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The relevance of a digestibility evaluation in the allergenicity risk assessment of novel proteins. Opinion of a joint initiative of COST action ImpARAS and COST action INFOGEST

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A B S T R A C T

The current allergenicity assessment of novel proteins is based on the EFSA GMO guidance. Recently, EFSA launched a new guidance document on allergenicity assessment of GM plants (2017). This document describes, amongst other topics, the new scientific and regulatory developments on *in vitro* protein digestibility tests. The EFSA GMO Panel stated that for *in vitro* protein digestibility tests, additional investigations are needed before any additional recommendation in the form of guidance can be provided. To this end, an interim phase is considered necessary to evaluate the revisions to the *in vitro* gastrointestinal digestion test, proposed by EFSA. This prompted the establishment of a joint workshop through two COST Action networks: COST Action ImpARAS and COST Acton INFOGEST. In 2017, a workshop was organised to discuss the relevance of digestion in allergenicity risk assessment and how to potentially improve the current methods and readouts. The outcome of the workshop is that there is no rationale for a clear readout that is predictive for allergenicity and we suggest to omit the digestion test from the allergenicity assessment strategy for now, and put an effort into filling the knowledge gaps as summarized in this paper first.

Abbreviations: AD, Atopic dermatitis; APC, Antigen presenting cells; BBM, Brush border membrane; DCs, Dendritic cells; GMO, Genetically modified organism; GI, Gastrointestinal; HPLC, High-pressure liquid chromatography; MS, Mass spectrometry; OVA, Ovalbumin; SPT, Skin prick test

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1. Introduction

The global food industry has a challenge to provide food for 9 billion people, at a sustainable level, by 2050, in the context of an increasing shortage of protein sources from known and traditional foods. By introducing novel protein sources, consumers will encounter new proteins, some of which may act as food allergens and thus significantly increase the probability of food allergy.

Food allergy is one of the most prevalent disorders in the Western world, with a prevalence around 5–8% in young children and 2–4% in adults (Sicherer and Sampson, 2014). Although severe, fatal reactions are rare, they are also very unpredictable. This results in a significant adverse impact on the quality of life of allergic individuals and their families, due to the potential for serious, potentially life-threatening allergic reactions (anaphylaxis). Food allergy has a substantial economic impact that directly impacts on society (e.g., medical care, dietary provision etc.) and indirectly due to illness, time and money spent for non-allergenic food purchase, etc. Also, the food industry regularly faces the financial and reputational consequences of food incidents and recalls, for instance, due to food allergen cross-contamination during production.

The food industry is tasked with (under national/pan-national regulations) the provision of safe food. Significant cost savings and improved resource utilization can be achieved when allergenic risk is predicted early in the development process of (novel) food proteins. However, the components of such a risk assessment have not yet been fully developed. The interplay between different factors, such as protein characteristics, mechanisms underlying allergic sensitization and individual risk factors remains unclear and validated predictive methods are lacking. The need for an allergen risk assessment for novel foods is set in the European legislation (EU-2015/2283). The allergenicity assessment suggested is based on EFSA GMO (Genetically Modified Organisms) guidance: “Allergenicity assessment of GM plants” published in 2011 and Implementing Regulation EU (No) 503/2013 (EFSA, 2011). Recently, EFSA launched a guidance document on allergenicity assessment of GM plants (Naegeli et al., 2017), which supplements the aforementioned guidance document. This new guidance document describes, amongst other topics, new scientific and regulatory developments on *in vitro* protein digestibility tests and suggested as an additional recommendation for a digestion test more investigation is needed. Therefore, an interim phase has been implemented to investigate the applicability and scientific relevance of the digestion test in allergenicity assessment.

This context was a perfect occasion for COST Actions ImpARAS and INFOGEST to join forces. COST Actions are bottom-up, pan-European research networks funded by the various research and innovation framework programmes, such as Horizon 2020. The **Imp**roved **A**llergenicity **R**isk **A**ssessment **S**trategy COST Action (ImpARAS, FA1402, www.imparas.eu) focusses on identifying gaps and generating new ideas and plans for improving the allergenicity assessment strategy. The COST Action INFOGEST stands for “Improving Health Properties of Foods by Sharing our Knowledge on the Digestive Process” and aimed to improve the current scientific knowledge on how foods are disintegrated during digestion and successfully promoted and implemented a harmonized digestion model (FA1005, www.cost-INFOGEST.eu/). In April 2017, a joint workshop was dedicated to the topic of the relevance of digestion in allergenicity risk assessment and how to improve the current methods and readouts. The outcome of the workshop is summarized in this paper including current knowledge, identified gaps and suggestions for future research.

Throughout the document we use the terms allergenicity, allergenic potential and immunogenicity. The definitions are defined as: i) allergenicity or allergenic potential: the potential of a material to cause sensitization and allergic reactions, frequently associated with IgE antibody and ii) immunogenicity: the ability of a material to elicit an immune response.

1.1. Current approach

The allergenicity risk assessment of novel proteins is mainly based on the guidelines mandated for GMOs using a weight-of-evidence approach, which involves an integrated case-by-case strategy. The safety evaluation incorporates as its main focal elements consideration of: the origin of the gene; sequence homology of the new encoded protein with known allergens; binding of the new protein by IgE from allergic individuals; and stability of the protein in a pepsin resistance test. No clear protocol for the pepsin resistance test is provided in the guidelines, thus many applicants use the method described by Astwood et al. (1996) or derivatives thereof, such as the protocol of Thomas et al. (2004) as that one has been adopted more widely in industry as a harmonized approach to the assay. However, this method has never been rigorously validated and there is still controversy regarding the potential link between the ability of a protein to resist the digestive process and its ability to induce an immune response. Presumably, it is not necessary for a protein to be intact when reaching the epithelial cells for this to happen. Fragments generated during digestion are frequently long enough to include at least 2 epitopes (i.e., with a molecular weight of 3.5 kDa) to cause sensitization (Lack et al., 2002; Mills et al., 2004). In a pioneering study, Astwood et al. used a rather crude incubation test with pepsin and compared the resistance to pepsin digestion of 16 known food allergens (e.g., ovalbumin (OVA)), β-lactoglobulin, Ara h2, β-conglycinin) and 9 common plant proteins considered to be non-allergenic (e.g., Rubisco Large Sub Unit and Small Sub Unit from spinach leaf, lipoxygenase from soybean seed, sucrose synthetase from wheat kernel, β-amylase from barley kernel or acid phosphatase and phosphofructokinase from potato tuber). Overall, major food allergens resisted the digestion process in contrast to non-allergenic proteins (mainly enzymes) that were rapidly digested (Astwood et al., 1996). In another study, impairment of the digestion process was shown to increase allergenicity of parvalbumin, the major fish allergen protein as measured by specific IgE and IgG1 levels and skin tests in a BALB/c mouse model, supporting the hypothesis that there is a link between resistance to digestion and allergenicity (Untersmayr et al., 2003). These results were later confirmed in human adults (Untersmayr et al., 2005a). However, when reviewing published literature on the digestion of pure allergens, Bøgh and Madsen did not find clear evidence of this link among a wider range of proteins (Bøgh and Madsen, 2016). Actually, the assessment of the allergenicity of digestion products, by either IgE-binding, elicitation or sensitising capacity, showed that digestion may abolish, decrease, have no effect, or even increase the allergenicity of food allergens. Fu et al. tested a number of comparable allergenic and nominally non-allergenic proteins with similar cellular functions for their ability to survive *in vitro* digestion. They selected 23 allergens and found no clear relationship between digestibility measured *in vitro* and allergenicity (Fu et al., 2002). This absence of link between the ability of a protein to resist digestion and allergenicity might be explained by the experimental conditions used to digest the proteins (enzyme: substrate ratio, pH and duration of the gastric phase, etc.) and also by differences in the analytical techniques that were used to characterise the digested product (e.g., ELISA, mass spectrometry, basophil activation tests, etc).

In 2013, a report on protein digestion commissioned by EFSA confirmed the contradictions found using the pepsin digestion test and suggestions for improvements were presented (Mills et al., 2013), as summarized below:

1) Lack of harmonisation of *in vitro* digestion test conditions that makes comparison of results difficult.

→ Needed: standardisation and optimization of digestion conditions (e.g., clear description of the source and specific activity of the proteases used; time course and sampling method employed; mixing method used during digestion; inactivation of further proteolysis)

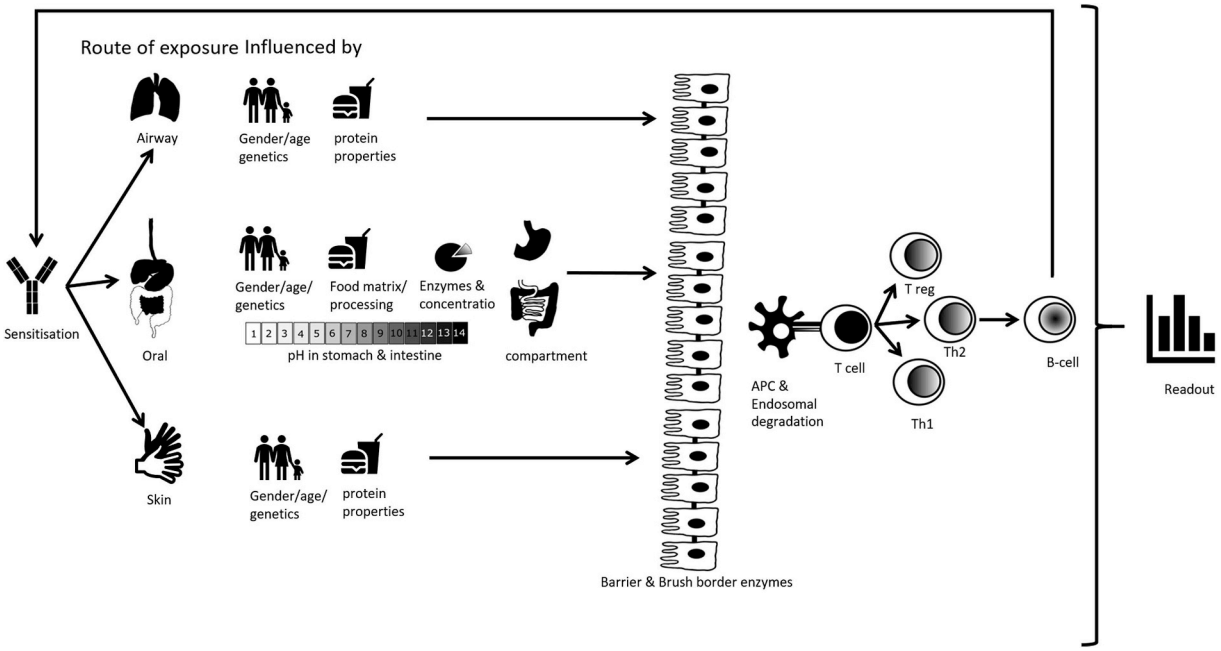

Fig. 1. Factors influencing food digestion and sensitization to food proteins.

2) No standardised method for monitoring and characterizing protein fragments and intact protein after digestion.
→ Needed: methods to characterise large resistant fragments that are more difficult to follow using LC-MS and standardised staining protocols for SDS PAGE.
3) Digestion protocol ("batch" assays) cannot replicate the dynamic digestion process that takes place in real life situations.
→ Needed: apply several analyses using pH ranges and pepsin: protein ratios such as those found physiologically.
4) Lack of consensus on comparators and standardised proteins to be included in digestion tests.
→ Needed: a "reference" set of allergenic and non-allergenic proteins.
5) At present digestion is not a sufficiently mature area of science to warrant inclusion in the risk assessment process.
→ Needed: interim phase to investigate the applicability of the digestion test in allergenicity assessment and to evaluate the revisions suggested.

During the interim phase, the old guidance on digestion remains in place (using solely pepsin digestion) and a two-year interim period was provided to further investigate (Naegeli et al., 2017): different test conditions to mimic digestion in healthy, impaired and elderly individuals and infants, to identify the interplay between pH, enzyme concentration and duration of the digestion, to consider intestinal digestion, and the use of a reference set of allergenic and non-allergenic proteins. Methods should be investigated to ensure reliable and robust digestion read-outs and end-points which enable a better comparison of test results. In addition, a definition of transience and persistence of digestion fragments should be defined, using concepts such as half-life. The report suggests that persistent peptide fragments with ≥9 amino acids in length are critical and may indicate that further assessment is required.

Based on the discussions during the ImpARAS-INFOGEST joined workshop, it was concluded that other factors in addition to those stated in the EFSA document might play a role as well in protein sensitization and digestion (See Fig. 1). These factors should be considered to establish a well-defined opinion about whether digestion resistance is relevant in an allergenicity assessment and if further optimization of the digestion protocol is worthwhile. The workshop members proposed that further information is needed relating to the following:

- **Characteristics of the normal versus atopic population** (stomach pH, enzyme concentrations) which may affect digestion of proteins.
- **Barrier function and sensitization routes** may be essential to determine if other routes of sensitization (e.g., skin, lung) are relevant in food allergy, which bypasses GI digestion.
- **influence of pH** on digestion and the factors that may impact this pH such as other diseases and medicine use which may influence gastric acid secretion as well as production of intestinal digestive juices and stomach and gastrointestinal pH.
- The role of **brush border membrane enzymes** in the digestion of proteins and peptide fragments focusing on luminal digestion products that can be further degraded by these enzymes, and may then be activated/inactivated before reaching the immune system.
- Involvement of **antigen presenting cells** (APC) in the digestion process. APC digest proteins internally with endolysosomal enzymes before presenting to antigen-specific T cells, suggesting that endolysosomal digestion should be as important as gastrointestinal digestion.
- How **matrix and processing** influence digestion intensity and kinetics and thus, the degradation of allergenic proteins, but also the generation of allergenic protein fragments. In theory, processing of an intact protein may result in different fragments to those observed without processing. Furthermore, re-aggregation of small non-sensitising fragments may occur resulting in larger entities which can be presented by APCs.
- The relevance of **dynamic vs. static digestion models** to closely mimic physiological digestion conditions.
- The importance of sensitivity and reproducibility of **digestion readouts** (e.g., kinetics, fragments, bioactivity of fragments, etc.).

In the following sections, these topics will be addressed in more detail.

2. How might physiological differences between atopic and non-atopic individuals affect allergen digestion and absorption?

Allergen digestion and absorption is a complex process which is often divided into three phases:

***Oral phase*:** Mechanical digestion in the oral cavity is an essential first step in increasing the potential surface area of food to enzymatic

action, initially to salivary amylase. The ability to chew food varies with age; as a response, infants are fed pureed foods which do not need to be chewed. Saliva is a mixture of components, which vary through early childhood and may be related to the introduction of solid foods in this age group (Manconi et al., 2013; Morzel et al., 2011). Absorption can also occur across the buccal mucosa without enzymatic degradation – something which has been demonstrated for peanut – with the potential to cause an immune response (Dirks et al., 2005). The latter study was performed in healthy, non-allergic adults. Whether absorption is different in atopic individuals is unknown. Saliva might also contain allergen-specific Immunoglobulin A (IgA), an antibody which binds allergen and potentially affects its absorption. Levels of allergen-specific IgA vary with age (Bottcher et al., 2002), although this may be confounded by allergen exposure. Of interest, a prospective study in 79 children with family history of atopy and 129 controls without an atopic family history found a higher occurrence of saliva IgA deficiency in the former, although no association was found between salivary IgA levels and the presence of clinical disease (van Asperen et al., 1985).

Gastric phase: Suppression of gastric acidity has been reported to be associated with increased protein absorption (Bloch et al., 1979; Kraft et al., 1967). This has led to concerns that gastric acid suppression may affect allergen denaturing (Untersmayr and Jensen-Jarolim, 2008) and might explain epidemiological data that antacid treatment in early childhood can increase the risk of food allergy (Mitre et al., 2018). Gastroesophageal reflux is common in infants with cow's milk protein allergy. However, there is a lack of data as to whether gastric pH levels are altered in atopic individuals, or whether the risk is purely due to iatrogenic use of medication. In support of the latter, pepsin secretion – considered important in the digestion of peanut allergens (Kopper et al., 2004) – appears to remain stable throughout childhood to mid-adulthood, but then may wane in old age (Feldman et al., 1996), yet this has not been associated with increased food allergy in old age. Furthermore, in an adult cohort, only a small percentage of food-allergic patients (7.7%) used antacids (Versluis et al., 2016). However, studies on the development of adult food allergy are rather scarce. Humans have a wide variation in their gastric secretions: pepsin concentration can vary over 10,000 fold between individuals (da Silva Gomes et al., 2003), and is further affected by the type of food consumed. But again, no data is available regarding differences in atopic versus non-atopic individuals.

Intestinal phase: Atopic status may affect allergen absorption through both IgE-mediated and –independent mechanisms. The incidence of non-IgE-mediated food allergy is up to 5% in infancy and can result in impaired epithelial barrier integrity, increasing allergen exposure and absorption. In laboratory studies, facilitated allergen uptake through the low affinity-IgE receptor may occur (Li et al., 2006), suggesting that sensitized atopic individuals may have different absorption kinetics for the allergens against which they produce antibody (Steele et al., 2012). Gut motility and intestinal blood flow will both affect digestion and absorption, though there are no data that these differ with atopic status; a greater effect is likely to be seen with everyday activities including exercise and alcohol intake, as well as medication use. Nonetheless, recent studies have identified changes in gut microbiota associated with food allergy status (Plunkett and Nagler, 2017), although it is difficult to assess the impact of reverse causality.

Hardly any information is available on physiological differences in the oral and gastric phase between atopic and non-atopic individuals. Already sensitized individuals may have an altered absorption kinetics for the allergens against which they produce antibodies.

3. How does the gastrointestinal pH influence allergen digestion?

The impact of pH on *in vitro* or *in vivo* gastrointestinal protein digestion of allergenic versus non-allergenic food proteins has not been thoroughly investigated. Based on currently available scientific literature, it is evident that the pH of the different gastrointestinal compartments has a substantial impact on food protein digestion.

Food is exposed to varying pH conditions within the gastrointestinal (GI) tract, which significantly influences protein denaturation, (affecting protein conformation). and local enzyme activity. In the mouth, food is macerated through chewing and comes into contact with saliva produced by submandibular, parotid and sublingual glands. Saliva has an average pH range of 6.2–7.6 (Marsh, 1994), although this is affected by oral health; for example, in patients with clinically healthy gingiva, saliva had a pH of 7.06 falling to 6.85 in patients with chronic generalized periodontitis and increasing to more alkaline pH with chronic generalized gingivitis (Baliga et al., 2013).

After a quick oesophageal passage, the food bolus enters the stomach where it is exposed to a low pH. Typical gastric pH in a fasted state is between 1.5 and 3.0 (Dressman et al., 1990; Evans et al., 1988; Ono et al., 2009), although this may vary from pH 1.0 to pH 8.0 even in healthy volunteers, with short periods of high pH peaks attributed to water intake, swallowing of saliva or reflux of duodenal content (Bergstrom et al., 2014; Koziolek et al., 2015). No gender differences have been reported (Dressman et al., 1990), but pH does vary with age due to reduced gastric fluid secretion in infants under 6 months (only 10–20% of adult volumes, when normalized to body weight) and also in older individuals (over age 65 years) compared to mid-adulthood (Agunod et al., 1969; Feldman et al., 1996; Hosking et al., 1975). In a recently published meta-analysis of eight different studies, the average gastric pH in fasted infants was pH 2.8 (Kamstrup et al., 2017). Gastric pH is higher in preterm infants (between 3.1 and 3.4), increasing to above pH 6 after an infant milk meal (Kamstrup et al., 2017; Michalek et al., 2011; Omari and Davidson, 2003). At least one study in healthy preterm infants reported an even more pronounced effect of feeding, increasing pH to pH 7 (Omari and Davidson, 2003). The food matrix also has a significant effect on gastric pH in adults, due to its inherent buffering capacity: gastric pH can increase up to 5.5, and then fall to pH 1.5 or below towards the end of the gastric emptying phase (Keller, 2012; Michalek et al., 2011). Disease also influences gastric acid secretion: hyperparathyroidism, Zollinger-Ellison syndrome and extensive small bowel resection are associated with enhanced gastric secretion (Keller, 2012; Layer et al., 1995; Seal et al., 1982). More commonly, gastric acid hyposecretion or achlorhydria is observed in chronic H. *pylori* infection, autoimmune gastritis or due to gastric resections or vagotomy (Keller, 2012). However, whether patients with these diseases are at a higher risk of food allergy is unknown.

Most studies linking the pH of gastrointestinal fluids and food protein allergenicity have, to date, focused on the gastric environment. The use of antacid medication to treat dyspepsia will increase fasting gastric pH > 5, even after relatively short periods of treatment (Banerjee et al., 2010; Bohle et al., 2006; Ono et al., 2009; Prichard et al., 1985). Given the association between antacid medication and the sensitization to food allergens (Scholl et al., 2005; Untersmayr et al., 2003, 2005a). Untersmayr et al. investigated the effect of different pH levels of simulated gastric fluid on the digestion of food proteins. Fish and milk protein digestion was completely inhibited upon shifting the pH of the simulated gastric fluid to either pH 2.75 for codfish or 3.0 for milk proteins (Untersmayr et al., 2005a, 2005b). Digestion of codfish proteins at pH 1.25 was associated with a reduced IgE-binding activity in a digestion-time dependent manner (Untersmayr et al., 2005b). With regards to elicitation of food allergy, low pH of the simulated gastric fluid used for digestion of codfish proteins was found to substantially influence their ability to trigger allergic reactions, evidenced by a significantly reduced histamine releasing activity of the digested samples (Untersmayr et al., 2005b). These findings were confirmed by a reduced

Table 1
Oral and gastrointestinal pH levels in health and under acid-suppressive medication.

Site	Average pH level	Age group	Fasted/fed status	Health status	Ref.
oral	7.06	Adults	not specified	healthy	Baliga et al. (2013)
	6.85	Adults	not specified	healthy	Baliga et al. (2013)
	7.24	adults	not specified	healthy	Baliga et al. (2013)
stomach	1.5–3	adults	fasted	healthy	(Dressman et al., 1990; Evans et al., 1988; Ono et al., 2009)
	5 > > 1.5	adults	fed	healthy	Kamstrup et al. (2017)
	2.8	infants	fasted	healthy	Kamstrup et al. (2017)
	6.4	infants	fed	healthy	(Layer et al., 1995; Seal et al., 1982)
	> 5	Adults/infants	fasted	PPI treatment	(Barbero et al., 1952; Bratten and Jones, 2009; Ono et al., 2009)
Small intestine	6.0–7.7	adults	fasted	healthy	Koziolek et al. (2015)
	5.6	adults	fed	healthy	Fredrikzon and Olivecrona (1978)
	7	infants	fasted	preterm	Untersmayr et al. (2003)
	6.5	infants	fed	preterm	Jensen-Jarolim and Untersmayr (2008)

capacity of digested proteins to trigger clinical reactions in codfish-allergic patients (Untersmayr et al., 2007). Increased pH of simulated gastric fluids does not only mimic the intake of anti-acid medication, but can also be used to simulate the situation of the immature infant digestion. By SDS-PAGE and ELISA, Menard et al. (2014) assessed the immunogenicity of milk proteins incubated in solutions of decreasing pH, starting at pH6.5 to mimic pH in piglets (Menard et al., 2014). Moreover, digestion resistant and immunogenic fractions of processed milk proteins were identified after simulating infant digestion (Dupont et al., 2010c). By comparing models for infant and adult digestion, a higher percentage of residual immunogenic egg proteins were observed after simulation of infant digestion (Dupont et al., 2010b).

Despite major efforts to establish a detailed record of pH in the different compartments of the GI tract, there remain a number of knowledge gaps, particularly with respect to intestinal pH distal to the stomach (Table 1). A better understanding about the influence of meal composition on pH and gastrointestinal transit time of the food bolus will be essential to better predict protein digestibility. Whether atopic individuals have altered pH in the different compartments of the GI tract due to inflammatory process, altered digestive fluid production etc. remains unknown. As has been demonstrated for H. *pylori*, infection and inflammation can be associated with altered local pH, which could influence protein degradation locally and therefore be associated with increased uptake of immunologically intact allergens. Finally, more work is needed to understand the impact of altered gastrointestinal pH on the overall microbiota composition, since the latter substantially influences immune response and oral tolerance induction (Chinthrajah et al., 2016; Imhann et al., 2016) as well as affect degradation of food proteins influencing their immunogenicity (Maiga et al., 2017).

Many parameters, including disease state, food matrix and medical prescriptions, can influence the pH in the stomach and have a substantial impact on protein digestibility. It is unknown if atopic individuals have altered gastrointestinal pH levels compared to healthy individuals. Furthermore other factors influenced by pH must be considered (e.g. microbiota, transit time through GI tract and protein absorption).

4. What is the role of barrier function and sensitization route?

Physical barriers form a key part of immune defences protecting the host from a harmful environment. These barriers include the skin and mucosal epithelial surfaces and maintain a variety of functions for excluding undesirable foreign materials. Immune cells, microbiota and anti-microbial peptides support this barrier function. However, when a physical barrier is disrupted, introduction of infectious, non-infectious, and potentially allergenic proteins may occur allowing for local and systemic invasion/immune response. The sensitization route then corresponds to the location where the encounter with an antigen will lead to specific Th2 cell activation. This encounter takes place at the epithelial interface of the organism with the external milieu, e.g., skin, pulmonary tract or GI tract epithelium, and the Th2 activation occurs in the corresponding draining lymph nodes. In this context, food allergy is considered to occur mainly after exposure to food antigens through the GI route.

The GI epithelium is a complex physical barrier allowing the transport of nutrients and small molecules while barring macromolecules, microbes and other non-digestible/non-absorbable materials. The epithelium interacts with food proteins after modification with gastric acids, gastric and pancreatic enzymes and brush-border proteases, leading mainly to peptides and amino acids, which are absorbed depending on size, polarity and shape (Snoeck et al., 2005). However, ingested food antigens can reach the epithelial GI barrier unmodified or as large immunogenic fragments, the quantity of which will depend on the protein (e.g., resistance to digestion), the food (e.g., matrix, processing, protein levels/contents), or host characteristics (e.g., defect in digestive function), as explained in previous sections. The epithelial cells create an effective highly regulated tight barrier allowing selective permeability to these antigens as part of immune tolerance (Menard et al., 2010; Watson et al., 2005). In this context, oral tolerance is the “default” response induced towards harmless ingested food antigens/fragments present in GI lumen. Oral tolerance results from an active immune process and relies on a controlled luminal food antigen sampling through intestinal epithelium and on the pro-tolerogenic intestinal mucosa environment, both necessary for the preferential induction of regulatory T cells in the mesenteric lymph nodes.(Adel-Patient et al., 2011; Hadis et al., 2011); (Hamad and Burks, 2017; Rezende and Weiner, 2017). This suppressive response then relies on an intact intestinal barrier function, which can be affected by i) microbial signals (e.g., pathogenic bacteria, lipopolysaccharides, helminth infection and Helicobacter pylori-associated gastritis) (Corrado et al., 1998; Snoeck et al., 2005), ii) the diet (e.g., high-fat diet), iii) medication (e.g., non-steroidal anti-inflammatory drugs), iv) some properties of luminal proteins (e.g., allergens presenting protease activity, such as proteins from house dust mite (Grozdanovic et al., 2016; Price et al., 2014; Tulic et al., 2016), v) aggregation state or chemical-induced structural changes (Bernasconi et al., 2006) vi) chemical/physical or even psychological stress. An abnormal quantity of undigested proteins may also result in an alarm signal at this site {Perrier and Corthesy (2011) #71}. When the GI barrier is disrupted, there may also be a change in intestinal permeability leading to sensitization and allergic reactions to food proteins in predisposed individuals (Perrier and Corthesy, 2011; Sicherer and Sampson, 2010), but it appears that other factors may lead to food sensitization at the GI site.

Intestinal permeability measured by the lactulose/mannose test in food-allergic individuals and those with food hypersensitivity on allergen-free diets for six months was higher compared with healthy

controls (Ventura et al., 2006) and remained increased in asymptomatic food allergic children on elimination diets (Jarvinen et al., 2013), although contradictory results have been described (Andre et al., 1987; Ventura et al., 2006). Increased permeability and Th2 microenvironments have also been described in sensitized individuals and during the elicitation phase of the allergic reaction. It is hypothesized that food particles and allergens cross the epithelial barrier and activate mast cells bound with specific IgE in sensitized individuals, which permits allergen passage in a setting of elevated IL-4 and CD23 on intestinal epithelial cells (Kaiserlian et al., 1993) that bind IgE-antigen complexes (Belut et al., 1980) and enhance transport. In allergic individuals, chymases, histamine, TNF-α, IL-13 and IL-8 from mast cells appear to regulate epithelial cell permeability and cause allergen molecule transport through the barrier (Clayburgh et al., 2006; Wang et al., 2005). Inflammatory mediators (e.g., cytokines, proteases) lead to further disintegration of barrier function and increased passage of allergens (Perrier and Corthesy, 2011). These events may lead to local and systemic symptoms in sensitized individuals exposed to the sensitising proteins, but may also lead to de novo-sensitization to intact proteins present in the same microenvironment (i.e., co-ingested proteins). However, despite data supporting increased permeability to large molecules in allergic children (Heyman et al., 1988), it is not entirely clear whether large protein allergens can pass through the barrier and cause allergic sensitization. More studies are needed to determine whether increased GI permeability is a risk factor for food allergy in certain atopic patients because intestinal permeability parameters are not predictive of food allergy (Perrier and Corthesy, 2011).

Additionally, non-GI sensitization to food allergens may occur. The ALSPAC birth cohort, a retrospective analysis, revealed that 5-year old peanut allergic children had severe atopic dermatitis (AD) in the first 6 months of life and 90% of the peanut allergic patients with severe AD had been exposed to peanut oil containing emollients through their inflamed/altered skin. These findings suggest that sensitization might occur to food antigens through the disrupted skin barrier in the absence of oral tolerance induction through GI tract exposure (Lack et al., 2003). Moreover, the risk of peanut allergy was positively associated with the level of peanut allergens in the environment, notably in case of severe AD (Brough et al., 2013, 2015). Previous studies demonstrated that milk, egg and fish proteins were also detectable in domestic dusts (Dybendal and Elsayed, 1994; Witteman et al., 1995). An additional argument for cutaneous sensitization to peanut is that circulating peanut-specific T cells from peanut allergic patients express cutaneous homing receptors (Chan et al., 2012; DeLong et al., 2011), whereas T cells from peanut-tolerant children express intestinal and cutaneous homing receptors (Chan et al., 2012).

Various human and animal studies demonstrate that altered skin permeability and/or skin inflammation increases the risk of sensitization to foods. AD is a risk factor for food allergy and pre-school AD is positively associated with IgE sensitization to foods and aeroallergens up to 16 years of age (Johansson et al., 2017). Furthermore, neonatal skin barrier dysfunction (assessed by trans epidermal water loss) predicts food allergy at 2 years of age, which supports transcutaneous allergen sensitization, even in infants without AD (Kelleher et al., 2016). Thus, a disrupted skin barrier even without symptoms may lead to sensitization and food allergy. Sensitization through the cutaneous route leading to a loss of oral tolerance underlies a new subtype of wheat-dependent exercise-induced anaphylaxis in adult Japanese women following sensitization through the use of soap containing hydrolysed wheat protein (Chinuki and Morita, 2012; Yagami et al., 2017). There are many associations between genetic skin barrier deficiencies and food allergy, such as the filaggrin gene mutation (Brough et al., 2014; Brown et al., 2011; Kelleher et al., 2015; Venkataraman et al., 2014), serine protease inhibitor SPINK5 gene variants (Ashley et al., 2017) or corneodesmosin mutations (Israeli et al., 2011; Oji et al., 2010).

IgE-dependent food allergy can be induced using various intact food antigens (e.g., OVA, peanut, tree nut, cashew, milk, wheat) in animals sensitized through skin exposure and then elicited through oral challenges. In these models, a high IgE level is induced after skin disruption and local inflammation induced by tape stripping (Bartnikas et al., 2013; Kondo et al., 1998; Noti et al., 2014; Strid et al., 2004), or after application on skin with constitutive barrier defect (e.g., filaggrin mutations (Fallon et al., 2009; Kawasaki et al., 2012), or using occlusive patches (Birmingham et al., 2007; Gonipeta et al., 2009, 2010), detergents (4% SDS (Muto et al., 2014),) or TSLP-inducers (Vitamin D analog) (Noti et al., 2014). The underlying mechanisms involve local IL-4 production by TSLP-elicited basophils, leading to Th2 polarization. Sensitization through skin can lead to a partial loss or prevention of oral tolerance induction (Strid et al., 2004), or no change at all (Muto et al., 2014). In most studies, sensitization was not observed when the barrier was unaltered. However, other studies showed that peanut or milk allergen exposure on healthy skin could lead to Th2 cell priming, which suggests that sensitization may occur through the GI route without a Th2 mucosal adjuvant. However, this appears to depend on the food matrix and allergen (Wavrin et al., 2014, 2015). Sensitization may also occur through exposure via the respiratory tract to various inhaled food proteins (e.g., wheat, shellfish, soy, peanut, egg, milk) at farms, restaurants, school, home or food industries (Ramirez and Bahna, 2009; Taylor et al., 2000). There are cases of sensitization via the respiratory route leading to food allergy, for example, a patient with an occupational inhalant pork allergy developed food allergy to pork and chicken (Hilger et al., 2010). In mice, Wavrin et al. observed more intense and frequent IgG1 and Th2 cell priming after respiratory exposure through non-altered epithelium than after skin exposure (Wavrin et al., 2014). This raises the possibility that exposure to environmental food allergens through altered or inflamed respiratory epithelia along with tobacco smoke, diesel particles, aeroallergen proteases, viruses and endotoxins might lead to food allergy. Sensitization via the respiratory tract or skin may result from allergens with particular physicochemical properties such as conformation, polarity and ability to cross the epithelial barrier that are independent of GI digestion. However, protein size may not be the key determinant as shown in studies testing the sensitization potency of bovine beta-lactoglobulin (18 kDa) and Ara h 1 (63 kDa) via skin or respiratory routes (Wavrin et al., 2014, 2015). Additional properties include the capacity to aerosolise, e.g., in steam upon heating, coating with particles or dust may enhance the interaction with the respiratory tract and resistance to acidic skin conditions (in skin sensitization) and the persistence of allergens on various surfaces.

Barrier function plays an important role in IgE-mediated food sensitization. While integrity of the GI epithelium is clearly critical, other barriers play an important role, particularly in early childhood, and therefore should be included in allergenicity assessment of novel foods.

5. What is the role of brush border membranes (BBM) enzymes

The internal surface of the small intestine is carpeted by villi, which are lined by a monolayer of columnar epithelial cells, mostly enterocytes. The apical surface of the enterocytes is constituted by a dense array of microvilli microscopically observable as the brush border membranes (BBM). The BBM contains dozens of hydrolytic enzymes embracing all the suites of digestive hydrolases, e.g., glycosidases, lipases and at least 20 different exo- and endo-peptidases with different activities and specificities (Picariello and Addeo, 2016; Woodley, 1994). The BBM peptidases are membrane-bound (glyco)proteins facing outward from the epithelium into the intestinal lumen and can also be released in vesicles budding from the membrane into the periapical space of enterocytes (Hooton et al., 2015). BBM hydrolases, either

anchored in the membrane or in vesicles, are responsible for a key step of the final degradation of nutrients in the pre-absorptive phase. BBM peptidases are essential to process the oligopeptides resulting from the upstream digestion step and may be crucial in preventing the internalization of potentially harmful "non-self" polypeptides. Furthermore, both as free enzymes and in BBM vesicles, hydrolases are active in the intestinal lumen and are involved in complete protein digestion by direct action on chyme or by activating pancreatic zymogens.

The ability of BBM peptidases to degrade or progressively hydrolyse peptides generated by GI digestion has been clearly demonstrated for a number of substrates, including peptides arising from gastro-pancreatic digestion of wheat (Shan et al., 2002), peanut proteins, caseins (Boutrou et al., 2008; Picariello et al., 2015) and ovalbumin (Claude et al., 2019).

Exopeptidases, which are the most represented and most active among the BBM peptidases, erode progressively peptides by cleaving off the external residues at both ends of the chain (N or C terminal), except when the terminal amino acid is a proline. At least three different endopeptidases split large polypeptides into smaller sequences, which are further degraded during a phase of secondary proteolysis. The primary role of BBM enzymes is to produce absorbable amino acids or di-/tripeptides, which can be further hydrolysed by enterocyte intracellular peptidases, at the same time limiting the uptake of potential immunogenic peptides to prevent the activation of the immune system. Active BBM peptidases are expressed at the level of duodenum, jejunum and ileum, though most of the peptide absorption occurs at the level of the jejunum, which indicates that food-derived peptides released by any digestion step are exposed to BBM enzymes prior to or during absorption in the GI tract. BBM peptidases are particularly active on sequences up to 20–25 amino acid residues. However, also combined with residual duodenal enzymes, they exhibit endoprotease activity and can cleave off even entire caseins or gliadins, though much more slowly when compared to low-sized polypeptides (Woodley, 1994, 2009).

Due to intrinsic structural stability or to process-induced modifications altering digestibility, some food-derived peptides might escape BBM hydrolysis and, in principle, might translocate unaffected across the epithelial barrier. Thus, immunologically active peptides can reach and interact with the intestinal immune system, either in the sensitization or elicitation phases of food allergy. The immaturity of the digestive system and the increased intestinal permeability during early infancy might result in a relatively high uptake of immunologically active polypeptides, perhaps underlying a contribution in sensitization.

Little information is available on the role of BBM enzymes on the allergenic potential of a protein. Only few workflows of *in vitro* digestion have included a step simulating BBM degradation. However, there are relevant results in experiments aimed at identifying protein fragments highly resistant to digestion in prolonged digestion models or over-digested food systems (Hausch et al., 2002; Shan et al., 2002). Although the immune pathogenesis of celiac disease differs from food allergies, there appears to be a critical role of BBM enzymes with food-derived peptides that contact the intestinal immune-competent system. For example, the immune dominant 33-mer fragment of α2-gliadin and similar gluten-derived peptides eliciting celiac disease are resistant to rat and human BBM hydrolysis (Hausch et al., 2002; Shan et al., 2002). Similarly, the susceptibility to BBM enzymes in *in vitro* digestion models has implicated a molecular basis of the reduced celiacogenic potential of diploid einkorn (*Triticum monococcum*) compared to tetra- and hexaploid wheat (Gianfrani et al., 2015). In the case of OVA, its ability to induce the degranulation of a humanised rat basophil leukemia (RBL) cell line activated with allergic patients' sera varies according to its state of aggregation. Degranulation in response to native OVA slightly decreases during the gastric phase, but is mostly abolished after duodenal digestion with no further change induced by BBM digestion (Claude et al., 2019). In contrast, OVA aggregates exhibit a low degranulation capacity in all stages of digestion. Adding a BBM enzyme digestion step did not substantially change degranulation capacity of OVA aggregates, suggesting that the peptide sequences causing degranulation were most likely already degraded by duodenal enzymes. (Claude et al., 2019). No digestion studies were found using BBM enzymes to distinguish non-allergenic vs. allergenic food proteins.

The contact of epitopes with immunocompetent cells such as DCs might occur in the intestinal lumen, before BBM enzymes degrade polypeptides. However recent studies appear to confirm that food allergens must cross the gut epithelium, at least, in the elicitation phase (Chinthrajah et al., 2016). Taken together, these data indicate that food protein digestibility and allergenic potential must be assessed with BBM enzymes during peptide digestion. Thus, simulating digestion with BBM hydrolases should be included in models of digestion simulating the entire digestive process, though an experimentally established consensus on the use of BBM enzymes is lacking due to a series of factors (Hooton et al., 2015; Picariello and Addeo, 2016) including the following:

- More information on the activity of the different peptidases is needed.
- Enzyme activity should be determined using validated methods, which are currently lacking.
- Intra- and inter-individual variability of the peptidase activity makes comparison between studies difficult.
- The enzymatic activity of BBM enzymes significantly varies along the different segments of the small intestine, while food-deriving material is progressively adsorbed. Estimating a physiological relevant peptidases-to-food substrate ratio is challenging.
- BBM enzymes are not commercially available and are laborious to purify.
- Representative and standardised enzymatic preparations are needed.
- More studies are needed to compare activity of pig, mouse and human BBM enzymes.
- Information on physiologically relevant conditions to use BBM enzymes in models of simulated digestion is necessary, because conditions adopted in previous papers appear without justification (Boutrou et al., 2008; Hausch et al., 2002; Petrilli et al., 1984; Shan et al., 2002).
- An overall clear role of BBM enzymes in the allergic response of proteins must be elucidated

Simulating digestion with BBM hydrolases should be included in models of digestion simulating the entire digestive process, though an experimentally established evidenced based consensus on the use of BBM enzymes is lacking.

6. What is the involvement of antigen presenting cells in the digestion process?

Antigen presentation by APC is a critical regulator of adaptive immunity (see Fig. 2). APC take up food proteins, process them and present the resulting peptide fragments in the context of MHC II molecules to T lymphocytes. Processing and degradation of antigens in specialized cellular compartments such as endosomes and lysosomes are essential for many aspects of antigen presentation (reviewed in (Roche and Furuta, 2015)). This degradation process is tightly regulated and involves activation of different proteases (e.g., cathepsin S and asparaginyl endopeptidase) which in turn promote the peptide MHC II complex assembly. Susceptibility of proteins to lysosomal proteolysis plays an important role in determining their immunogenicity (Thai et al., 2004; Toda et al., 2011). There seems to be a highly regulated balance between antigenic protein proteolysis and complete

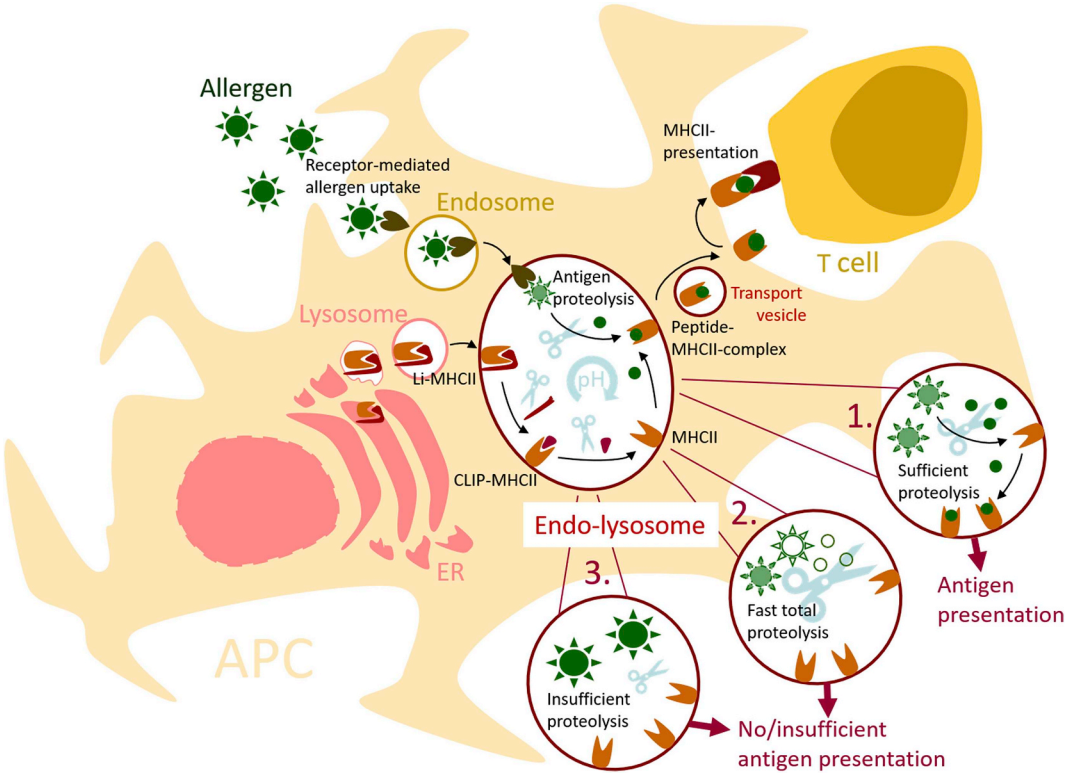

Fig. 2. Model of endo-lysosomal allergen processing and presentation. Allergens are captured by the APC by receptor-mediated uptake, followed by transport into the cell in endosomes. ER derived lysosomes containing MHCII αβ dimers coupled to the invariant chain (Li-MHCII) merge with this endosome to form the endo-lysosome. In this cellular compartment, Li is proteolytically removed from MHCII, leaving class II-associated invariant chain peptide (CLIP) at the binding groove of MHCII, which is enzymatically removed, facilitating peptide binding onto MHCII. In addition, at a lowered pH, allergens are proteolytically degraded into peptides, which can bind MHCII. These peptide-MHCII complexes are transported to the cellular membrane by transport vesicles where they can lead to MHCII presentation to T cells. In the endo-lysosome, sufficient proteolysis of allergens leads to peptide binding to MHCII and antigen presentation (1). If allergens are quickly totally degraded (2), or resistant to proteolysis (3), no or insufficient antigen presentation occurs

degradation of proteins and destruction of peptides bound to MHC II in the antigen processing compartment of the APC. The stability to endolysosomal proteolysis leads to low-density of MHC II-peptide complexes on DCs which favours Th2 responses (Buatois et al., 2003; Delamarre et al., 2003). Indeed, efficient Th2 cell activation requires high stability in the early endosome, but efficient degradation in a lower pH environment in the late endolysosomal compartment (Machado et al., 2016). However, both extremes, *e.g.,* very high and very low protein stability to proteolysis seems to result in inadequate quantities of T cell peptides needed for efficient antigen presentation.

In vitro assays determining endolysosomal degradation may be an efficient tool providing information on the immunogenicity or allergenicity of proteins and information about peptide clusters harbouring T cell epitopes. In allergy research, two assays have been used to simulate antigen processing in the lysosomal/endolysosomal compartment upon uptake by professional APC (Delamarre et al., 2005, 2006; Egger et al., 2011). The first method involves the coupling of proteins to latex beads, which allows for tracking of intracellular, phago-lysosomal protein degradation over time in APC by flow cytometry (Hoffmann et al., 2012; Savina et al., 2010). This method correlates with phagosomal functions, antigen degradation and APC processing and loading. This method was used to assess and compare degradation of several food allergens, including OVA, peanut Ara h 1, Ara h 2, Ara h 3 and Ara h 6 and ß-lactoglobulin. Intracellular protein degradation was higher for Ara h 1 and Ara h 3 than for Ara h 2 and 6, which might correlate with the magnitude of T cell activation by these allergens (Smit and Pieters, 2017). In addition, modification of ß-lactoglobulin by glycation changed intracellular degradation, which most likely caused this protein to be more available for proteolysis, leading to decreased immunogenicity (Perusko et al., 2018). The second method is used more often than the first and focuses on the fractions containing endolysosomal proteases from APC. The DC line, JAWS II, as well as mouse bone marrow- and human blood-derived DCs are most frequently used because of a high similarity in composition and proteolytic activity of isolated endolysosomal fractions (Egger et al., 2011). However, proteolytic activity differs between proteases from different cell types, with highest protease efficiency in macrophages > DCs > B cells, though, highly similar peptide profiles were observed despite kinetics differences (Hofer et al., 2017; Roche and Furuta, 2015). Typically, proteins are incubated with lysosomal fractions at 37 °C at low pH which simulates the endolysosomal environment and degradation kinetics is monitored by gel electrophoresis and proteolytic peptides are analysed by mass spectrometry. The lysosomal fractions contain several endo- and exo-proteases like cathepsins and legumins, which are capable of enzymatically processing the intact protein. A major role is attributed to cathepsin S (Egger et al., 2011; Pablos et al., 2018). The importance of cathepsin S was shown by specific inhibition during endolysosomal degradation, which resulted in a drastic reduction of proteolytic activity and peptide generation (Wildner et al., 2017). A key feature of endolysosomal degradation is the identification of peptide clusters containing important T cell epitopes (Gadermaier et al., 2011; Mutschlechner et al., 2010; Schulten et al., 2011). Thus, *in vitro* approaches may help to downscale the number of synthetic peptides for T cell assays, thereby minimizing the use of material from patients. This assay was used to study the degradation of several food proteins, which correlated with immunogenicity and allergenicity. For instance, a hypoallergenic variant of peach Pru p 3, which was susceptible for simulated endolysosomal degradation displayed low *in vivo* immunogenicity and low IgE reactivity (Toda et al., 2011). However when comparing allergenic lipid transfer proteins, Pru p 3 showed higher

stability in the endolysosomal degradation assay than comparators Api g 2 and Art v 3, accompanied by a constant supply of relevant peptides (Gadermaier et al., 2011). Furthermore, in all studies, previously identified immunodominant T cell epitopes of Pru p 3, were represented within obtained peptide clusters (Schulten et al., 2009). Immunization with native Ara h 6 from peanut induced only limited IgG levels and presented high resistance to *in vitro* cathepsin digestion compared to the immunogenic heat-treated proteins, which showed lower stability (Guillon et al., 2016). Lipid binding led to a stabilizing effect during endolysosomal degradation of Ara h 1 from peanut, Sin a 2 and Sin a 3 from mustard and authors suggest this might contribute to their potent allergenic capacity (Angelina et al., 2016). Comparative analyses of allergenic and non-allergenic molecules of the 2S albumin, tropomyosin and collagen protein family indicated a slightly higher stability of non-allergenic representatives (personal communication G. Gadermaier).

There are several knowledge gaps associated with the extent of allergen endolysosomal degradation and the methods to study it. For example, studying allergen endolysosomal degradation reflects sensitization at the T cell level, but it does not provide information for direct predictions about protein-IgE binding. Both methods are useful tools for studying the intracellular processing of allergens and estimating protein immunogenicity or allergenicity (Egger et al., 2011; Perusko et al., 2018). However, because endolysosomal degradation is performed with purified molecules, the influence of lipid or small molecule binding is unknown and requires further investigation. Furthermore, the context for allergen uptake and presentation might also play a role in the observed outcome of immunogenicity. Additionally, organ-specific pre-processing in the lung, skin or gut has not been considered. Assuming that sensitization to food allergens takes place in the GI tract, enzymatic digestion prior to endolysosomal processing should be considered.

In vitro assays determining endolysosomal degradation may be an efficient tool to provide information on the process leading to immunogenicity and allergenicity of proteins. However, available methods are until now proven to be particularly valuable for comparisons of protein isoforms, modified variants, and different batches. Additional standardized tests in line with in vivo models should be performed to improve the ability to predict the allergenicity of a protein.

7. What is the influence of the food matrix and processing on digestion?

The structure of food and its processing can significantly impact digestion kinetics (Parada and Aguilera, 2007) and can have an impact on food protein allergenicity. For example, there are reports that show that susceptibility to digestion, a measure of protein stability, relates to allergenic potential (Bøgh and Madsen, 2016). Despite the debate on this subject, the pepsin resistance test proposed by Astwood et al. (1996) has been widely used on pure proteins, without taken the effect of the food matrix and processing in to account. This is probably due to the complexity of interpreting the data generated with complex mixtures. However, as evidence emerges on the effect of different types of processing on various measures of allergenic potential, typically some form of immunoreactivity such as IgE binding, it is becoming clear that there are many factors at play. These include exposure and routes of sensitization as well as the type of food and the way that it is processed. We do not eat protein, we eat food and that means that the food matrix is always highly relevant. Recent examples of this notion are studies looking at the cellular entrapment of nutrients in raw or cooked tree nuts, showing that a significant proportion of the nutrients are never released and consequently pass through the GI tract without being absorbed (Grundy et al., 2015).

Foods are often subjected to different kinds of processing before being consumed. Simple peeling of the apple can lower exposure to allergens when consumed, as there are high allergen concentrations in the skin. Heating proteins, for example, can cause them to unfold and lose secondary and tertiary structure. This in turn may lead to an increase in surface hydrophobicity that is sufficient to drive aggregation. Unfolding and loss of structure can increase the accessibility of proteases to their substrate and thus make proteins more digestible. Excessive aggregation may also have the reverse effect depending on the density of the aggregates. For example, the whey protein β-lactoglobulin is resistant to pepsin in its native state but heating can significantly increase its susceptibility to pepsin hydrolysis. However, if it is heated in a way that large aggregates are formed such as when the pH is close to its isoelectric point (around pH 5), then pepsin resistance is again increased (Macierzanka et al., 2012). Enzymatic crosslinking of heat denaturated β-lactoglobulin can increase sensitising power even though it might lower elicitation (Stojadinovic et al., 2014). The extent to which thermal processing affects allergenic potential depends upon the protein in question. Some proteins such as caseins or Bet v 1 homologues are only weakly folded, whereas others such as the lipid transfer proteins (LTPs) have a more rigid structure. The former group are heat-resistant but are easily digested while the latter group are more resistant, yet both groups can be allergenic. Heating of the Bet v 1 homologue from apple, Mal d 1, significantly lowered IgE binding capacity although T-cell reactivity was increased (Bohle et al., 2006) and yet the apple LTP, Mal d 3, is highly resistant to thermal processing (Sancho et al., 2005). The effect of thermal processing (raw vs. roasting or boiling) on peanut allergens has been widely investigated. The results show that the IgE-binding capacity of purified Ara h 1 and Ara h 2/6 was altered by heat treatment (Blanc et al., 2011; Vissers et al., 2011). However, no significant difference in IgE immunoreactivity of Ara h 2/6 was observed between whole protein extracts from raw and roasted peanuts suggesting that native IgE-reactive protein is still present in the seed despite thermal treatment (Vissers et al., 2011). A decrease in allergenicity of boiled peanuts resulted mainly from a transfer of low-molecular-weight allergens into the water during cooking (Mondoulet et al., 2005).

Heating is only one type of food processing, albeit the most common. A range of other processes such as high pressure (HP), ultrasound, etc. have been considered as potential methods to reduce allergenicity. HP treatment of apple (Husband et al., 2011) showed that Mal d 1 was subject to loss of rabbit IgG reactivity as soon as the apple tissue was disrupted, although it was remarkably resistant to both thermal and HP processing. The other major allergen in apple, Mal d 3, was found to be resistant to thermal processing in apple, which is in contrast to behaviour in solution. However, the combination of HP and heat treatment significantly reduced its IgG reactivity. The IgG reactivity of Mal d 3 strongly correlates with a loss of secondary structure of the protein (Johnson et al., 2010). Pectin was found to protect Mal d 3 from thermal denaturation in solution and is a possible candidate for the protective effect of the fruit and highlights the importance of the food matrix as discussed below. Already it is becoming clear that the evidence is inconclusive, even for a single allergen. Only hydrolysis can completely remove the allergenic potential of a protein (Verhoeckx et al., 2015). Additionally, structural homology does not reliably predict the effect of processing on allergenicity, so that individual food allergens should be tested and interactions with other proteins, fat, and carbohydrates in the food matrix should be considered (Nowak-Wegrzyn and Fiocchi, 2009).

Foods are composed of carbohydrates, fat, proteins and various micronutrients, collectively designated the food matrix. The food matrix may affect the kinetics of digestion of proteins. This is not least because the buffering capacity of high protein content foods will increase initial gastric pH and this will decrease pepsin activity as well as alter GI motility through GI hormone-controlled feedback. High fat can

have similar effects on GI motility depending on when lipid empties into the duodenum. Different types of food matrices (cellular, baked, emulsified, etc.) can have different effects (Mandalari et al., 2014; Nowak-Wegrzyn and Fiocchi, 2009), which may occur over a range of length-scales from molecular interactions such as glycation to encapsulation and factors affecting GI motility. Often it is not clear whether the matrix or the processing has a role to play such as when egg or milk allergic patients can tolerate baked foods containing the relevant allergens. For example, Lemon-Mulé et al. (Lemon-Mule et al., 2008) reported that 70% of children with IgE-mediated egg allergy tolerated baked egg in the form of a muffin (heated at 176 °C for 30 min). The authors state that "in the case of egg white, interactions with the food matrix at high temperatures are important for decreasing allergenicity". These are similar results to those obtained in children presenting with IgE-mediated milk allergy (Nowak-Wegrzyn et al., 2008). In more recent work on egg allergy, cake was tolerated by 88% of children and omelette food challenge was passed in 74% of cases. This suggests that the food matrix was only important in a small number of cases and that the important factor was the heating (Miceli Sopo et al., 2016). Both factors may be important for digestion kinetics (Mulet-Cabero et al., 2017). At the molecular scale, a study on the effect of glycation on β-lactoglobulin, showed that high levels of glycation decreased proteolysis and, consequently, increased IgG and IgE reactivity of hydrolysates, regardless of the carbohydrate used. Protein aggregation during the advanced stages of Maillard reaction had a masking effect on protein epitopes, counteracting the negative effect of the lower digestibility of glycated protein on its allergenicity (Corzo-Martinez et al., 2010). On the other hand, differences in the response to allergens embedded in different matrices may be a result of differences in gastric residence times. A small study using fat continuous (chocolate) or water continuous (mousse) food showed that the chocolate had a longer residence time and caused a higher threshold (delayed response) to an oral challenge to peanut (Mackie et al., 2012).

The progress in food allergy research has allowed us to assess risk on a population scale and while processing and the food matrix have an effect on digestion kinetics and allergenicity, we are a long way from understanding the effects to the extent that we can predict the direction and magnitude of any change in these parameters and properties.

Processing and the matrix may increase, decrease or abolish the allergenic potential of food proteins, but whether this is a result of altered digestibility remains to be investigated. Current evidence does not provide any clear rules regarding how matrix and processing affect digestion and allergenicity. The same processing of one protein can increase allergenicity, while with another protein allergenicity is decreased.

8. What are the advantages of static or dynamic *in vitro* digestion models?

The most physiologically-relevant way for studying food digestion is to use human volunteers. However, clinical trials are expensive and sometimes might be ethically questionable especially when invasive strategies are used to collect effluents through nasogastric probes for instance. Animal models have other drawbacks and this is why a large variety of *in vitro* digestion models mimicking the human GI tract has become very popular. Both static and dynamic *in vitro* digestion models are used.

Static models consist of a series of bioreactors that mimic the physicochemical and enzymatic environment the food will meet when entering different compartments of the GI tract (mouth, stomach, small intestine). It is static because every step is fully completed after the next one starts, thus, there is no flow of food between the different compartments until the step is completed. Additionally, the pH, concentration of digestive enzymes, bile salts, etc. are constant in the different compartments. Static models are easy to use, inexpensive and reproducible. INFOGEST COST Action experts reviewed the correlation between data from digestion models and *in vivo* experiments (Bohn et al., 2017) and found that the static models predict end-point values like glycaemic index, bio-accessibility of a limited number of nutrients or digestion-resistant peptide, address underlying molecular mechanisms (Mandalari et al., 2009b) and are used to compare large series of samples with identical conditions for screening purposes (Dupont et al., 2010a). In animal science, static *in vitro* digestion models have been shown to be reliable predictors of end-point values such as protein and amino acid digestibility (Ekmay et al., 2017) but, to our knowledge, have not been used for showing the persistence of large protein fragments in the gastrointestinal tract that could be able to stimulate the immune system and cause food allergy. The downside is that these models may be oversimplified and might lack physiological relevance and are not useful for following dynamic events in the GI tract such as the kinetics of macronutrient hydrolysis.

Dynamic *in vitro* digestion models recreate the dynamic aspects of food digestion. These systems simulate food transit within the different compartments of the gut. The pH is regulated, digestive enzymes and bile are injected in real time and nutrient absorption is possible in the small intestine for some of them. INFOGEST experts confirmed that these systems mimic human physiology when defined parameters are available to control the digestive process (Dupont et al., 2018). However, systems available on the market might be expensive and are not always adapted to solid foods requiring a thorough mixing of the food to avoid plugging of the system. Most of them also require significant amounts of food and digestion enzymes and may not be relevant for studying the fate of purified expensive nutrients or bioactives. It is not yet clear whether studying the dynamics of the digestion is needed for assessing food allergy sensitization or whether end-point measurements are sufficient. Dynamic *in vitro* digestion models are more appropriate for investigating the fate of allergenic foods in the GI tract, assessing the resistance of allergens to digestion and determining the effect of matrix and food composition. However, for pure expensive single allergens, preliminary studies with validated *in vitro* static models might provide valuable information with the added advantage of being cheap and providing information on the resistance of a protein to GI digestion. As a compromise between simple, inexpensive static models and more complex and costly dynamic systems, semi-dynamic systems that combine a dynamic gastric step with a static intestinal step were developed. The gastric step, including a gradual pH decrease and pepsin secretion, substantially impacts on the variability of protein digestion. This has been clearly shown for the digestion of skim milk for example (Egger et al., 2019)). Indeed, during the gastric phase of a static digestion, the pH is set at 3.0 and is therefore close to the optimal pH of pepsin (pH 2.0). Consequently, proteins like caseins are fully degraded after 30 min of gastric digestion with this model. In contrast, a dynamic gastric condition decreases gradualy the stomach pH from 6.8 to 3.0 in 42 min resulting in a slower breakdown of caseins and a proportion of intact caseins are still visible after 60 min of digestion. Semi-dynamic systems combine the dynamics of the gastric digestion and are still reasonably simple to apply. Because the GI tract is changing over lifetime, static and dynamic models mimicking the digestive systems of specific populations have been developed such as static (Dupont et al., 2010b; Menard et al., 2018) and dynamic (de Oliveira et al., 2016a,b; Menard et al., 2014) models of the infant gut. Similarly, the GI tract of the elderly has been modelled (Levi and Lesmes, 2014). However, there are no models for disease and atopic individuals, nor models integrating a complex microbiota and its metabolic activity.

The gradual hydrolysis of the main milk proteins present in a rehydrated skim milk powder was compared after *in vitro* digestion with the dynamic DIDGI® digestion system developed at INRA (Menard et al.,

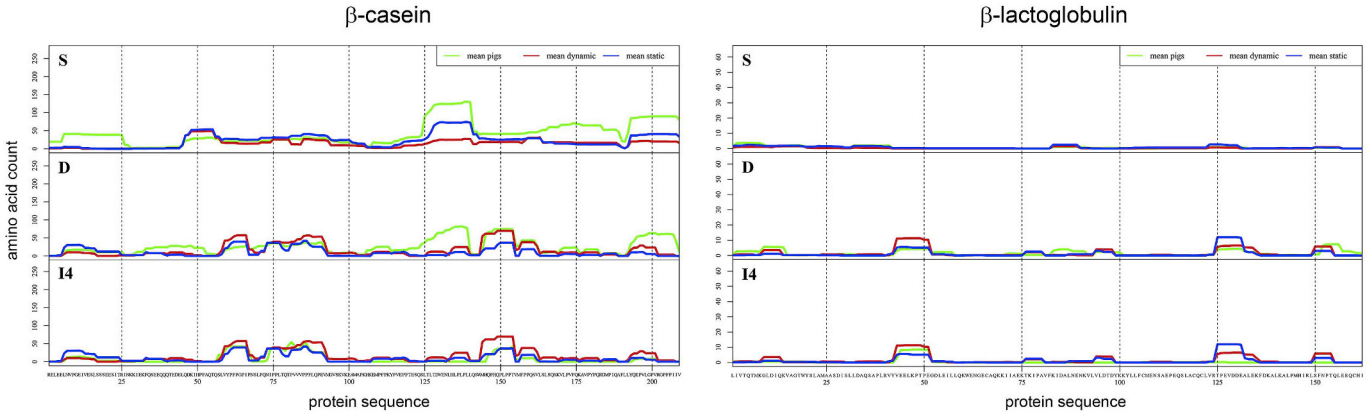

Fig. 3. Qualitative comparison of dynamic (red line), static (blue line) and *in vivo* pig (green line) digestion for β-casein and β-lactoglobulin. The same skim milk powder was subjected to the three different digestion models. The resulting peptide patterns were measured by mass spectrometry and endpoints of the *in vitro* digestion models were compared to the stomach (S) sample from pigs, while the intestinal endpoints were compared with pig samples from the duodenal (D) and the last section of the small intestines (I4). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

2014) and the static harmonized INFOGEST method (Minekus et al., 2014). Protein hydrolysis was analysed by gel electrophoresis (SDS-PAGE), peptide patterns were established by mass spectrometry (MS) and free amino acids quantified with high-pressure liquid chromatography (HPLC). Overall, protein hydrolysis had a similar qualitative behaviour in static and dynamic digestion models (Fig. 3). In the gastric phase, the peptide patterns were similar, correlation between peptide patterns from static and dynamic *in vitro* digestion calculated for αs1-casein achieved a factor of 0.77. Moreover, the peptide patterns obtained at the gastric and intestinal endpoints with both *in vitro* digestion protocols had a good approximation to *in vivo* results previously collected on pigs (Egger et al., 2019) and were correlated with a factor of 0.71 (static) and 0.88 (dynamic). Gradual peptide generation was comparable under both *in vitro* digestion conditions. However, the formation of a coagulum and a cream layer in the gastric phase that occurs during milk digestion *in vivo*, was absent in the static model due to the absence of mixing, but was present in the dynamic model. Therefore, dynamic *in vitro* digestion models should be chosen to study kinetic processes occurring during digestion, notably for whole food including matrix. At the intestinal endpoint, the total correlation calculated over all proteins was 0.89 between static and dynamic models, 0.81 (static-*in vivo*), and 0.85 (dynamic-*in vivo*). Moreover, at the level of free amino acids, the dynamic protocol resulted in a gradual release of free amino acids that was closer to the *in vivo* situation. This observation can be explained by the gradual addition of active enzymes during the digestion process. This appears to be the first study comparing experiments conducted with static and dynamic *in vitro* digestion models with *in vivo* data and more studies are needed to investigate the difference between the models and relevance to humans on other types of food matrices.

Ideally, the best way to investigate digestion of allergens would be to submit the allergen-containing food to a validated and standardised *in vitro* digestion protocol. However, pH and enzyme conditions should be adapted when allergy sensitization is linked to an impaired digestion system (e.g., higher pH, less enzymes). This would enable a detailed assessment of the decomposition of the proteins and the detection of critical and even questionable allergenic peptides. Notably, dynamic or semi-dynamic models handle possible matrix effects and the buffering capacity of the food when mixing conditions are carefully chosen. When such foods are not available, like in the case of pure allergens, dedicated *in vitro* static models could be an alternative with the limitation of the end-point of gastric or intestinal digestion, without providing information about the fate of the molecules during the gastric or intestinal step. A consensus model for allergenicity testing agreed by experts in the field is crucial. Finally, the ongoing development of microsystems to miniaturize the GI process might be added value for the *in vitro* digestion of pure allergens because the quantities of proteins that will be required will be extremely low. In this field, microfluidics might speedup the development of miniaturized *in vitro* digestion models (Marze et al., 2014).

Ideally, the best way to investigate digestion of allergens would be to submit the allergen-containing food to a validated and standardized in vitro digestion protocol, where the pH and enzyme conditions are adapted to atopic individuals. It is not yet clear whether studying the dynamics of the digestion is needed for assessing food allergy sensitization or whether end-point measurements are sufficient. Dynamic or semi-dynamic models must be used when matrix effects and the buffering capacity of the food must be considered.

9. What is the importance of digestion readout: *intact proteins and fragments?*

Resistance to digestion in the GI tract has been considered a general feature of 'complete' food allergens, because for a protein to sensitise via the GI tract, the protein must survive as intact protein or as large peptide fragments to be efficiently recognised by the immune system. In 1996, Astwood and colleagues concluded, that resistance to digestion is an effective parameter for distinguishing food allergens from non-allergenic proteins (Astwood et al., 1996). However, there is still no clear definition on when to consider a protein resistant or susceptible to digestion (Bøgh and Madsen, 2016) and no consensus about the importance of the resistance of peptide fragments during the digestion process. However, the length, the persistence, the abundance as well as the rearrangement of peptide fragments during the digestion process, may be important for potential residual allergenicity. Furthermore, there is no clear-cut size above which peptide fragments may act as 'complete' allergens and below which they will not. Though a general opinion appears to be that the lower size limit for allergenicity of peptide fragments is around 3.5 kDa (FAO/WHO, 2001; Huby et al., 2000; Lack et al., 2002; Poulsen and Hau, 1987) or 30–32 amino acids. Recently, the EFSA GMO panel suggested that stable peptide fragments ≥9 amino acids might possess residual allergenic potential due to efficient peptide binding to HLA-DQ (Naegeli et al., 2017). Indeed, an assessment of the *in vitro* digestibility may provide valuable insights on the resistance of a given protein or a whole food to digestion in the GI tract. However, the use of appropriate analysis methods for assessment

of residual intact protein and derived fragments is essential.

Almost all studies evaluating resistance of food allergens to digestion assessed the survival of intact protein, fewer studies examined the stability of peptide fragments emerging during the digestion process and even fewer identified the size distribution profile of the generated peptide fragments (Bøgh and Madsen, 2016; Di Stasio et al., 2017a, 2017b). The majority of studies evaluating the resistance of proteins and arising peptide fragments, did so by SDS-PAGE alone or in combination with Western blotting methods, although other methods using HPLC or MS have been used as well (Bøgh and Madsen, 2016).

Several studies have stressed that the choice of methods used for detection of residual intact allergen and emerging peptide fragments may heavily impact the experimental outcome and therefore the interpretation of the results of the digestibility tests. Some methods allow for detection of the amount of residual intact protein or stable peptide fragments emerging from the digestion process, whereas other methods only determine the presence or absence of proteins and peptides, with more or less sensitivity and specificity. For example, a study investigating the digestibility of the milk allergen β-lactoglobulin, showed that no intact β-lactoglobulin was left after digestion according to SDS-PAGE, while HPLC analysis showed that approximately 10% residual intact β-lactoglobulin remained (Mandalari et al., 2009b). Similarly, an evaluation of the residual intact kiwi allergen Act d 2 after digestion using SDS-PAGE revealed that no intact protein was left, while trace amounts of residual intact protein were observed when evaluated by HPLC (Bublin et al., 2008). This clearly indicates that the two analytical methods for assessment of intact protein have different detection limits and that the methods used for detection of emerging peptide fragments may heavily impact on the experimental outcome. For example, in a study investigating the digestion products of kiwi allergens, stable peptide fragments were detected by Western blotting with sera from allergic patients, but not with a Coomassie staining of SDS-PAGE (Lucas et al., 2008). In another study, it was shown that neither staining of SDS-PAGE nor western blotting with patient sera resulted in the detection of any peptide fragment generated during the digestion of the avocado allergen Prs a 1. However, stable peptide fragments were clearly detected when using more sensitive and specific methods such as MS in combination HPLC, skin prick test (SPT), or inhibitory immunoblot and ELISA (Diaz-Perales et al., 2003).

Not only the type of assay, but also its precise experimental protocol and reagents may influence the interpretation of the outcome of the digestibility test. For instance, Thomas et al. (2004) reported that the type of electrophoresis gel and the fixation techniques when performing SDS-PAGE might influence the detection of stable peptide fragments. Furthermore, in an inter-laboratory ring trial evaluating digestibility of milk allergens, it was shown that the digestion assay was robust, but sampling and electrophoretic methods had a significant impact on reproducibility (Mandalari et al., 2009a). Collectively, these results stress the importance of evaluating residual intact protein and generated peptide fragments hereof in assays with appropriate sensitivities and robustness, and that the use of more than one method is worthwhile. To date, no single method is suitable for the detection of both intact proteins and peptide fragments of different sizes in an effective manner, and consequently, combinations of methods may provide more realistic digestibility test results (Di Stasio et al., 2017b; Di Stasio et al., 2017a; Naegeli et al., 2017). Other precautions should be considered when preparing digestion products for protein-chemical analysis to preserve structural integrity, because peptides tend to form specific formations which is critical for the allergenic potential of digestion products (Bøgh et al., 2012). Importantly, additional studies are needed for improved recommendations of readouts for analyses of digestion products. Thanks to the recent advances in -omics strategies, new and improved analytic methods, which are continuously being established and will eventually improve high-resolution analyses of digestion products.

No single method is suitable for the detection of both intact proteins and peptide fragments of different sizes. Additional studies are needed for improved recommendations of readouts for the analyses of digestion products (intact protein, fragments and aggregates hereof).

10. What is the importance of digestion readout: *bioactivity/ allergenicity?*

An important aspect when evaluating the digestibility of allergenic proteins or foods is to assess whether the digestion products may retain allergenic potential after degradation in the GI tract. Certain food allergens are highly susceptible to digestion, but their digestion products may be allergenic (Bøgh and Madsen, 2016). This stresses the importance of using biologically relevant and specific methods, in addition to classical biochemical methods to assess allergenic potential of peptide fragments generated during digestion. Allergenic potential is defined by three distinct features, the ability of a protein to bind IgE antibodies, to elicit an allergic reaction and the capacity to sensitise *de novo* (Aalberse, 2000). This emphasises that the allergenic potential of digestion products should be evaluated on these different levels. Only proteins and/or derived digestion products possessing all three features are acknowledged as complete allergens, as defined by Aalberse (2000). Notably, not all allergens are complete allergens, even in their intact form. Well-recognised examples of incomplete food allergens are proteins homologous to the birch pollen allergen Bet v 1, that may elicit allergic reactions but normally do not sensitise through the GI tract due to their high sensitivity to digestive proteases (Schimek et al., 2005). However, the major peanut allergen Ara h 1, a very labile protein that is easily digested to small peptide fragments, retains its ability to act as a complete allergen after simulated GI digestion, being able to bind IgE, elicit reaction as well as to sensitise (Bøgh et al., 2009; Eiwegger et al., 2006). This relies on the capacity of generated peptides to reassemble into specific structures.

Several studies have evaluated the IgE binding capacity of digestion products generated after *in vitro* digestion (Bøgh and Madsen, 2016). For food allergens, the IgE binding ability of digestion products can easily be addressed when patient sera containing specific IgE to the parent protein is available. The IgE binding capacity may be evaluated by different methods, whereof immunoblotting and ELISAs are the most common methods of choice. However, the results from these assays may differ. For example, the IgE binding capacity of digestion products from avocado and hazelnut was not detectable using immunoblotting, but was measurable when using an inhibition ELISA and EAST (Diaz-Perales et al., 2003; Vieths et al., 1999). This discrepancy could be explained by differences in sensitivity of the methods. The evaluation of the IgE binding capacity of food allergens after digestion has resulted in abrogation, reduction, unchanged or even increased IgE binding capacity. For example, the assessment of IgE binding capacity of peanut allergen Ara h 3 and caviar allergens demonstrated that no residual IgE binding capacity was left after digestion (Untersmayr et al., 2003; van Boxtel et al., 2008). In contrast, IgE binding capacity of the egg allergen ovomucoid and of cod allergens was retained after digestion, although reduced compared to the parent proteins (Takagi et al., 2005; Untersmayr et al., 2005b; Urisu et al., 1999; Yamada et al., 2000). In other studies, IgE binding capacity of grape allergen Vit v 1 and milk allergen β-lactoglobulin showed that IgE binding capacity of the digestion products was similar to the parent allergens (Selo et al., 1999; Vassilopoulou et al., 2006), whereas IgE binding capacity of β-lactoglobulin and kiwi allergens digestion products in another study was increased in comparison to the parent proteins (Haddad et al., 1979; Lucas et al., 2008). Only a few studies found a correlation between IgE binding capacity and the size of the digestion products (Bøgh and

Table 2
Summary of the discussion on relevance of digestion in allergenicity testing and the identified gaps.

	Digestion is relevant for allergenicity testing	Digestion is not relevant for allergenicity testing
What is known?	• Everything goes through the mouth, digestion increases exposure and is therefore relevant in risk assessment • Digestion is related to sensitization through GI tract • Many stable proteins (in stomach) are causing severe allergic reactions. The labile allergens are causing mild reactions and are mostly cross reactive with pollen. • When the pH in the stomach is altered (elevated), more people get sensitized (infants, antacids) • Poor predictability of current tests: too much focus on disappearance of intact protein, not on appearance of allergenic/sensitising fragments. • Processing and food matrix influence digestion • endolysosomal digestion in APC and by BBM enzymes should be considered	• Digestion is not predictive since some known allergenic proteins are stable while others are labile. This is also seen for non-allergenic proteins • Fast allergic reactions are already seen when food is in the mouth of a patient. The food did not enter the stomach yet and is thus not subjected to proteolysis. • Where does sensitization takes place? Other routes (skin, lung) of exposure might play a role in food allergy. • Barrier is important • Intact protein enters the intestinal compartment due to gastric emptying • In humans digestion is depending on many individual factors (age, medication, food consumption habits etc.), which are not constant throughout the life. Tests can only be predictive for the general population, but never for an individual, due to these variations.
What are the gaps	• Evidence role of elevated pH on digestion mechanism and what is the pH in the GI tract of atopic individuals? • Rational to use a certain enzyme: protein ratio and pH in digestion assay. • The role of microbiota in food allergy development/sensitization/food digestion/gastrointestinal barrier function? • The exact mechanisms of trans-epithelial and transcutaneous transport that allow passage of food allergens to migrate and interact with the immune system, to determine whether there are differences between allergenic and non-allergenic proteins. • Knowledge regarding site/mechanisms of sensitization (lung, skin, gastrointestinal) • The role of endolysosomal digestion, can we use it as a standalone model or should it be incorporated in a digestion strategy? • The role intestinal BBM enzyme hydrolysis in allergic responses, can we use it as a standalone model or should it be incorporated in a digestion strategy? • A better understanding about the influence of meal composition on pH levels and gastrointestinal transit time of the food bole will be essential to better predict protein digestibility. • Information on how processing influence digestion. Do other fragments occur or is just the kinetics changed? • Evidence that a physiological model is more predictive than static model. • Information on what should be the readout: kinetics, endpoint products, bioactivity and which methods can be used to measure these endpoints. • Information on how well peptide fragments of different sizes elicit and sensitise? • Information on how non-allergenic proteins behave and what are good reference proteins.	

Madsen, 2016). Moreover, various outcomes were seen for the same allergens as a consequence of exact digestion and evaluation methods used, as exemplified for β-lactoglobulin, but also based on the specificity of the sera used to assessed IgE-binding capacity.

Few studies have investigated residual eliciting capacity of allergens, i.e., the ability of the allergens to cross-link IgE antibodies on effector cells after digestion. Assessing the eliciting capacity is a measure of the biological/functional relevance of digestion products generated during the digestion process and is more than just an ability to bind IgE. Elicitation *in vitro*, *ex vivo* and *in vivo* methods include humanised RBL assay, histamine release from human basophils (BAT) or skin prick test (SPT) and have led to different outcomes depending on the allergen. An abrogation of eliciting capacity after digestion was shown for the apple allergen Mal d 1, the hazelnut allergen Cor a 1 and the celery allergen Api g 1 which abrogate the eliciting capacity after digestion (Schimek et al., 2005), whereas the eliciting capacity was retained, though in a reduced manner, for the avocado allergen Prs a 1, the cow's milk allergens β-lactoglobulin and α-casein, cod allergens and cherry allergens (Diaz-Perales et al., 2003; Morisawa et al., 2009; Scheurer et al., 2004; Untersmayr et al., 2005b, 2007). The digestion products from peanut allergen Ara h 1 and the grape allergen Vit v 1 retain a similar eliciting capacity as the parent proteins (Eiwegger et al., 2006; Vassilopoulou et al., 2006).

While evaluation of IgE binding and eliciting capacity can be undertaken with human samples, sensitization studies cannot be studied in humans for ethical reasons. Although no animal model has been validated for use in sensitization assessment, they are regarded as good alternatives to human, due to development of IgE responses with similar specificity (Fritsche, 2009). Only few studies have evaluated the residual sensitising capacity of digestion products. For example, the sensitising capacity of digestion products from β-lactoglobulin, showed that the digestion process abolished the sensitising capacity of β-lactoglobulin, while digestion products from the peanut allergen Ara h 1 retained sensitising capacity compared to the parent protein (Bøgh et al., 2009, 2013). Partial hydrolysis of β-lactoglobulin only reduced its tolerising potency that relies on induction of specific Treg cells in the GALT, whereas it was suppressed by extensive trypsin hydrolysis. This probably results from complete destruction of T cell epitopes during hydrolysis and lead to ignorance by the GI immune system.

Based on current knowledge of residual allergenicity of allergens after digestion, digestion may abrogate, reduce, leave unchanged or even increase allergenic responses compared to the parent protein or food. Untersmayr et al. (2005b) showed that while digestion products may retain IgE binding capacity they may not retain eliciting capacity, underlining that even though allergens may retain some allergenicity after digestion, this may not necessarily tantamount to functional/biological relevance.

Furthermore, digestion products containing peptides of sizes previously stated to be too low to be allergenic might still induce an allergic response (Bøgh et al., 2009; Bøgh and Madsen, 2016; Eiwegger et al., 2006) which suggests that small digested peptide fragments should be evaluated. Additional studies are then needed to further address the relevance of allergenicity assessment of digestion products for improved recommendations for the specific assays for assessment of IgE binding, eliciting and sensitising capacity.

Specific biological assays for the assessment of IgE binding, eliciting and sensitising capacity of digestion products should be used to determine the biological relevance of IgE binding and to detect activity of very small amounts of small and large protein fragments after digestion. This will improve our understanding of the relevance of digestion in allergenicity assessment.

11. Discussion

Currently, stability in a pepsin resistance test is one of the main pillars for assessing the allergenic potential of (novel) proteins, though its predictive ability remains unknown. For this reason, EFSA announced an interim phase to investigate the applicability of the digestion test in allergenicity assessments and to evaluate potential revisions to improve the current *in vitro* GI digestion test. In 2017, COST Actions

ImpARAS and INFOGEST organised a workshop addressing the relevance and applicability of the pepsin resistance test in allergenicity assessment of proteins and how to improve the test. The main conclusions and gaps are listed in Table 2 and are summarized below.

Allergic symptoms may be experienced within seconds of exposure to the oral mucosa (elicitation) and sensitization is possible via different routes; respiratory, skin and oral. The contributions of these routes to food allergy and food sensitization have not been thoroughly investigated. Allergenic proteins may cause allergy due to cross-reactivity with pollen (e.g., Bet v 1 homologues), while others can sensitise via the skin (e.g., peanut) or lungs (e.g., shrimp). Atopic compared to non-atopic individuals may have different absorption kinetics for allergens against which they produce antibody, which suggests that barrier status might be more important than digestion. Sensitization and food allergy may occur when the barriers are compromised and whole allergens or their large sized fragments pass through the barrier and interact with the immune system; this can be amplified by a variety of “extrinsic” factors. However, it is not completely clear if an enhanced intestinal permeability is the cause or the result of an immune-mediated adverse reaction to foods (Price et al., 2013). While there is active research in the area of barrier function, there remain many knowledge gaps least of which are i) the exact mechanisms of trans-epithelial and transcutaneous transport that allow passage of food allergens to migrate and interact with the immune system, ii) differences between allergenic and non-allergenic proteins that enable preferential transport, and iii) dependence on physicochemical properties that determine resistance to GI digestion. In the context of sensitization through skin or respiratory tract, it appears that a digestion resistance may not be informative at all for assessing allergenic properties of food proteins.

The pH of the different GI compartments has a substantial impact on food protein digestion. This is exemplified by changes in pH associated with disease, use of antacids, and the effects of age with infancy or old age contributing to the individual digestive capacity, which might predispose to adverse reactions to food. However, it is not clear if this occurs with allergenic proteins or all proteins. Factors other than an altered pH (e.g., antacids) may play an important role in food allergy and food sensitization as Versluis et al. showed that only a small percentage of food allergic patients (7.7%) used antacids (Versluis et al., 2016). In addition, the GI microbiome substantially influences immune response and oral tolerance induction (Chinthrajah et al., 2016; Imhann et al., 2016). Recent studies have identified changes in gut microbiota associated with food allergy (Plunkett and Nagler, 2017). However, it is difficult to assess the impact of reverse causality. One critical factor is the lack of reports addressing gastric pH or gastric/intestinal secretions in atopic individuals, which is necessary for understanding the mechanisms underlying food allergy, which will aid the development of the digestion assays.

In addition to GI pH, microbiota, other mechanisms in the GI tract may have a substantial impact on how the immune system encounters food proteins. Protein hydrolysis takes place in the GI tract due to enzymes produced by the brush border membranes. Many peptidases are among these brush border enzymes, though their role in the allergic response to proteins remains unclear they should not be neglected. For instance, BBM enzymes may have a great impact on the size of the proteins crossing the barrier and may improve the predictive ability of the digestion test and should not be omitted in digestion models simulating the entire digestive process. Consensus on how to use BBM enzymes in experiments is currently lacking and needs further investigation.

APC are involved in the processing and presenting of proteins to the immune system. Intact proteins/protein fragments are, after crossing the barrier (GI, airway or skin) or due to direct sampling from the gut lumen, taken up by APC and subjected to endolysosomal degradation, which has not been extensively studied. High protein stability might to some extent depend on the protein family and (enhanced) allergenicity. However, a limited number of (food) allergens have been studied and correlated to T cell reactivity and allergenicity. More studies are needed to draw conclusions on the benefit of endolysosomal degradation as a standalone assay or if this should be incorporated in an assay covering the whole digestion cascade from stomach, intestinal, BBM enzyme hydrolysis to endolysosomal degradation.

The effect of processing and matrix is important but not currently incorporated into digestion assays despite the fact that both may affect the allergenicity of food proteins. Whether the effect on allergenicity occurs via altered digestibility due to pH changes in the stomach or structural changes of the protein remains to be investigated. Processing of one protein may increase while another decreases allergenicity, which might be an explanation for the unpredictability of the digestion assays. The effect of matrix and processing on allergenicity requires more investigation and might be incorporated eventually in digestion assays, though it will not be possible to incorporate these factors in a simple (static) digestion models. Alternatively, in dynamic or semi-dynamic models, possible matrix effects and the buffering capacity of the food when mixing conditions are carefully chosen might improve model predictability. Pure allergenic proteins can be digested using dedicated *in vitro* static models, with their limitation that these models consider the end-point of gastric or intestinal digestion, without giving information about fate of molecules during the gastric or intestinal step (digestion kinetics). A consensus model for allergenicity testing agreed by experts in the field is crucial.

While a model should reflect the human situation, it is more important that it is predictive, but this is only possible when the method is more physiologically relevant than the currently used methods. A static pepsin digestion is not physiologically relevant and thus a more dynamic system that addresses factors such as matrix and kinetics is crucial, however the usage of these models for their predictive ability in allergenicity assessment still needs to be demonstrated. The inclusion of BBM enzymes, transport across the barrier and endolysosomal degradation might be important to further enhance the predictive ability of the assays but will increase the complexity, the costs and thereby reduce its usefulness as a screening assay.

Furthermore, it is important to make decisions on the readout of the system before deciding on a digestion assay or strategy including criteria for defining a protein as an allergen. Key readouts should be: residual intact protein, peptide fragments and residual allergenicity of the formed digestion products, which should be evaluated with assays with appropriate sensitivities and robustness, and more than one method should be used. Before being recommended for use in allergenicity risk assessment of food proteins, methodology for the evaluation of the digestion products generated during the digestion process should be standardised for profiling intact proteins, large peptide fragments and small peptides. Moreover, it is vital to correlate allergenicity with other properties such as size to improve predictive ability of allergenicity. The EFSA opinion document (Naegeli et al., 2017) suggests the use of the terms transient and persistent for digestion fragments based on concepts such as half-life and that a persistent peptide fragment with ≥ 9 amino acids in length is critical and may indicate that further assessment is required. However, all proteins will form 9 amino acids fragments after digestion with pepsin followed by chymotrypsin and trypsin. The quantity of 9 amino acids fragments depends on the size of the protein. Indeed 9 amino acids fragments bind T cell epitopes, but 9 amino acids length is important for inducing allergy, and for inducing other immunological responses (Th1 and tolerance) as well and will therefore be difficult to use as a discriminating factor for allergenicity. Because hydrolysis/breakdown of any protein will result in fragments ≥ 9 amino acids (as tested using PeptideCutter, data not shown), whether they are allergenic or not, therefore additional tests will always be needed to discriminate between those posing a risk and others. This suggests that digestion is not sufficiently discriminating and could be omitted from the current allergenicity risk assessment strategy without loss. A better understanding of digestion and reliable, reproducible models that predict allergenicity are crucial. Especially key

is determining i) why digestion abrogates, reduces, increases or does not affect food protein allergenicity compared to the parent protein or food, ii) why digestion products may retain IgE binding capacity but not retain eliciting capacity and iii) why digestion products containing small peptides may be allergenic. Research is necessary for addressing the relevance of allergenicity assessment of products resulting from the digestion process and recommendations are needed on the use of specific assays in assessment of IgE binding, eliciting and sensitising capacity.

12. Conclusion

The main conclusion of the meeting and the extensive literature search that followed is that protein digestion is relevant for allergenicity of some proteins, but not for all. Many other factors in addition to digestion in the stomach might play more pivotal roles and some of these factors may have a great impact on digestion and should be included in the digestion assay strategy. However, these factors complicate the design and implementation of a simple, suitable and predictive digestion assay/strategy enormously, especially because it is not clear yet how these factors exactly influence digestion and how these factors can be included. Moreover, there is no rationale on which to base a clear readout that is predictive for allergenicity exclusively and the exact route of exposure and mechanisms behind food sensitization and food allergy are not fully understood yet. Therefore, we suggest to omit the digestion test from the allergenicity assessment strategy for now and put an effort into filling the knowledge gaps. Finally, any digestion assay developed to support the allergenicity assessment of novel dietary proteins should be validated and produce results that can distinguish known allergens from non-allergens with a reasonable level of selectivity.

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