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Review

Approaches to assess IgE mediated allergy risks (sensitization and cross-reactivity) from new or modified dietary proteins

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ABSTRACT

The development and introduction of new dietary protein sources has the potential to improve food supply sustainability. Understanding the potential allergenicity of these new or modified proteins is crucial to ensure protection of public health. Exposure to new proteins may result in *de novo* sensitization, with or without clinical allergy, or clinical reactions through cross-reactivity.

In this paper we review the potential of current methodologies (*in silico*, *in vitro* degradation, *in vitro* IgE binding, animal models and clinical studies) to address these outcomes for risk assessment purposes for new proteins, and especially to identify and characterise the risk of sensitization for IgE mediated allergy from oral exposure. Existing tools and tests are capable of assessing potential crossreactivity. However, there are few possibilities to assess the hazard due to *de novo* sensitization. The only methods available are *in vivo* models, but many limitations exist to use them for assessing risk. We conclude that there is a need to understand which criteria adequately define allergenicity for risk assessment purposes, and from these criteria develop a more suitable battery of tests to distinguish between proteins of high and low allergenicity, which can then be applied to assess new proteins with unknown risks.

1. Introduction

Proteins from new or alternative sources could strongly improve the sustainability of food protein supply. This can be achieved via different ways, e.g. through development of new protein sources, improvement of crops, by providing solutions to technical challenges during manufacturing, by providing new nutritional sources, as well as by valorising unused side products. The EU General Food Law requires that consumers have access to safe and wholesome food of the highest standard (Regulation EC no 178/2002). Before introducing new or modified foods or food ingredients into the market, safety assessments must be performed to determine that the new product will not result in harm to the consumer and to protect public health. Food allergy is a relatively frequent disease in humans and, when it occurs, dietary proteins are usually the major contributor for the development and elicitation of allergic reactions.

Methodologies and principles of risk assessment in food safety have developed and become harmonized to a large extent worldwide over the past half century. The risks addressed are mainly those posed by chemical, microbiological and physical hazards. Food allergy was a latecomer to the field of food safety hazards and real progress in the development of methods ensuring consumer protection is of rather

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recent date. In the first decade of this century, knowledge has accumulated on the sensitivity of food allergic individuals from observed thresholds during clinical food challenges, leading to the development of methods for assessing the risk to food allergic consumers from oral exposure to *known* allergenic food proteins already present in the diet (Kruizinga et al., 2008; Spanjersberg et al., 2007, 2010). Quantitative risk assessment methodologies are now available for assessing risks regarding the unintentional consumption of regulated (EC 1169/2011) major food allergens (Crevel et al., 2014; Remington et al., 2015). Similar methods may also be applied to assessing potential risks due to cross-reactivity between new or modified proteins and known allergens in persons with manifest food allergies (Verhoeckx et al., 2016). However, new or modified proteins can also pose a risk of *de novo* sensitization leading to the development of new food allergies.

This paper aims to discuss how current methodology can identify potential risks to consumer health from *de novo* IgE mediated sensitization or from a potential clinical manifestation of allergy provoked by new or modified food proteins. These include foods derived from genetic modifications or DNA recombinant technology (GMs), and Novel Foods as regulated in Europe by EC 1829/2003, complemented with EC 503/2013, and by EC 2015/2283, replacing the original EC 258/97, respectively. We discuss how existing model systems, assays and other methodologies contribute to the risk analysis process. The focus is on IgE mediated allergy.

2. Allergenicity risk assessment

Risk is defined as a function of the probability of an adverse health effect and the severity of that effect, consequential to a hazard(s) in food (CODEX, 2013). For the purposes of the current discussion, the hazard is the potential of a material to induce an IgE-mediated immune response (to be an allergen) with the adverse health outcome manifested as sensitization or as development of a clinical allergy. There are two phases in food allergy - the induction (sensitization) phase and the effector (elicitation) phase, for which separate risk assessments might be applicable. Assessment in the elicitation phase will mainly address the probability of eliciting an allergic (cross-) reaction and the severity of that reaction upon exposure to a defined amount of allergenic protein. The sensitization phase consists of priming the immune system, leading to the formation of specific IgE antibodies. The presence of IgE antibodies is a prerequisite, but is not the only requirement for the development of clinical allergy. There is no clear relationship between the intensity and degree of sensitization and its progression into clinical allergy. The probability of induction of sensitization is therefore higher than the risk of development of allergy. Taking sensitization as the hazard metric will inevitably make the assessment more conservative, and over-protective than choosing the development of allergy.

Most model systems and assays developed with the aim of investigating the allergenicity of new or modified food proteins focus on hazard assessment. They were mostly developed based on the characteristics of known allergens. It is unknown if these methodologies would also be predictive for completely new proteins or foods which have not previously been encountered.

By definition, the risk due to an identified hazard depends on the quantity of the hazard needed to cause adverse effects and on the actual level and/or pattern of exposure to that hazard. Although the impact of exposure for the elicitation phase has been explored and methods for quantitative risk assessment for *known* allergens have been developed, knowledge on how exposure influences the development of the sensitization phase is limited. In theory, all foods or proteins newly introduced into the food chain can cause *de novo* sensitization (Houben et al., 2016). Hazard is the intrinsic property and potency to induce *de novo* sensitization, modulated by the genetic disposition of the consuming individual. Exposure depends on many variables including: the amount consumed, the frequency and pattern of consumption, the concentration and stability of the protein in the food, co-exposure to adjuvants and the matrix when prepared to be consumed. For example, the expression levels of proteins currently introduced into GM-crops for e.g. pesticide resistance are typically very low, and once incorporated into food products their presence, and hence exposure, is practically negligible. Such low exposure, together with an absence of measurable allergenic potential presents a very low risk of allergenicity from such commercialized GM crops.

For protein sources intended for nutritional purposes, the quantities ingested will be much more significant. There are examples where the introduction of a new or novel proteinaceous food into the food chain has been reported (however rarely) to cause allergic reactions. Hoff et al. (2003) described an asthmatic patient who experienced an allergic reaction to quorn, a brand name for foods containing mycoprotein from the mold *Fusarium venenatum*. This was confirmed by double-blind placebo-controlled oral challenge and the authors concluded that this was not due to a primary sensitization, but due to cross-reactivity with aero-allergens. Lupin was introduced into the food chain in the late nineties in the EU (Peeters et al., 2007). According to Hieta et al. (2009), allergic reactions to lupin occurred most frequently among patients with other food allergies, mainly to legumes, indicating that lupin allergy occurs largely due to cross-reactivity. However, others showed that lupin allergy is not always due to cross-reactivity, but can be the result of primary sensitization through oral exposure (Lindvik et al., 2008; Peeters et al., 2007), and also by prior inhalation of lupin flour (Prieto et al., 2010). Similar to lupin, kiwi fruit was introduced to the EU in the 20th century and soon after kiwi allergy was first reported (Fine, 1981). Upon further investigation, kiwi allergy can result from cross-reactivity with pollen and latex (Diaz-Perales et al., 1999; Gall et al., 1994) or through primary sensitization (Alemán et al., 2004).

As discussed above, exposure to a protein may result in *de novo* sensitization or allergy, or allergic reactions through cross-reactivity. Definition of the relevant risk: e.g. the probability of sensitization, the probability of allergy development, the risk of allergic reactions in the allergic and in the general population, etc., will be crucial when developing future methodologies and guidance.

3. Current guidance

Expert scientific bodies have produced guidance for allergenicity assessment of new proteins and food sources that are introduced into the human diet (CODEX, 2003, 2009; EFSA, 2010, 2011; FAO/WHO, 2001). Table 1 summarises data requirements. Key guidance documents on how to assess potential allergenicity were first drawn up to address proteins introduced using recombinant DNA (GM-)techniques (CODEX, 2003), which can be considered as the basic standard, with amended improvements as suggested by the advancement of scientific knowledge and methodologies. As no single property is recognized as predicting food allergy in humans, all documents recommend a weight of evidence approach. This approach takes into account various elements and observations judged to be important to the potential of a protein to cause an allergic reaction, and focusses on knowledge gained from the investigation of known food allergens. Common key elements in all published guidance are sequence homology, susceptibility to enzymatic degradation by digestive enzymes (pepsin), and specific IgE binding. For particular situations, further data, including *in vivo* studies (e.g. human), might be considered. The endpoint of the assessment is a conclusion about the likelihood of the GM-derived protein being an allergen (CODEX, 2003). This approach is also applicable to the assessment of added food enzymes (EFSA, 2014a).

The approaches developed for GM foods have also been used to assess the potential of new non-GM proteins or protein-enriched food products introduced onto the market for their potential to cause allergic reactions (Meredith, 2005; Poulsen, 2004; Putten et al., 2011) (see examples in the Annex). In the EU, the scientific guidance documents for GM foods have formally evolved through EFSA, and only recently has a separate EFSA panel developed specific guidance for non-GM

Table 1
Key elements for allergenicity assessment of new foods from existing guidance documents.

Element	GM Foods: transgenic proteins	Non-GM Foods (single proteins, extracts, whole foods)
Background	History of exposure and safety of gene product and sources	Comprehensive literature review Food composition, particularly its protein(s), its source, the production process, and available experimental and human allergenicity data. Case reports of allergic reactions and/or allergenicity studies (*in vitro*, in animals, in humans)
Protein analysis	*In silico* amino acid sequence comparisons to known allergens Physico-chemical properties e.g. resistance to pepsin degradation *in vitro*	Protein Content (ACNFP, 2011) (Total, specific) Degree of amino acid sequence homology with known allergens Immunological tests (e.g. Western blotting with human sera) Molecular weight of the potentially allergenic protein, heat stability, sensitivity to pH, digestibility by gastrointestinal proteases
Human testing	Specific IgE binding studies using well-characterised sera from individuals allergic to the identified source or skin-prick testing with relevant subjects	Detection of specific IgE antibodies Skin prick testing Double blind placebo controlled food challenge studies
Other possibilities	Animal models, *in vitro* biological assays (EFSA, 2014b) Inclusion of the expression of endogenous allergens into the comparative compositional analysis of the GM plant and its appropriate comparator (EFSA, 2010, 2011, 2017) Post-launch monitoring	Demonstration (characterisation, manufacturing process, literature, human data, animal data, *in vitro* data) that derivatives of foods considered as allergens are unlikely to trigger adverse reactions and are exempt from labelling (EFSA, 2013). Post-launch monitoring

novel foods. Improved coordination both within and between national and international agencies is needed to advance future assessments. Potential allergenicity of GM-expressed proteins is initially assessed by amino acid sequence comparisons to known allergens, which may trigger further investigation. This approach can also be applied to single, defined non-GM proteins, although may be less appropriate for complex novel non-GM foods because there is no specific transgene product to compare. If the novel food is unrelated to any other species containing known major allergenic proteins or is a complex food mix from a little-known source an investigation into the phylogenetic relationship with characterised food commodities could inform about the necessity for any further work.

Three examples of novel ingredients evaluated by EFSA under the Novel Foods Regulation 258/97/EC (European Commission, 1997) demonstrate a lack of consistency in the criteria applied for allergenicity assessment (Annex 1). Briefly, the use of 'Ice Structuring Protein (ISP) type III HPLC12 preparation' as food ingredient was deemed to be safe after extensive investigation, including a human oral immunogenicity study, as the protein was originally isolated from ocean pout. Fish belong to the regulated major allergens (EFSA, 2008). Rapeseed protein isolate was assessed as "safe under the proposed conditions of use" and approved despite the conclusion that a risk of sensitization to rapeseed, as well as the risk of cross-reactivity in subjects allergic to mustard, cannot be excluded. Interestingly, the Opinion does not discuss the potential public health impact of the introduction of a new allergenic substance into the diet. Rapeseed protein as a novel food ingredient was authorised to be placed on the market (L 196/27) (EFSA, 2013) provided that the labelling of foods containing the ingredient is devised in such a way that people with mustard allergy are able to avoid consumption of those foods. In 2005, EFSA rejected a dossier on the use of chia as a food ingredient with the justification that it, among other things, lacked information with regard to the potential allergenicity of chia (seeds and ground) (EFSA, 2005). In 2009, the dossier was resubmitted containing an additional bibliographic search for cases of allergy to chia. This was deemed sufficient to approve the chia seeds with no restrictions for potential allergenicity (EFSA, 2009). These three examples highlight that a more harmonized guidance on allergenicity assessment of protein-containing novel foods (proteins, protein isolates, or protein-rich foods) is needed, as well as greater clarity on the criteria used by the Authority to reach its conclusions.

4. Background information: history of (safe) use

Consideration of the 'history of use' (Constable et al., 2007) of a protein source, or a traditional food, is useful to provide important background information on how to develop a strategy for assessing the potential to elicit an allergic reaction, and to provide clues for optimal risk management. A history of safe use describes the existing safety profile including any known health effects, patterns of use, processing properties and protein characteristics. Similarly, the first step to estimate the potential of a single protein to cause an allergic reaction is to consider its origin, extent and mode of previous exposure and to assess the familiarity of its use. For a protein introduced into a GM crop, an understanding of the potential allergenic profile of the donor organism from which the gene is derived can provide guidance for further investigations, although for all practical purposes, proteins from sources with known IgE mediated allergy are unlikely to be considered to be brought forward into the market place.

Importantly, this knowledge (see Table 2) cannot determine if a novel protein has the potential for *de novo* sensitization via ingestion, unless allergy has been demonstrated under other circumstances (e.g. inhalation). It can however be used as a starting point and indication if there is a possibility of cross-reactivity.

5. Protein-centred investigations

5.1. Bioinformatics approaches

Based on the current guidelines, a novel protein can be considered as a putative allergen if it shares greater than 35% sequence identity with a known allergen over a sliding window of at least 80 amino acids (CODEX, 2009). There is however little solid scientific basis for a general application of this rule, which has not been formally validated, as far as we know. The conservatism of the greater than 35% identity over 80 amino acids approaches and applicability to all protein families has been questioned (Herman et al., 2015; Silvanovich et al., 2009). The 80 amino acid sliding window approach is thought to be selected to correspond to the typical size of a protein domain containing IgE epitopes (Herman et al., 2009). The threshold of 35% was intended to identify proteins that share similar functions, since many common plant allergens fall within a few functional categories (Taylor, 2002). However, the 35% threshold is considered as conservative, as cross-reactivity usually requires more than 50–70% identity in the antibody-binding region of interest (Aalberse, 2000). Two of the most widely used tools for sequence alignment are FASTA (Pearson and Lipman, 1988) and BLAST (Altschul et al., 1990) that use certain weighting parameters to assess the significance of sequence alignments (Ladics et al., 2011). The E-score (FASTA) and E-value (BLAST) represent the probability that the alignment might occur by chance. The calculation of E-score incorporates parameters such as amino acid identity (i.e.,

Table 2
Information relating to source organisms/whole foods and single proteins (or Description of history of safe use).

Whole foods/Source Organism		Single Proteins	
Element	Considerations	Element	Consideration
Characterisation and Identity	Does it belong to a food group known to be allergenic? Are some varieties more/less allergenic than others? Is the allergen known?	Source organism	Can a history of safe use be described?
Details of use	Is the food cooked or eaten raw? Are certain parts of the plant avoided? Is the food an extract (e.g. oil, flour? Is protein present as consumed?) What is the impact of processing?	Protein familiarity	Sequence homologies to other proteins within the same protein family OR with other protein families OR known allergens? Is the protein well conserved (sequence & structure & function)?
Human Exposure Pattern	Timing and frequency of consumption? Geographical/cultural/environmental influences? Staple food or minor ingredient?	Dietary Exposure	Is the protein abundant in nature – is there significant consumption of the protein (or highly related proteins)?
Known Health Effects	What is the level of scientific evidence for elicitating allergic reactions? Severity? Anecdotal reports or confirmed by clinical investigation?	Processing	Impact of digestive enzymes and/or temperature on protein stability. Is the protein easily processed; no undesired products generated

extent to which two amino acids are invariant), amino acid similarity (i.e., the extent to which different amino acids may share biophysical features, such as molecular charge state), gaps in the alignment, length of the alignment, scoring matrix (which applies scoring values for aligning two amino acids together), and size of the database. It should be noted that the developers of the FASTA software tool provide specific guidance on their intended and correct usage of the software. Pearson (2016) states "*In evaluating the search results, the expectation or E-value is the most reliable and sensitive indicator of likely sequence homology. For protein:protein alignments, if the E-value is less than* 10^{-6}, *the sequences are almost certainly homologous*." Alignment of the whole sequence of the novel protein and allergens in the database, without the restriction of the length of the sliding window has been proposed as a relevant alternative approach (Herman et al., 2015; Silvanovich et al., 2009). However, although the literature referenced above suggests a greater scientific basis for E-score than for the greater than 35% identity/80 AA sliding window (Herman et al., 2015; Silvanovich et al., 2009), more detailed studies concerning the relevance of using E-score (E-value) for predicting allergenic cross-reactivity would be of benefit. One possibility would be hypothesis-driven investigations with relevant human serum samples.

A full and detailed *in silico* search for allergen IgE cross-reactivity requires a comprehensive, well-curated allergen database. The allergen database most often used currently in the assessment of novel proteins is the Allergen Online database from the Food Allergy Research and Resource Program (FARRP; www.allergenonline.org). It is updated annually and curated based on predefined criteria (Goodman et al., 2016). On the same concept, a new database called COMPARE is being built by the Health and Environment Science Institute (HESI) and aims to be a publicly accessible and transparent resource of allergens (HESI, 2016). Other allergen repositories exist, with large variability in the number of allergens listed and the information available (Gendel, 2009; Schein et al., 2007), e.g. Allergome, IUIS. Interestingly, some of them contain information or links to information on 3D structure or structural domains on the allergen (e.g. the Allergen Database for Food Safety, http://allergen.nihs.go.jp/ADFS/), whereas others include identified IgE-epitopes and/or functional motifs (e.g. the Structural Database of Allergenic Proteins, SDAP; http://fermi.utmb.edu/SDAP/sdap_ver.html). Other databases, such as AllFam, classify allergens into protein families (http://www.meduniwien.ac.at/allfam/). Unfortunately, not all of these online databases are regularly updated. In addition, the data are generally not in a detailed format that is easy to incorporate with other databases/methods (Ivanciuc et al., 2011). Moreover, information regarding 3D structure and IgE-epitopes is limited for a large number of allergens.

Beyond these approaches based on the Codex Alimentarius guidelines (CODEX, 2009), several other methods for cross-reactivity prediction exist. These include alignment-free methods based on the main physicochemical properties of proteins (Dimitrov et al., 2014), detection based on filtered length-adjusted allergen peptides (Martinez Barrio et al., 2007), pairwise sequence similarity vectorization (Muh et al., 2009) or amino acid and dipeptide composition of proteins (Saha and Raghava, 2006). EFSA recommends to quantify sensitivity and specificity to evaluate the performance of bioinformatics methods (EFSA, 2010). However, such performance assessments require at least clear definitions of positives and negatives, applicable to all methods. The available bioinformatics approaches have different methodologies, use diverse positive and negative sets of control proteins and have widely varying validation procedures due to the lack of conventional criteria for non-allergenic proteins. All these factors hinder the performance evaluation and efficiency comparison of the existing bioinformatics approaches and the development of more accurate methods for allergenicity prediction. However, one of the fundamental problems is that there are currently no defined structural characteristics distinguishing allergens from non-allergens.

In conclusion, existing bioinformatics approaches can aid to identify potential cross-reactivity of a protein new to the diet with known allergens. When a significant primary sequence alignment is obtained *in silico*, it is interpreted as a possibility that the novel protein could be recognized by IgE in consumers with the corresponding allergy. In this case, the next step in the allergenicity assessment requires the *in vitro* testing of the novel protein with patient serum for verification of the cross-reactivity. In practice, it is more likely that the protein will not be used in a commercialized product.

The current approaches take into account only potential similarities of the introduced protein with known allergens regarding the primary structures, but not the conformational epitopes. Investigations to identify allergenic 3D motifs and development of algorithms for structure prediction and comparison (e.g.Iterative Threading Assembly Refinement (I-TASSER; http://zhanglab.ccmb.med.umich.edu/I-TASSER/)) is needed to understand if such information would be relevant to predict IgE cross reactivity.

In the context of this present discussion, a major limitation of the current *in silico* methods is that they cannot predict *de novo* sensitization and cannot differentiate between sensitization and elicitation of allergy. Progress will require methods that exploit an understanding of the molecular mechanisms of allergy to define adverse outcome pathways (AOP). Once knowledge about the routes of entry, the pathways to

antigen-presenting cells, the mechanisms of allergen presentation and recognition by T and B cells is improved, it could be used to facilitate the search for peptide fragments that characterise novel proteins with *de novo* sensitizing and/or allergenic potential.

5.2. Enzymatic degradation assays

The evaluation of the resistance of recombinant proteins to degradation by digestive enzymes is a key part of the current allergenicity assessment of GM crops (CODEX, 2009; EFSA, 2011; Goodman et al., 2008). This test is based on the postulate and preliminary observations that resistance to gastric digestion differed between two sets of proteins derived from foods: commonly allergenic and rarely allergenic (Astwood et al., 1996). Thus such resistance might be an intrinsic feature of allergens and therefore, a new protein that is resistant to gastric digestion (or is partially degraded into stable fragments of sufficient size) has the potential to interact with the immune system, whilst a protein that is rapidly and completely degraded is unlikely to interact and evoke an immune response. Degradation of the protein will likely influence the effective dose and severity of reaction in the elicitation phases. However, for sensitization purposes, the issue is rather what type of immune response is triggered by a specific protein, and how that is influenced by enzymatic degradation. Tolerance is also an active immune response, and requires the protein to interact with the immune system. Local mucosal IgA and IgG responses to food proteins are common. For sensitization, avoidance of interaction with the immune system may not be one of the most important criteria, but rather what would trigger a class shift and a more systemic immune response. In fact, hypotheses have been proposed stating that the intrinsic feature of allergens is not the resistance to digestion in the gastrointestinal tract, but rather the resistance to degradation within antigen-presenting cells (Foster et al., 2013), producing stable degradation fragments that constitute T-cell binding epitopes (Toda et al., 2011). Further investigation is needed to determine a correlation between allergenicity and susceptibility to endosomal degradation.

Enzymatic degradation tests evaluate the susceptibility of purified proteins to degradation in simple pepsin resistance assays or the more sophisticated simulated gastric and/or intestinal fluid assays, according to a standardized protocol. In the simulated gastric fluid (SGF) test (Thomas et al., 2004), the protein is incubated in the presence of pepsin (10 units/μg of protein) at pH 1.2 at 37 °C for between 0.5 and 60 min. The simulated intestinal fluid (SIF) test differs from the SGF test in that the pH is close to neutral (7.5) and the digestion is mediated by pancreatin (a mixture of duodenal enzymes). In both tests, the degradation of the protein and potential appearance of digestion fragments are usually evaluated by SDS-PAGE after various incubation times. Depending on experimental design and the question to be answered, it is possible to use Western blots (immunoblots), chromatography methods, e.g. FPLC, HPLC, or mass spectrometry, for more detailed examination of the fragments. Goodman et al. (2008) proposed to classify a protein as rapidly degraded if > 90% is degraded in less than 2 min, and as stable if it is still detectable by a validated method after 60 min of incubation. Additionally, *in silico* tools such as PeptideCutter (http://web.expasy.org/peptide_cutter/) are available to predict enzymatic degradation of a protein before conducting laboratory experiments.

It is crucial to understand what can be expected of these models. The correlation of digestibility tests with allergenicity potential is not absolute (Bøgh and Madsen, 2016; EFSA, 2017). Some food allergens are stable for up to 60 min in SGF, while non/low allergenic proteins are rapidly digested (Astwood et al., 1996; Fuchs and Astwood, 1996; Thomas et al., 2004). However, some non-allergenic proteins may also be relatively stable to digestion, whilst some allergens may be rapidly degraded (Fu et al., 2002; Herman et al., 2007). Therefore, enzymatic degradation tests are recognized as not absolutely predictive. (Foster et al., 2013). In addition, the size of the remaining fragments after digestion that would be considered as being without allergenicity potential is not well defined. The minimum size of peptides which might act as B-cell receptor epitopes and cause IgE cross-linking is not clear, but require the presence of at least two epitopes which can only be accommodated in peptides greater than 9 amino acids in length (EFSA, 2017).

The SGF test is limited in its capacity to replicate human digestion, as it does not represent all physiological conditions of the gastric digestion. In various cases, including early childhood or elderly age, during a meal or while taking antacid medication, the pH of the stomach increases above 4 and/or the gastric pepsin concentration and activity decrease (Bourlieu et al., 2014; Chen et al., 2008; Grassi et al., 2011; Minekus et al., 2014; Untersmayr et al., 2005). More physiologically relevant tests have recently been developed (Mills et al., 2013). Static digestion models are available which consist of combinations of simulated gastrointestinal proteolysis processes, including a gastric phase, a duodenal phase, and sometimes an oral phase (Mandalari et al., 2009; Minekus et al., 2014). A recent international consensus method described by Minekus et al. (2014) and later validated by three inter-laboratory trials using skimmed milk powder demonstrates the importance of applying standardized methods allowing data comparison and discussion across laboratories (Egger et al., 2016).

Dynamic digestion models include physical processing and temporal changes in luminal conditions for getting closer to the *in vivo* digestion process (Björck et al., 2012; Mitea et al., 2008). These physiological models study the digestion of compounds of interest including allergens, the effect of food matrix, and the effect of food processing. However, they were not developed, nor evaluated, for their relevance in the prediction of protein allergenicity. Furthermore, they also demand considerably more resources.

The interpretation of these enzymatic degradation assays must take into account that they do not provide a direct measure of allergenicity, but provide corroborative data on an endpoint associated with allergenicity (cross reactivity). Importantly, there has been little work on the applicability of these assays to proteins contained in a complex food matrix, rather than purified proteins. Such models could help understanding the possible effects of food matrix and/or food processing on the digestibility and allergenicity of the proteins.

5.3. Impact of processing

The physicochemical properties (solubility, stability, conformation, and matrix interaction) of a particular protein impact on how and to what extent the immune system might be exposed to allergenic polypeptides. For example, heat stability of novel proteins has been mentioned as an influencing factor in guidance documents (CODEX, 2009; EFSA, 2011). However, the effects of protein function/conformation loss on clinical allergenicity are not consistent: they can have no effect, may increase, or reduce the allergic reaction (Privalle et al., 2011). The respective outcome depends on the allergen and allergen family, the sensitization profiles of the allergic patients, or the heating procedure used (Verhoeckx et al., 2015). Currently, assessment of the allergenicity potential for proteins after processing is not generally performed, and comparisons between differently processed allergens and different products are difficult to make. Processing may influence, but does not necessarily eliminate the allergenic potential of dietary proteins (e.g. baked egg vs raw egg) (Leonard et al., 2015). Heat stability tests of novel food proteins generally show a low correlation between protein stability to heat and potential to elicit reactions, and provide limited information as part of the allergenicity risk assessment of novel proteins. However, consideration of how food is processed and prepared for consumption is important when preparing material for testing in experimental studies to investigate hazard identification and characterisation of new or modified proteins, and to determine the extent of exposure.

Table 3
Considerations in designing an*in vitro/in-vivo* clinical testing plan.

Parameter	Considerations
Test material	Exposure from its intended commercial food use must be a pre-requisite for considering clinical tests. The safety of the test material (viral, bacteriological, toxicological) has to be assessed prior to clinical testing. Test solutions used in SPT should comply with requirements for medicinal products under observation of GCP standards. The intended use of the protein or protein preparation (with multiple proteins present) in food, and expected processing steps for its final commercial use must be considered when selecting the form of protein to be tested: extracts, purified protein or processed forms. Preferably, all possible processing methods should be taken into account in the assessment. Potential differences in allergenicity of processed forms can be tested *in vitro* and possibly by SPT.
Food matrix effects	Dilution of protein and matrix fat content can change the test outcome and possibly lead to incorrect interpretation of results (Mackie et al., 2012; Schulten et al., 2011) in *in vitro*, SPT, and in double blind placebo controlled studies. Prevention of possible reactions to matrix proteins could be achieved by introducing as few additional allergens as possible into the challenge test material and by making sure that the tested individual can consume all ingredients without problems.
Selection of individuals	Selection of the subjects based on expected cross-reactivity, co-sensitization or *de-novo* sensitization (see text). Individuals selected for allergy testing of a novel or modified protein should come from a group at high-risk for developing food allergies. Inclusion of control groups of atopic patients, without co-sensitization to homologous proteins, and preferably healthy individuals to ensure the relevance of any observed reaction.
Study design	Clear description of the outcome parameter and the number of individuals needed to make a sound statistical basis. The outcome can be sensitization, allergy, or severity of allergy. If one outcome is regarded to be more important than another, this could result in the selection of individuals from a group that is most valuable for answering that particular question. The IgE reactivity profile measured for an individual will be compared to the same person's IgE reactivity profile to a *reference food* or similar proteins (*reference proteins*) to which a confirmed allergy exists (subjects in the high risk group). The IgE reactivity will be compared to the reactivity of the non-allergic control group.

6. *In vivo* centred investigations

At present, *in vivo* models are the only methods able to to assess the hazard (and thus risks) due to *de novo* sensitization. However the current tests have several limitations, whether in animals or humans, to characterise tolerance vs allergenicity and further, the potency and dose of responses. Proper study design, including the appropriate choice of controls, test materials and subject selection should in theory provide a comprehensive allergenicity overview for new dietary proteins using *in vivo* methods (see Table 3).

All *in vivo* studies, including animal studies and clinical trials, exist within an ethical framework which must be taken into account not only in the deployment of existing methods, but also in the development of new ones. An evaluation of the evidence generated in previous steps, as well as a complete toxicological evaluation of the test substance(s) is required prior to animal studies or clinical trials.

6.1. Animal models

Animal models are widely used in research laboratories to study sensitization to food proteins, and are the only model in which *de novo* sensitization has been demonstrated, although their performance is strongly dependent on experimental design and conditions (choice of species/strain, route of exposure, adjuvants, etc). They are not generally used in the risk assessment of a new protein/new food, as they do not permit reliable characterisation of the hazard, and validated models do not exist. This potential use, but acknowledged limitation, is reflected in the guidelines for GM crops. CODEX (2009) states that animal models may be used as part of a risk assessment strategy as scientific knowledge and technology evolve. In 2010, an EFSA opinion concluded: "*Animal models are in general considered not validated and inconclusive for the assessment of the sensitizing potential of a novel protein*" (EFSA, 2010). A year later the EFSA GM-crop guideline opens for the possibility of using animal models stating that although *"in vivo tests on animal models have not been validated so far for regulatory purposes, they may be considered useful to provide additional information* e.g. *on the potential of the newly expressed protein for de novo sensitization*" (EFSA, 2011). In practice, a positive result may thus be able to assert a potential for allergenicity, but a negative one would not be able to exclude it.

6.1.1. The continuing challenge of predicting a rare event in humans using a small number of experimental animals

Food allergy is a prevalent disease but an allergic reaction to a single protein is a relatively rare event in humans when considering the number of known allergenic proteins in contrast with the total number of known proteins. The default response to a new dietary protein is to develop oral tolerance through hypo-responsiveness in order to protect the organism from producing a harmful response to an innocuous substance. Oral tolerance also causes hypo-responsiveness to subsequent local or systemic exposure to the same protein or cross-reacting proteins (Kim and Surh, 2015; Kroghsbo et al., 2011). The greatest challenge in developing animal models is to avoid the default reaction "oral tolerance" in a way that preserves as much as possible of the normal physiology and protein chemistry so that it will predict sensitization in a meaningful way. One way to experimentally overcome oral tolerance is to circumvent the GI tract by dosing intraperitoneally (i.p) or subcutaneously (s.c.), but this has the disadvantage of potentially overestimating sensitization because the digestive tract is circumvented (Ladics et al., 2010). When using the oral route, it is important to bypass the oral cavity by dosing intragastrically (i.g.) as dosing in the mouth tends to induce oral tolerance (Madsen and Pilegaard, 2003). In mice, cholera toxin used as adjuvant enhances absorption through the gastrointestinal tract (GI) and also has a stimulating effect on the immune system (Frossard et al., 2015). The drawback of using an adjuvant is potentially overestimating sensitization. In Brown Norway rats, specific IgE can be induced by food allergens using i.g. dosing without adjuvant. The disadvantage of this model is the relatively low IgE response induced, decreasing the sensitivity and the possibility to study symptoms after challenge (Knippels and Penninks, 2003; Kroghsbo et al., 2014b). The advantage of the oral route is that the influence of the food matrix and processing methods (crude preparation, real food, purified protein) may be studied. The disadvantage is that oral dosing requires larger amounts of the test protein than i.p. or s.c. dosing (Kroghsbo et al., 2014a).

When using rodents one should be aware that there are strain differences in the ability to mount an IgE response to a specific protein (Ladics et al., 2010), just as there are differences between different human beings. This applies as much to the question of whether an immune response occurs, as to the issue of ranking different proteins when trying to characterise the hazard (Blaikie and Basketter, 1999). Diet is also a critical issue, and there is clear evidence of potential epigenetic effects, insofar as the ability of a given generation to mount an allergic immune response is influenced by the parental diet and even

that of earlier generations. To be able to induce a relevant immune response the animals need to be bred on a diet without the antigen or cross-reacting antigens for 2–3 generations as tolerance interferes with the response (Kroghsbo et al., 2011).

6.1.2. Hazard characterisation

Much of the effort when developing animal models for food allergy has been focused on the potential for a protein to sensitize i.e. induce a specific IgE response. Many foods are able to induce specific IgE and allergic symptoms in humans. Some of these cause allergic reactions in very few subjects although they contain storage proteins and are frequently eaten e.g. potato and maize (Informall). They are potentially hazardous but the risk connected to these foods is very small. Optimally, animal models should be able to predict not only if a food protein can induce specific IgE, but also its potency in doing so. There are several parameters for characterizing the allergenic potential of a food or protein, for instance: (i) the protein sensitizes many subjects when exposed, (ii) the symptoms elicited are frequently severe, (iii) the doses eliciting symptoms are low, and (iv), the protein sensitizes at a low dose. The first three parameters are difficult to study in any model, including animal models. Results may depend on the nature of the exposure (route, adjuvant, matrix, etc.), with either sensitization or tolerance as a possible outcome.

Most allergenic proteins are present in native foods in relatively high concentrations (e.g. casein in milk, ovalbumin in egg, parvalbumin in fish, etc.), leading to high exposure of consumers under normal conditions of consumption. In theory, this exposure results in induction of tolerance, although if exposure is not high and/or frequent enough, or does not occur within an appropriate time-window it will lead to sensitization in predisposed individuals (Du Toit et al., 2015). Indeed, experience from animal models as well as some epidemiological evidence of non-food allergens shows that the dose-response for food allergens may not be linear (Custovic, 2015). Increasing the dose of allergenic protein does not necessarily increase the induction of IgE (Kroghsbo et al., 2014a). Data from animal models have also indicated that there may be an optimum dosage and frequency for sensitization (and a dose below which the immune system ignores the protein), probably depending on the specific allergen (Vinje et al., 2009, 2011), but more research is needed in this area. Recent studies of peanut allergy prophylaxis in high risk infants suggest that these observations from animal studies may also be true for human sensitization (Du Toit et al., 2015). In the absence of alternatives, some well-defined animal models have been used to address highly specific questions, such as, comparing the sensitizing capacity of related proteins (Kroghsbo et al., 2011) or investigating how modification of a particular food or protein influences sensitization (Kroghsbo et al., 2014b).

6.1.3. Future for animal models in risk assessment

There are still many unknowns regarding sensitization to food allergens in humans, including dose-response relationships and potency. There have been many attempts to develop predictive animal models for food allergy using different dosing routes with or without adjuvants and with or without multiple doses to include dose-response as a source of information (Bøgh et al., 2016; Ladics et al., 2010). The major challenge is to correctly rank potential novel food allergens as a function of potency, based on the potency rank order of known food allergens, a challenging task when it is difficult in any case to categorise proteins in this respect. Up to now no useful, reliable and validated animal model (or any model) that meets this criterion has been developed.

6.2. Clinical approaches to allergenicity testing of new protein sources

While clinical studies are primarily considered in the context of cross-reactivity, circumstances exist where they can be used to investigate the sensitization potential of a protein. The framework in which the Human Repeat Insult Patch Test (HRIPT) is used to rule out the potential for skin sensitization (using low molecular weight allergens) in contact allergy, could also be appropriate for new protein sources. In that framework, the assay is set up with the hypothesis that a certain level of exposure to a chemical will not cause sensitization, (i.e. the exact opposite of the basis of animal tests). This same principle is the message of a sensitization and oral tolerance study to peanut performed in mice, which is more tailored toward the higher molecular weight allergens in food allergy (Strid et al., 2004).

Clinically relevant allergy can result from cross-reactivity or *de novo* sensitization. It is important to realize that while many individuals can be sensitized to a dietary protein, only a proportion experience clinical symptoms upon re-exposure, and are therefore considered allergic to that protein (an allergen). Both CODEX and EFSA guidance indicate that if homology to known allergens, and/or pepsin resistance is observed, then immunological tests using human sera, such as specific IgE binding studies, are recommended. This type of test, similar to routine clinical diagnostic tests utilizing the detection and quantitation of specific IgE (e.g. by ImmunoCAP™), in general only indicates the presence or absence of sensitization to a known protein, but not clinical allergy. IgE antibody levels to some specific individual proteins (e.g. Ara h 2, Cor a 9 and 14) can predict the likelihood of a reaction on challenge (Klemans et al., 2013; Masthoff et al., 2013), but these results are specific for a particular (clinical) population and vary by study (Calvani et al., 2015). They also do not predict reaction severity.

Before entering into any trial using human sera or designing a clinical trial it is very important to evaluate the existing information on the test material, the intended use and the food matrix. The selection of participants in a clinical study depends primarily on the hypothesis to be tested.

6.2.1. Subject selection

To select appropriate individuals for allergenicity testing, it is important to realize that food allergic patients are very different from one to another. They usually are allergic to more than one food and with different specific IgE titers, different recognition patterns of specific allergens and varying degrees of severity of the allergy. Moreover, food allergy can be cross-reactive (with inhalant or food allergens) but not always. Food-allergic patients can be only plant food-allergic, animal food-allergic or both. In addition a proportion of patients are only sensitized but not clinically allergic. In specific cases food allergic reactions only occur after exercise (e.g. in wheat dependent exercise induced allergy, WDEIA) (Scherf et al., 2016).

Depending on the research question, one has to select the appropriate group of subjects, and do a systematic clinical and lab work-up to fully characterise the (food) allergies of the patients. In individuals with known clinical allergy to a specific food, ***cross reactivity*** can occur when a novel protein has a high amino acid sequence homology to a known allergenic protein, epitope or component as is in the food causing the allergy (Verhoeckx et al., 2016). There are many examples of clinically relevant cross reacting proteins in the literature, such as Bet v 1, LTP, profilin and others (Hoffmann-Sommergruber and Mills, 2009; Werfel et al., 2015).

People with a high risk of ***de novo sensitization*** to novel proteins are more difficult to identify. Individuals selected to investigate the *de novo*-sensitization to test materials should come from a group that is at high-risk for developing food allergies because of the presence of atopic symptoms or a family history of allergy. This can be either with a broad sensitization and/or allergy pattern or with more specified spectrum of sensitizations/allergies and the specific population needs to be decided based on the research question. As with cross-reactivity, other factors such as physical activity in food dependent exercise induced allergy might be taken into account.

Individuals can be exposed and become sensitized via other routes than the digestive tract. Therefore, individuals working in an occupational setting with respiratory or dermal exposure to the new protein or

previous dermal exposure (e.g. in case the studied protein is already present as ingredient in cosmetic products) are a potentially at risk group which in assessments could provide valuable insights into *de novo* sensitization and subsequent reactions.

6.2.2. In vitro investigations with ex-vivo materials from allergic individuals

6.2.2.1. IgE binding. IgE-binding studies should be conducted if a novel protein is closely phylogenetically related to a known allergen or if specific amino acid sequence homology has been shown with known allergens. Positive results in binding studies can be regarded as indication for a possible cross-reactivity or co-sensitization with known allergens. This requires sera from clinically verified food-allergic individuals and has to be done in collaboration with experienced clinical partners.

Serum must optimally be obtained from well-characterised, at-risk allergic patients who have a convincing history of allergic reactions to a particular allergenic food, with a positive ImmunoCAP™ (> 0.35 kU/L) and/or skin prick test (SPT), AND preferably a positive double-blind placebo-controlled food challenge (DBPCFC) to the allergenic food. When DBPCFC is not possible, patients with sensitization above the 95% PPV level could be used or patients with a recent (within one year) reaction due to accidental exposure to a food allergen that could be clearly identified.

Negative controls are needed for a proper serum screen of the novel protein. If results with serum from at-risk persons are ambiguous a broad panel of sera from individuals with different allergy profiles can be used (i.e. pollen, plant, animal) and examined with different analytical methods (ELISA, RAST, immunoblot) (Verhoeckx et al., 2016).

6.2.2.2. Functional basophil activation tests. It is important to remember that IgE-binding in a serum screen does not automatically indicate clinically relevant allergy. *In vitro* functional IgE-binding options include the basophil activation test (BAT), which requires only 1 mL of blood from the at-risk individuals, no cell separation and measures the activation markers on the basophil surface (i.e. CD63 and CD203c) with flow cytometry after allergen stimulation (Chirumbolo et al., 2008). Previously, BAT has been shown to discriminate between allergic and non-reactive individuals sensitized to peanut (Santos et al., 2014). However, van Erp et al. (2017) recently showed that due to spontaneous releasers and non-responders BAT is not more informative than component specific ImmunoCAP. The BAT has the advantage that denatured proteins extracted using stringent conditions (e.g. urea and SDS/DTT) can be investigated, which would not be possible (dermal irritation) in direct clinical testing (see below). More research is needed regarding the applicability of BAT, as a number of issues including nonspecific activations and release can occur. There is also a Rat Basophilic Leukaemia cells (RBL) assay available. However, strict serum requirements regarding specific/non-specific IgE ratios and nonspecific activation and release limit the widespread applicability of BAT and RBL tests for investigation of new proteins.

6.2.3. Clinical testing options

6.2.3.1. Skin prick testing (SPT). SPT is the least invasive first step in clinical testing. Protein extracts of the new protein source (in all processed forms) and of the single purified protein should be applied. Unfortunately, solvents usable in skin testing are limited by the need to remain within acceptable physiological parameters (e.g. pH, irritancy, general safety – microbiological and chemical, etc.). Insoluble or difficult to solubilize proteins can only be tested *in vitro* or *ex or in vivo* in, for example, basophil activation testing or prick-to-prick methods. For prick-to-prick applications the test protein is transferred from the source directly to the skin without previous extraction. The prick-to-prick method lacks standardization, but can be very informative (Bolhaar et al., 2005; Henzgen et al., 2008). SPT can be performed before the oral challenge (as below) to get information on clinically relevant sensitization in the study participants to (parts of) the tested product.

6.2.3.2. Oral challenge. IgE-mediated food allergy is the elicitation of allergic symptoms upon ingestion in sensitized persons. Oral challenge is the only test to demonstrate unequivocally the presence of true food allergy. The “gold standard” for confirming food allergy is a DBPCFC with the tested food protein or food product. The double blinding allows both patient and clinic staff to evaluate signs and symptoms as objectively as possible.

6.2.3.3. Oral human immunogenicity studies. Longer term ingestion studies with the protein or protein source of interest (e.g. Crevel et al. (2007)) can also be informative to identify potential immunogenicity and therefore the possibility of allergenicity. These studies should be of sufficient duration to allow any antibody response to become manifest. As discussed earlier, such studies can only be conducted once the general safety of the test material has been ascertained and following an assessment of the weight of evidence in accordance with guidelines described earlier. In this context their use is similar to the Human Repeat Insult Patch Test to confirm the absence of sensitizing potential in materials applied to the skin. However, it must be noted that the appropriate number of subjects should be included to ensure proper predictive power and this would likely be more than the 50–200 individuals used in the Human Repeat Insult Patch Test due to the relative low expected frequency of food allergy.

In summary, with careful selection of appropriate subject and control groups, based on the study design, clinical evaluation is a powerful tool to predict allergenicity of new and existing proteins.

7. Discussion

Food allergy is a relatively rare event in humans as the default reaction to a dietary protein is to develop oral tolerance. Adaptive immune responses, such as allergy, consist of two phases: sensitization and elicitation, which must be analysed separately. Quantitative approaches to assess the risk posed by substances eliciting reactions in already sensitized individuals are proving very successful (Allen et al., 2014; Crevel et al., 2014). However, dose responses in relation to sensitization look to be non-linear, probably because exposure may lead to either tolerance or sensitization. To complicate matters further the relationship between sensitization and elicitation is complex. The manner in which the population is exposed to new proteins also impacts on the risk of potential sensitization and allergenicity.

Guidelines developed so far focus on hazard analysis, and on mainly structural characteristics for the potential to elicit an allergic reaction, in already sensitized individuals. The available tools analyse for properties of known allergens, and function best for cross-reactivity. None of the available methods have been formally validated for their predictive abilities, and there are only few standardized methodologies available (in comparison to OECD testing protocols for toxicological testing of chemicals). The current weight-of-evidence approach seems supported empirically and successfully for decision-making, to the extent that no cases of human allergic responses have been reported to any foods derived from approved agricultural biotechnology products (Ladics, 2008). Current approaches that have required assessing safety through a weight-of-evidence approach to approve GM crop commercialization appear to be well suited to protecting consumers. One key piece of evidence in terms of food allergy is that consumers in countries extensively cultivating and producing foods from GM crops (e.g., the US) have levels of food allergy indistinguishable from other world areas with similar socio-economic profiles, but with negligible consumption of foods from GM crops (e.g., the EU) (James, 2015; Nwaru et al., 2014; Sicherer, 2011). Exposure to these products has been low in comparison to that expected for proteins used for nutritional reasons. Novel foods intended as protein sources may thus pose different challenges, in that complex mixes of proteins are expected, and exposure scenarios will be

very different.

Current focus is on applying existing tools and tests to assess the risks due to cross-reactivity/co-sensitization but little is available for a strategy to identify and characterise with reasonable certainty the risks arising from *de novo* sensitization. No single test is available (or expected in the near future) for predicting or for characterising the *de novo* sensitization potencies of new proteins. The only tests that can identify if a protein will induce specific IgE is *in vivo* models in animals or humans, although as discussed in this document, there are many limitations to characterise the potency and dose responses, and uncertainties to characterise tolerance vs allergenicity. In addition, there are ethical aspects to consider. As yet, no *in silico* or *in vitro* approach can be used to identify the potential of a protein to sensitize, and subsequently elicit a clinical reaction. An overall mechanistic model (or adverse outcome pathway, AOP) for food allergy does not yet exist, Human allergic responses are complex, and while extensive research has been conducted, predictive models remain elusive. Arguably, the (very) limited ability to predict sensitization restricts the development and use of novel protein sources, which is crucial to make our future food supply more sustainable. There is a need for a comprehensive, systematic testing and assessment strategy to identify and characterise the risks associated with allergic reactions due to *de novo* sensitization to specific proteins, which incorporates relevant aspects of exposure, intrinsic protein properties and matrix/processing effects. New potential allergenic hazards are currently managed through avoiding exposure. Not authorizing the introduction of an identified new allergen into the marketplace, or by identifying an indication for allergenicity early in development (which may lead to cancelling the project) avoids exposure of the whole population. Labelling will alert consumers with existing sensitivities to the presence of a potential hazard (e.g. rapeseed protein isolate and individuals with existing mustard seed allergies). However it does nothing to mitigate the public health impact of a protein with high food sensitizing potency. In the absence of methodologies to determine sensitization potential and potency, a post-launch monitoring exercise may be considered to provide an early warning of any undue allergenicity developing after introduction of a novel food into the market and thereby permit the initiation of risk management measures. Possibilities and limitations for such resource intensive post-launch monitoring in the context of novel foods and unintended health effects has been critically discussed (Hepburn et al., 2008) and the special case of allergenicity has been reviewed (Wal et al., 2003).

Development of a coherent risk assessment strategy would benefit greatly from a clear definition of criteria for distinguishing between proteins of high and low allergenicity (i.e. ability to induce IgE, potency to induce IgE, expected prevalence of IgE-sensitization, expected prevalence of allergy, expected exposure, expected eliciting potency, expected frequency of reactions, expected frequency of severe reactions). Appropriate tests could then be applied, or developed as needed, to investigate the relevant protein characteristics. The COST Action network (ImpARAS, www.imparas.eu) has recently started to discuss these criteria from first principles and will continue with the broader subject of improving strategies for allergen risk assessment throughout 2016–2018/9.

For any chosen approach it will be important to demonstrate that the methodologies are able to distinguish between allergens of different potency and rank them appropriately. The current general lack of systematic data to rank existing, known allergenic proteins impairs the necessary work to validate any of potentially alternative methodologies. This applies to both the potential to sensitize and subsequently elicit reactions, although good progress has been made with regard to the risk assessment of elicitation by known allergens. As scientific knowledge progresses, it should be possible to improve the methodologies used in allergenicity risk assessment, e.g. new possibilities due to the development of bioinformatics tools, and relevant *in vitro* biological tests. It will be vital to identify those approaches, methods and technologies on which future research efforts should be focussed, taking into account their current performance, but also the scope for their evolution into predictive approaches.

Although knowledge of historical use and exposure is useful to focus assessments for potential cross-reactivity, it is of little value when investigating completely novel proteins and their inherent risk for *de novo* sensitization. The consideration of exposure levels is a key element for assessing consumer risk, and clinical studies could have an important role for assessing the allergenic potential of new dietary proteins. However, we should not wait until we have fully defined all the clinical adverse outcome pathways (AOPs) related to food allergy, but rather select a battery of tests which can distinguish between low and high allergenic proteins, defined on the basis of accepted criteria for allergenicity (see above). The battery of currently available tests could be run using a panel of selected known strong, moderate, weak and (virtually) non-allergenic proteins to help to identify the most efficient testing strategy for allergenicity differentiation. A better understanding of AOPs could guide the development of better *in vitro* and *in vivo* allergenicity testing methods. Therefore, it is important to leave room for flexibility and improvement of methodologies within any regulation or guidance, as our progression of knowledge will aid with the development and improvement of tests and tools.

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Appendix A. Supplementary data

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