72.8.4368-4375.
- (226) Bernstein, J. A.; Bernstein, I. L.; Bucchini, L.; Goldman, L. R.; Hamilton, R. G.; Lehrer, S.; Rubin, C.; Sampson, H. A. Clinical and Laboratory Investigation of Allergy to Genetically Modified Foods. *Environ. Health Perspect.* **2003**, *111* (8), 1114–1121.
- (227) Bucchini, L.; Goldman, L. R. Starlink Corn: A Risk Analysis. *Environ. Health Perspect.* **2002**, *110* (1), 5–13.
- (228) Rathinam, M.; Singh, S.; Pattanayak, D.; Sreevathsa, R. Comprehensive in Silico Allergenicity Assessment of Novel Protein Engineered Chimeric Cry Proteins for Safe Deployment in Crops. *BMC Biotechnol.* **2017**, *17* (1), 64 DOI: 10.1186/s12896-017-0384-z.
- (229) Goodman, R.; Hileman, R.; Silvanovich, A.; Rice, E.; Holleschak, G.; Astwood, J.; Hefle, S. A Bioinformatics Assessment of the Potential Allergenicity of the Introduced Proteins in Genetically Engineered Crops. In *ALLERGY: AN EXPANDING CHALLENGE IN THE 21ST CENTURY*; Kim, YY and Lee, S., Ed.; MEDIMOND S R L: VIA MASERATI 5, 40128 BOLOGNA, 00000, ITALY, 2002; pp 115–120.
- (230) Guerrero, G.; Moreno-Fierros, L. Characterization of the Intranasal Immunogenicity of Cry Proteins in Balb/c Mice. *Clin. Immunol.* **2002**, *103* (3, 2, S), S99–S100.
- (231) Guerrero, G.; Moreno-Fierros, L. Immunogenicity and Potential Use as Carriers Vaccines of Cry Proteins Administered by Intranasal Route to Balb/c Mice. *Clin. Immunol.* **2002**, *103* (3, 2, S), S102–S103.
- (232) Hileman, R. E.; Silvanovich, A.; Goodman, R. E.; Rice, E. A.; Holleschak, G.; Astwood, J. D.; Hefle, S. L. Bioinformatic Methods for Allergenicity Assessment Using a Comprehensive Allergen Database. *Int. Arch. Allergy Immunol.* **2002**, *128* (4), 280–291 DOI: 10.1159/000063861.
- (233) Bernstein, J. A.; Bernstein, I. L.; Bernstein, D. I. Allergenicity of Cry9c: An Unresolved Issue. *J. Allergy Clin. Immunol.* **2001**, *108* (4), 655–656 DOI: 10.1067/mai.2001.118513.
- (234) Vázquez-Padrón, R. I.; Moreno-Fierros, L.; Neri-Bazán, L.; Martínez-Gil, A. F.; De-la-Riva, G. A.; López-Revilla, R. Characterization of the Mucosal and Systemic Immune Response Induced

- by Cry1Ac Protein from *Bacillus Thuringiensis* HD 73 in Mice. *Brazilian J. Med. Biol. Res.* **2000**, *33* (2), 147–155 DOI: 10.1590/S0100-879X2000000200002.
- (235) Moreno-Fierros, L.; García, N.; Gutiérrez, R.; López-Revilla, R.; Vázquez-Padrón, R. I. Intranasal, Rectal and Intraperitoneal Immunization with Protoxin Cry1Ac from *Bacillus Thuringiensis* Induces Compartmentalized Serum, Intestinal, Vaginal and Pulmonary Immune Responses in Balb/c Mice. *Microbes Infect.* **2000**, *2* (8), 885–890 DOI: 10.1016/S1286-4579(00)00398-1.
- (236) Betz, F. S.; Hammond, B. G.; Fuchs, R. L. Safety and Advantages of *Bacillus Thuringiensis*-Protected Plants to Control Insect Pests. *Regul. Toxicol. Pharmacol.* **2000**, *32* (2), 156–173 DOI: 10.1006/rtp.2000.1426.
- (237) Vázquez-Padrón, R. I.; Moreno-Fierros, L.; Neri-Bazán, L.; de la Riva, G. A.; López-Revilla, R. Intra-gastric and Intraperitoneal Administration of Cry1Ac Protoxin from *Bacillus Thuringiensis* Induces Systemic and Mucosal Antibody Responses in Mice. *Life Sci.* **1999**, *64* (21), 1897–1912 DOI: 10.1016/S0024-3205(99)00136-8.
- (238) Vázquez, R. I.; Moreno-Fierros, L.; Neri-Bazán, L.; De La Riva, G. A.; López-Revilla, R. *Bacillus Thuringiensis* Cry1Ac Protoxin Is a Potent Systemic and Mucosal Adjuvant. *Scand. J. Immunol.* **1999**, *49* (6), 578–584.
- (239) Dunn, S. E.; Vicini, J. L.; Glenn, K. C.; Fleischer, D. M.; Greenhawt, M. J. The Allergenicity of Genetically Modified Foods from Genetically Engineered Crops: A Narrative and Systematic Review. *Ann. Allergy. Asthma Immunol.* **2017**, *119* (3), 214–222.e3 DOI: 10.1016/j.anai.2017.07.010.
- (240) Bernstein, I. L.; Bernstein, J. A.; Miller, M.; Tierzieva, S.; Bernstein, D. I.; Lummus, Z.; Selgrade, M. K.; Doerfler, D. L.; Seligy, V. L. Immune Responses in Farm Workers after Exposure to *Bacillus Thuringiensis* Pesticides. *Environ. Health Perspect.* **1999**, *107* (7), 575 DOI: 10.2307/3434400.
- (241) Rahnama, H.; Nikmard, M.; Abolhasani, M.; Osfoori, R.; Sanjarian, F.; Habashi, A. A. Immune Analysis of Cry1Ab-Genetically Modified Potato by in-Silico Analysis and Animal Model. *Food Sci. Biotechnol.* **2017**, *26* (5), 1437–1445 DOI: 10.1007/s10068-017-0181-4.
- (242) Birch, A. N.; Casacuberta, J.; De Schrijver, A.; Gralak, M. A.; Guerche, P.; Jones, H.; Manachini, B.; Messean, A.; Naegeli, H.; Nielsen, E. E.; Nogue, F.; Robaglia, C.; Rostoks, N.; Sweet, J.; Tebbe, C.; Visioli, F.; Wal, J.-M.; Modified, E. P. G. Scientific Opinion on Application (EFSA-GMO-NL-2011-96) for the Placing on the Market of Genetically Modified Insect-Resistant and Herbicide-Tolerant Cotton GHB119, for Food and Feed Uses, Import and Processing under Regulation (EC) No 1829/2003 from Baye. *EFSA J.* **2016**, *14* (10), e04586 DOI: 10.2903/j.efsa.2016.4586.
- (243) Torres-Martínez, M.; Rubio-Infante, N.; García-Hernández, A. L.; Nava-Acosta, R.; Ilhuicatzí-Alvarado, D.; Moreno-Fierros, L. Cry1Ac Toxin Induces Macrophage Activation via ERK1/2, JNK and P38 Mitogen-Activated Protein Kinases. *Int. J. Biochem. Cell Biol.* **2016**, *78*, 106–115 DOI: 10.1016/j.biocel.2016.06.022.
- (244) Torres-Martínez, M.; Rubio-Infante, N.; Lilia Garcia-Hernandez, A.; Nava-Acosta, R.; Ilhuicatzí-Alvarado, D.; Moreno-Fierros, L. Cry1Ac Toxin Induces Macrophage Activation via ERK1/2, JNK and P38 Mitogen-Activated Protein Kinases. *Int. J. Biochem. Cell Biol.* **2016**, *78*, 106–115 DOI: 10.1016/j.biocel.2016.06.022.
- (245) Farias, D. F.; Viana, M. P.; Oliveira, G. R.; Santos, V. O.; Pinto, C. E. M.; Viana, D. A.; Vasconcelos, I. M.; Grossi-de-Sa, M. F.; Carvalho, A. F. U. Food Safety Assessment of Cry8Ka5

- Mutant Protein Using Cry1Ac as a Control Bt Protein. *Food Chem. Toxicol.* **2015**, *81*, 81–91 DOI: 10.1016/j.fct.2015.04.008.
- (246) Panel, E.; Organisms, M. Scientific Opinion on an Application (EFSA-GMO-BE-2011-98) for the Placing on the Market of Herbicide-Tolerant Genetically Modified Soybean FG72 for Food and Feed Uses, Import and Processing under Regulation (EC) No 1829/2003 from Bayer CropScience. *EFSA J.* **2015**, *13* (7), 4167 DOI: 10.2903/j.efsa.2015.4167.
- (247) Finamore, A.; Roselli, M.; Britti, S.; Monastra, G.; Ambra, R.; Turrini, A.; Mengheri, E. Intestinal and Peripheral Immune Response to MON810 Maize Ingestion in Weaning and Old Mice. *J. Agric. Food Chem.* **2008**, *56* (23), 11533–11539 DOI: 10.1021/jf802059w.
- (248) Abolins, S.; King, E. C.; Lazarou, L.; Weldon, L.; Hughes, L.; Drescher, P.; Raynes, J. G.; Hafalla, J. C. R.; Viney, M. E.; Rile, E. M. The Comparative Immunology of Wild and Laboratory Mice, *Mus Musculus Domesticus*. *Nat. Commun.* **2017**, *8* (May), 1–13 DOI: 10.1038/ncomms14811.
- (249) Hammond, B.; Kough, J.; Herouet-Guicheney, C.; Jez, J. M. Toxicological Evaluation of Proteins Introduced into Food Crops. *Crit. Rev. Toxicol.* **2013**, *43* (SUPPL.2), 25–42 DOI: 10.3109/10408444.2013.842956.
- (250) Mendelsohn, M.; Kough, J.; Vaituzis, Z.; Matthews, K. Are Bt Crops Safe? *Nat. Biotechnol.* **2003**, *21* (9), 1003–1009 DOI: 10.1038/nbt0903-1003.
- (251) ILSI Research Foundation. A Review of the Food and Feed Safety of the Cry1Ab Protein. **2016**, 1–15 DOI: 10.13140/RG.2.2.15073.10081.
- (252) Dhama, K.; Saminathan, M.; Jacob, S. S.; Singh, M.; Karthik, K.; . A.; Tiwari, R.; Sunkara, L. T.; Malik, Y. S.; Singh, R. K. Effect of Immunomodulation and Immunomodulatory Agents on Health with Some Bioactive Principles, Modes of Action and Potent Biomedical Applications. *Int. J. Pharmacol.* **2015**, *11* (4), 253–290 DOI: 10.3923/ijp.2015.253.290.
- (253) EFSA. Scientific Opinion on the Evaluation of Allergenic Foods and Food Ingredients for Labelling Purposes. *EFSA J.* **2014**, *12* (11) DOI: 10.2903/j.efsa.2014.3894.
- (254) U.S. Food and Drug Administration. Guidance for Industry: Immunogenicity Assessment for Therapeutic Protein Products. 2014.
- (255) Dong, J. C.; Kobinger, G. P. Hypothesis Driven Development of New Adjuvants. *Hum. Vaccin. Immunother.* **2013**, *9* (4), 808–811 DOI: 10.4161/hv.22972.
- (256) Ruiters, B.; Shreffler, W. G. Innate Immunostimulatory Properties of Allergens and Their Relevance to Food Allergy. *Semin. Immunopathol.* **2012**, *34* (5), 617–632 DOI: 10.1007/s00281-012-0334-8.
- (257) Jahn-Schmid, B.; Pickl, W. F.; Bohle, B. Interaction of Allergens, Major Histocompatibility Complex Molecules, and T Cell Receptors: A 'Ménage à Trois' That Opens New Avenues for Therapeutic Intervention in Type I Allergy. *Int. Arch. Allergy Immunol.* **2011**, *156* (1), 27–42 DOI: 10.1159/000321904.
- (258) EFSA. Scientific Opinion on the Assessment of Allergenicity of GM Plants and Microorganisms and Derived Food and Feed. *EFSA J.* **2010**, *8* (7), 1700 DOI: 10.2903/j.efsa.2010.1700.
- (259) Katsara, M.; Minigo, G.; Plebanski, M.; Apostolopoulos, V. The Good, the Bad and the Ugly: How Altered Peptide Ligands Modulate Immunity. *Expert Opin. Biol. Ther.* **2008**, *8* (12), 1873–1884 DOI: 10.1517/14712590802494501.

- (260) Naegeli, H.; Birch, A. N.; Casacuberta, J.; De Schrijver, A.; Gralak, M. A.; Guerche, P.; Jones, H.; Manachini, B.; Messéan, A.; Nielsen, E. E.; Nogué, F.; Robaglia, C.; Rostoks, N.; Sweet, J.; Tebbe, C.; Visioli, F.; Wal, J.; Eigenmann, P.; Epstein, M.; Hoffmann-Sommergruber, K.; Koning, F.; Lovik, M.; Mills, C.; Moreno, F. J.; van Loveren, H.; Selb, R.; Fernandez Dumont, A. Guidance on Allergenicity Assessment of Genetically Modified Plants. *EFSA J.* **2017**, *15* (6) DOI: 10.2903/j.efsa.2017.4862.
- (261) U.S. Food and Drug Administration. Assay Development and Validation for Immunogenicity Testing of Therapeutic Protein Products - Guidance for Industry. 2016.
- (262) U.S. Food and Drug Administration. Guidance for Industry: Immunogenicity- Related Considerations for Low Molecular Weight Heparin Guidance for Industry. 2016.
- (263) Bryce, P. J. Balancing Tolerance or Allergy to Food Proteins. *Trends Immunol.* **2016**, *37* (10), 659–667 DOI: 10.1016/j.it.2016.08.008.
- (264) Vojdani, A. Lectins, Agglutinins, and Their Roles in Autoimmune Reactivities. *Altern. Ther. Health Med.* **2015**, *21 Suppl 1*, 46–51.
- (265) P, A. H. An Overview of Immunologic Adjuvants - A Review. *J. Vaccines Vaccin.* **2012**, *04* (01), 1–4 DOI: 10.4172/2157-7560.1000167.
- (266) Aucouturier, J.; Dupuis, L.; Ganne, V. Adjuvants Designed for Veterinary and Human Vaccines. *Vaccine* **2001**, *19* (17–19), 2666–2672 DOI: 10.1016/S0264-410X(00)00498-9.
- (267) Batista-Duharte, A.; Lindblad, E. B.; Oviedo-Orta, E. Progress in Understanding Adjuvant Immunotoxicity Mechanisms. *Toxicol. Lett.* **2011**, *203* (2), 97–105 DOI: 10.1016/j.toxlet.2011.03.001.
- (268) McSorley, S. J.; Ehst, B. D.; Yu, Y.; Gewirtz, A. T. Bacterial Flagellin Is an Effective Adjuvant for CD4+ T Cells In Vivo. *J. Immunol.* **2002**, *169* (7), 3914–3919 DOI: 10.4049/jimmunol.169.7.3914.
- (269) Ebrahimi, S. M.; Tebianian, M. Role of Mycobacterial Heat Shock Protein 70 (MHSP70) as Genetic Vaccine Adjuvants. *World Appl. Sci. J.* **2011**, *14* (10), 1569–1575.
- (270) Grohmann, U.; Bronte, V. Control of Immune Response by Amino Acid Metabolism. *Immunol. Rev.* **2010**, *236*, 243–264 DOI: 10.1111/j.1600-065X.2010.00915.x; 10.1111/j.1600-065X.2010.00915.x.
- (271) Marrack, P.; Scott-Browne, J.; MacLeod, M. K. L. Terminating the Immune Response. *Immunol. Rev.* **2010**, *236* (1), 5–10 DOI: 10.1111/j.1600-065X.2010.00928.x.
- (272) Nimmerjahn, F.; Ravetch, J. V. Antibody-Mediated Modulation of Immune Responses. *Immunol. Rev.* **2010**, *236*, 265–275 DOI: 10.1111/j.1600-065X.2010.00910.x.
- (273) Scientific Opinion on the Evaluation of Allergenic Foods and Food Ingredients for Labelling Purposes. *EFSA J.* **2014**, *12* (11), 3894 DOI: 10.2903/j.efsa.2014.3894.
- (274) Bryce, P. J. Balancing Tolerance or Allergy to Food Proteins. *Trends Immunol.* **2016**, *37* (10), 659–667 DOI: 10.1016/j.it.2016.08.008.
- (275) U.S. Food and Drug Administration. Guidance for Industry: Immunogenicity Assessment for Therapeutic Protein Products [Excerpts]. *Biotechnol. Law Rep.* **2013**, *32* (3), 172–185 DOI: 10.1089/blr.2013.9927.
- (276) Verhoeckx, K.; Broekman, H.; Knulst, A.; Houben, G. Allergenicity Assessment Strategy for

- Novel Food Proteins and Protein Sources. *Regul. Toxicol. Pharmacol.* **2016**, *79*, 118–124 DOI: 10.1016/j.yrtph.2016.03.016.
- (277) Brehm, M. A.; Cuthbert, A.; Yang, C.; Miller, D. M.; DiIorio, P.; Laning, J.; Burzenski, L.; Gott, B.; Foreman, O.; Kavirayani, A.; Herlihy, M.; Rossini, A. A.; Shultz, L. D.; Greiner, D. L. Parameters for Establishing Humanized Mouse Models to Study Human Immunity: Analysis of Human Hematopoietic Stem Cell Engraftment in Three Immunodeficient Strains of Mice Bearing the IL2rynull Mutation. *Clin. Immunol.* **2010**, *135* (1), 84–98 DOI: 10.1016/j.clim.2009.12.008.
- (278) Matzinger, P. The Evolution of the Danger Theory. *Expert Rev. Clin. Immunol.* **2012**, *8* (4), 311–317 DOI: 10.1586/eci.12.21.
- (279) Matzinger, P. Tolerance, Danger, and the Extended Family. *Annu. Rev. Immunol.* **1994**, *12* (1), 991–1045 DOI: 10.1146/annurev.iy.12.040194.005015.
- (280) Vance, R. E. Cutting Edge Commentary: A Copernican Revolution? Doubts About the Danger Theory. *J. Immunol.* **2000**, *165* (4), 1725–1728 DOI: 10.4049/jimmunol.165.4.1725.
- (281) Vance, R. E. Cutting Edge Commentary: A Copernican Revolution? Doubts About the Danger Theory. *J. Immunol.* **2000**, *165* (4), 1725–1728 DOI: 10.4049/jimmunol.165.4.1725.
- (282) Netea, M. G.; Quintin, J.; Van Der Meer, J. W. M. Trained Immunity: A Memory for Innate Host Defense. *Cell Host Microbe* **2011**, *9* (5), 355–361 DOI: 10.1016/j.chom.2011.04.006.
- (283) Netea, M. G.; Joosten, L. A. B.; Latz, E.; Mills, K. H. G.; Natoli, G.; Stunnenberg, H. G.; O'Neill, L. A. J.; Xavier, R. J. Trained Immunity: A Program of Innate Immune Memory in Health and Disease. *Science (80-.)*. **2016**, *352* (6284), aaf1098–aaf1098 DOI: 10.1126/science.aaf1098.
- (284) Töpfer, E.; Boraschi, D.; Italiani, P. Innate Immune Memory: The Latest Frontier of Adjuvanticity. *J. Immunol. Res.* **2015**, *2015*, 1–7 DOI: 10.1155/2015/478408.
- (285) Franceschi, C.; Salvioli, S.; Garagnani, P.; de Eguileor, M.; Monti, D.; Capri, M. Immunobiography and the Heterogeneity of Immune Responses in the Elderly: A Focus on Inflammaging and Trained Immunity. *Front. Immunol.* **2017**, *8* DOI: 10.3389/fimmu.2017.00982.
- (286) Grignolio, A.; Mishto, M.; Caetano Faria, A. M.; Garagnani, P.; Franceschi, C.; Tieri, P. Towards a Liquid Self: How Time, Geography, and Life Experiences Reshape the Biological Identity. *Front. Immunol.* **2014**, *5* (APR), 1–17 DOI: 10.3389/fimmu.2014.00153.
- (287) Meyer, U.; Feldon, J.; Dammann, O. Schizophrenia and Autism: Both Shared and Disorder-Specific Pathogenesis via Perinatal Inflammation? *Pediatr. Res.* **2011**, *69* (5 PART 2), 26–33 DOI: 10.1203/PDR.0b013e318212c196.
- (288) Huurre, A.; Kalliomäki, M.; Rautava, S.; Rinne, M.; Salminen, S.; Isolauri, E. Mode of Delivery - Effects on Gut Microbiota and Humoral Immunity. *Neonatology* **2008**, *93* (4), 236–240 DOI: 10.1159/000111102.
- (289) Franceschi, C.; Bonafè, M.; Valensin, S.; Olivieri, F.; De Luca, M.; Ottaviani, E.; De Benedictis, G. Inflamm-Aging. An Evolutionary Perspective on Immunosenescence. *Ann. N. Y. Acad. Sci.* **2000**, *908* (1), 244–254 DOI: 10.1111/j.1749-6632.2000.tb06651.x.
- (290) Franceschi, C.; Grignolio, A. Immunosenescence within an Evolutionary Perspective. In *IMMUNOLOGY TODAY Three Historical Perspectives under Three Theoretical Horizons*; Bononia Univeristy Press: Bologna, 2010; pp 79–99.

- (291) Santoro, A.; Ostan, R.; Candela, M.; Biagi, E.; Brigidi, P.; Capri, M.; Franceschi, C. Gut Microbiota Changes in the Extreme Decades of Human Life: A Focus on Centenarians. *Cell. Mol. Life Sci.* **2018**, *75* (1), 129–148 DOI: 10.1007/s00018-017-2674-y.
- (292) Mishto, M.; Liepe, J. Post-Translational Peptide Splicing and T Cell Responses. *Trends Immunol.* **2017**, *38* (12), 904–915 DOI: 10.1016/j.it.2017.07.011.
- (293) Giuliani, C.; Garagnani, P.; Franceschi, C. Genetics of Human Longevity Within an Eco-Evolutionary Nature-Nurture Framework. *Circ. Res.* **2018**, *123* (7), 745–772 DOI: 10.1161/CIRCRESAHA.118.312562.
- (294) Garagnani, P.; Bacalini, M. G.; Pirazzini, C.; Gori, D.; Giuliani, C.; Mari, D.; Di Blasio, A. M.; Gentilini, D.; Vitale, G.; Collino, S.; Rezzi, S.; Castellani, G.; Capri, M.; Salvioli, S.; Franceschi, C. Methylation of ELOVL2 Gene as a New Epigenetic Marker of Age. *Aging Cell* **2012**, *11* (6), 1132–1134 DOI: 10.1111/accel.12005.
- (295) Horvath, S.; Garagnani, P.; Bacalini, M. G.; Pirazzini, C.; Salvioli, S.; Gentilini, D.; Di Blasio, A. M.; Giuliani, C.; Tung, S.; Vinters, H. V.; Franceschi, C. Accelerated Epigenetic Aging in Down Syndrome. *Aging Cell* **2015**, *14* (3), 491–495 DOI: 10.1111/accel.12325.
- (296) Horvath, S.; Pirazzini, C.; Bacalini, M. G.; Gentilini, D.; Di Blasio, A. M.; Delledonne, M.; Mari, D.; Arosio, B.; Monti, D.; Passarino, G.; De Rango, F.; D'Aquila, P.; Giuliani, C.; Marasco, E.; Collino, S.; Descombes, P.; Garagnani, P.; Franceschi, C. Decreased Epigenetic Age of PBMCs from Italian Semi-Supercentenarians and Their Offspring. *Aging (Albany, NY)*. **2015**, *7* (12), 1159–1170 DOI: 10.18632/aging.100861.
- (297) Vanhooren, V.; Desmyter, L.; Liu, X.-E.; Cardelli, M.; Franceschi, C.; Federico, A.; Libert, C.; Laroy, W.; Dewaele, S.; Contreras, R.; Chen, C. N-Glycomic Changes in Serum Proteins During Human Aging. *Rejuvenation Res.* **2007**, *10* (4), 521–531a DOI: 10.1089/rej.2007.0556.
- (298) Bürkle, A.; Moreno-Villanueva, M.; Bernhard, J.; Blasco, M.; Zondag, G.; Hoeijmakers, J. H. J.; Toussaint, O.; Grubeck-Loebenstein, B.; Mocchegiani, E.; Collino, S.; Gonos, E. S.; Sikora, E.; Gradinaru, D.; Dollé, M.; Salmon, M.; Kristensen, P.; Griffiths, H. R.; Libert, C.; Grune, T.; Breusing, N.; Simm, A.; Franceschi, C.; Capri, M.; Talbot, D.; Caiafa, P.; Friguet, B.; Slagboom, P. E.; Hervonen, A.; Hurme, M.; Aspinall, R. MARK-AGE Biomarkers of Ageing. *Mech. Ageing Dev.* **2015**, *151*, 2–12 DOI: 10.1016/j.mad.2015.03.006.
- (299) Zeevi, D.; Korem, T.; Zmora, N.; Israeli, D.; Rothschild, D.; Weinberger, A.; Ben-Yacov, O.; Lador, D.; Avnit-Sagi, T.; Lotan-Pompan, M.; Suez, J.; Mahdi, J. A.; Matot, E.; Malka, G.; Kosower, N.; Rein, M.; Zilberman-Schapira, G.; Dohnalová, L.; Pevsner-Fischer, M.; Bikovsky, R.; Halpern, Z.; Elinav, E.; Segal, E. Personalized Nutrition by Prediction of Glycemic Responses. *Cell* **2015**, *163* (5), 1079–1095 DOI: 10.1016/j.cell.2015.11.001.

Glossary and Abbreviations

Glossary:

- *Adaptive memory*: Adaptive memory is long-term, antigen-specific ability of T and B lymphocytes to respond more rapidly and more efficiently to a specific antigen upon second encounter.
- *Adjuvanticity*: the ability to modify the immune response, being adjuvants substances that, when co-administered with an antigen increase the immune response to the antigen and therefore might increase the allergic response. Adjuvant immunobiological functions:
 - i) improve the immunogenicity of highly purified or recombinant antigens (protein or peptide);
 - ii) increase the innate immune response to antigen by interacting with pattern recognition receptors (PRRs) on or within accessory cells;
 - iii) provide physical protection to antigens which grants the antigen a prolonged delivery;
 - iv) increase the capacity to cause local reactions at the injection site (e.g. during vaccination), inducing greater release of danger signals by chemokine releasing cells such as helper T cells and mast cells;
 - v) help in the translocation of antigens to the lymph nodes where they can be recognized by T cells.
- *Allergenicity*: The ability of an antigen to induce an abnormal immune response, which is an overreaction and different from a normal immune response in that it does not result in a protective/prophylaxis effect but instead causes physiological function disorder or tissue damage
- *Antigenicity*: The ability of an antigen to induce an immunological response when it is encountered by the human body. Antigenicity involves two types of immune characteristics, immunogenicity and allergenicity
- *Epitope*: All parts of an antigen that can be recognized and bind to either immune cells (e.g., T cells and B cells), free molecules (e.g., antibodies), or cell-surface proteins [e.g., major histocompatibility complex (MHC)]
- *Immunogenicity*: Immunogenicity refers to the ability of an antigen to trigger normal and protective immune responses after being encountered by the human body. In particular, we describe the immunogenicity of an antigen using the following three aspects:
 - i) the ability to defend the immune system (immunological defense), which is the ability to repel an exogenous antigen and to fight against infection;
 - ii) the ability to keep the immune system stable (immunological homeostasis), which is the ability of the body to recognize and eliminate damaged tissue, inflammation and/or senescent cells, and
 - iii) the ability to kill and to remove abnormally mutated cells so as to monitor and inhibit the growth of malignancies in the body (immunological surveillance).
- *Immunological biography*: The individual immunological history encompassing the capacity to take into account both the qualitative as well as the quantitative and the temporal aspects of the immunological stimuli that each individual – as an accumulator and elaborator of antigenic stimuli – is exposed to in our lifelong history.
- *Innate memory*: Innate memory is the ability of an organism to adapt its immune response depending on a previous infections or vaccination, mediated by NK cells and monocytes/macrophages. This immunological re-programming can result in non-specific

suppression (tolerance) or increased innate immune response (training) against reinfection by the same or different pathogens.

- *Nonspecific effects*: “Nonspecific” immune effects are induced by a vaccination or infection, against unrelated and antigenically diverse infectious agents. Nonspecific effects are mediated by cross-reactive lymphocytes and innate memory cells, and might be either beneficial or detrimental, depending on the type of memory of the cells involved.
- *Trained immunity/memory*: Trained immunity/memory is the enhanced nonspecific protection against infections after previous exposure to certain microbial components (e.g., β -glucans), possibly involving epigenetic and metabolic re-programming in the cell.
- *Tolerance*: Tolerance is the refractory state of monocytes/macrophages, involving epigenetic remodelling, induced by microbial components (e.g., LPS). Upon subsequent challenge, even with a high dose of LPS, a less robust induction of pro-inflammatory cytokines ensues.
- *Liquid self*: According to Grignolio *et al.*²⁸⁶, the full integration of the immune response mechanisms into the host body’s ecosystems, i.e., in adding the temporal, as well as the geographical/evolutionary and environmental dimensions.
- *Self*: According to Burnet, who introduced the term in 1949, the immunological self, coincided with all the cell surface pattern recognitions that are ignored by the normal immune defensive action, namely the biological constituents peculiar to each individual. Since Burnet, there have been roughly a dozen different attempts to find an answer, but none has found a general acceptance. They can be grouped in six major answers as follows. The self is:
 - i) everything encoded by the genome;
 - ii) any tissue under the skin accessible to lymphocytes, including structures encoded by commensal genomes or excluding immune “privileged” sites such as brain, cornea, and testes;
 - iii) the set of peptides complexed with the MHC;
 - iv) specifics like APCs and thymic epithelium or soluble molecules of B lymphocytes;
 - v) a set of bodily proteins that exist above a certain concentration;
 - vi) the immune network itself, variously conceived.

Abbreviations:

AI	Artificial intelligence
APC	Antigen-presenting cell
Bt	<i>Bacillus thuringiensis</i>
FAO	Food and Agriculture Organization of the United Nations
Food	The term food it is meant for food and feed
HDP	Host defence peptide
HLA	Human Histocompatibility Complex
IS	Immune System
LPS	Lipopolisaccharide
MHC	Major Histocompatibility Complex
PCPS	Proteasome-catalyzed peptide splicing

QC	Quality Control
URL	Uniform Resource Locator
WHO	World Health Organization
WoS	Web of Science