Are physicochemical properties shaping the allergenic potency of animal allergens?

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Abbreviations

2D – secondary structure,

3D – tertiary structure,

4D – quaternary structure,

BAT - basophil activation test,

DBPCFC - double-blind placebo-controlled food challenge,

EAST - enzyme allergosorbent test,

ELISA - enzyme-linked immunosorbent assay,

HPP - high-pressure processing,

HHP - high hydrostatic pressure,

IgE – immunoglobulin E,

IgG – immunoglobulin G,

OFC - open food challenge,

PEF - pulsed electric fields,

PTM - post-translational modifications,

PUV - pulsed ultraviolet,

RAST - radioallergosorbent test,

RBL - rat basophilic leukaemia,

SPT - skin prick tests,

Th1, Th2 – T helper cell type 1 or 2,

WHO/IUIS - World Health Organization/International Union of Immunological Societies

Abstract

Key determinants for the development of an allergic response to an otherwise 'harmless' food protein involve different factors like the predisposition of the individual, the timing, the dose, the route of exposure, the intrinsic properties of the allergen, the food matrix (e.g. lipids) and the allergen modification by food processing. Various physicochemical parameters can have an impact on the allergenicity of animal proteins. Following our previous review on how physicochemical parameters shape plant protein allergenicity, the same analysis was proceeded here for animal allergens.

We found that each parameter can have variable effects, ranging on an axis from allergenicity enhancement to resolution, depending on its nature and the allergen. While glycosylation and phosphorylation are common, both are not universal traits of animal allergens. High molecular structures can favour allergenicity, but structural loss and uncovering hidden epitopes, can also have a similar impact. We discovered that there are important knowledge gaps in regard to physicochemical parameters shaping protein allergenicity both from animal and plant origin, mainly because the comparability of the data is poor. Future biomolecular studies of exhaustive, standardized design together with strong validation part in the clinical context, together with data integration model systems will be needed to unravel causal relationships between physicochemical properties and the basis of protein allergenicity.

Keywords: plant allergens, protein families, allergenicity, food processing, allergen integrity.

Introduction

Presently, food allergies are a very concrete public health problem, reaching near-epidemic proportions in some regions of the world. The number of allergic reactions requiring medical treatments, and often hospitalisation, has multiplied over the past few years creating an important economic burden in several developed countries [1]. Understanding the mechanisms underlying this health condition is mandatory for better diagnosis and management of food allergies. With the increasing number of populations moving across the world, the local frequency of certain food allergies might significantly change. Additionally, with globally linked market places, the sensitised/allergic individuals are currently exposed to very different types of foods.

Food-allergic reactions are caused by the immunorecognition of specific proteins, following the breakdown of immunologic and clinical tolerance to an ingested food antigen(s). It is important, not only to explore the physiological mechanisms underlying food allergy, but also to evaluate the structural properties of food allergens and how they are affected by current/novel food processing technologies [2,3]. At present, there is an impressive number of publications available, exploiting different physicochemical parameters of several allergens and thus, providing a local overview of their impact on those proteins. However, some questions remain to be answered in the broad context: (i) which physicochemical parameters affect mostly the allergenicity of food proteins? (ii) do the same parameters fit every allergen, independently of its origin? (iii) do homologous proteins have the same behaviour towards specific physicochemical properties?

Our previous work reviewed those questions for plant allergens [4]. Same as for plant allergens, physicochemical parameters play also a critical role in the allergenicity of animal proteins. For this review, we gathered and analysed available publications reporting evidence about the impact of different physicochemical characteristics on the

allergenicity of animal protein families. Also, we aimed at identifying common features among distinct protein families of plant and animal origin in the light of the physicochemical parameters' potential to affect protein allergenicity. For this purpose, we will first make a general description regarding each animal allergen family (biological function, chemical and structural composition, and clinical relevance) to establish their importance within the context of this review. Secondly, the collected evidence will be discussed under each physicochemical property topic, since the objective of this work is to evaluate how each physicochemical parameter shapes protein allergenicity across protein families and within family members.

Animal Allergen Families

The latest statistical data provided by AllFam database in 2017 [5,6] indicates 445 allergenic proteins from animal sources, with 94% (*n*=421) of them being included in the WHO/IUIS (World Health Organization/International Union of Immunological Societies) nomenclature database [7]. These animal allergens were described on exposure routes via ingestion, inhalation, and/or contact [5,6]. Like for plant food allergens (*n*=436 proteins), animal allergens (*n*=410 molecules) are also distributed by families of proteins (*n*=71). However, more than 70% of the animal allergenic molecules are known food allergens, which are restricted to four families of proteins, namely the tropomyosins, the EF-hand family (parvalbumins), the ATP-guanido phosphotransferase (arginine kinases), and the alpha/beta-caseins.

Tropomyosins

Tropomyosins are present in all eukaryotic cells, except for plants. They are composed of a variety of actin-binding proteins with the main function of actin cytoskeleton regulation,

156 which is of major relevance for both muscle and non-muscle cells [8]. Structurally,
157 tropomyosins have an average length of approximately 284 residues, corresponding to
158 coiled-coil homo- or hetero-dimers that form a polymer along the length of actin (Table
159 1). They consist of two parallel α-helices with two sets of seven alternating actin-binding
160 sites, 34-38 kDa, being only functional as dimers [9]. Tropomyosins are important
161 contractile proteins that are highly conserved in both vertebrates and invertebrates but
162 only considered as allergens in invertebrates [10,11], representing up to 1% of their
163 muscle mass [12,13]. One exception of this allergenicity rule seems to be fish
164 tropomyosins [14-16].
165 Tropomyosin family ranks the first position in terms of the total number of allergens
166 (*n*=64) identified in animals [5,6], with 25 of those being registered in the WHO/IUIS
167 allergen nomenclature database [7] as food allergens, mainly belonging to crustaceans
168 (crab, prawn, lobster), molluscs (oyster, snail, abalone, squid), fish (tilapia, catfish,
169 salmon) and fish nematodes (worms). Interestingly, shrimp allergic individuals clinically
170 cross-react with a novel tropomyosin from mealworm, the larvae of a beetle (*Tenebrio*
171 *molitor*), evidencing that tropomyosin is one of the cross-reacting allergens [17]. In the
172 invertebrate family, tropomyosins are considered as panallergens (universal proteins
173 responsible for IgE cross-reactivity to a large quantity of related and unrelated allergenic
174 sources) [10,11,18], as well as, major allergens in several species.
175 Tropomyosins are the third most prevalent cause of food-induced anaphylaxis [19], but
176 they are also important respiratory allergens from crustaceans, arthropods, house dust
177 mites and helminths [20]. Among the priority foods, the eliciting doses (ED) associated
178 with the consumption/contact of these species (e.g. crustaceans) are in general high as
179 compared to strong food allergens such as peanuts, namely 26.2, 280 mg of protein for
180 ED01 and ED05, , respectively, or up to 2.5 g for ED10 (for comparison ED10 peanut

2.8 mg protein) [21-23]. Most of the allergic reactions are related to major allergen tropomyosin. Therefore, a small dose of the tropomyosins is sufficient to trigger severe and systemic clinical symptoms that may include immediate cutaneous reactions, oral allergy syndrome (OAS), digestive symptoms, anaphylaxis and asthma [24].

Parvalbumins

Parvalbumins are calcium-binding proteins, belonging to the second largest family of animal food allergens (*n*=46) [5,6]. These proteins have evolved into two distinct evolutionary lineages, being classified as α- and β-parvalbumins. Although presenting similar conformational structures, α- and β-parvalbumins differ in their isoelectric points (pI) (α-: pI ≥5; β-: pI ≤4.5) and molecular weights, as well as, in their primary structures, affinities for Ca^{2+}- and Mg^{2+}-binding, cell-type-specific expression and physiologic functions [25,26].

Parvalbumins are sarcoplasmic muscle IgE-binding proteins, small in length (approximately 109 amino acids and 10-12 kDa), acidic pI (3.9-5.5) and Ca^{2+}-binding (Table 1) [27,28]. They are relevant contractile proteins, representing 1-3% of muscle mass in invertebrates or fish, respectively [13,29]. Structurally, parvalbumins belong to the EF-hand family [30], characterised by the presence of three typical helix-loop-helix domains, organised in a globular tri-dimensional conformation (Table 1). Two of these domains (CD and EF domains) are capable of binding divalent metal ions (Ca^{2+} or Mg^{2+}), while the third one (AB domain) forms a cap that covers the hydrophobic surface of the functional domain pair [31].

Parvalbumin is the main fish allergen sharing similar biochemical and immunochemical characteristics across fish species consumed in different parts of the world [28,32-34]. Most fish allergies are triggered by parvalbumins [24,35] with allergenic homologs being

expressed in fish at variable levels [29]. Cartilaginous fishes (e.g. rays), mainly consisting of α-parvalbumins, are tolerated by most bonefish (β-parvalbumins) allergic patients, due to their low allergenic capacity [36]. α-Parvalbumins are generally not considered allergenic because of their proximity to human homologs [30]. However, this dogma has been challenged with α-parvalbumins being identified as food allergens in frog (Ran e 1), chicken (Gal d 8) and crocodile (Cro p 2) meats [7,37-40].

Gad c 1 was the first β-parvalbumin identified as a fish allergen in Baltic cod, being functionally related to the regulation of calcium switching in muscular-skeletal cells [41-43]. Since then, several allergenic β-parvalbumins (Clu h 1, Cten i 1, Cyp c 1, Gad c 1, Gad m 1, Lat c 1, Lep w 1, Onc m 1, Pan h 1, Ras k 1, Sal s 1, Sar sa 1, Sco s 1, Seb m 1, Thu a 1 and Xip g 1) have been identified mainly in fish species (Atlantic herring, grass carp, common carp, Baltic codfish, Atlantic codfish, barramundi, turbot fish, trout, catfish, Indian mackerel, salmon, pilchard, Atlantic mackerel, redfish, tuna, and swordfish, respectively), although two have been found in frog (Ran e 2) and crocodile (Cro p 1) [7].

Most fish species express two or more β-parvalbumin isoallergens that diverge in their amino acid sequences (e.g. salmon β1- and β2-parvalbumins share 64% of protein identity). Patients might have IgE-repertoires for all allergens or isoallergens [28,44]. Also, dimeric and polymeric forms of parvalbumin with high molecular weight (aggregates of approximately 24 and 48 kDa) have been reported to show IgE-reactivity [45,46]. Due to their capacity to sensitise through the gastrointestinal tract, β-parvalbumins are classified as a class I or complete food allergens [47]. However, upon handing and food processing, they can induce sensitisation by inhalation (occupational allergy) [48,49], thus they are both food and respiratory allergens. Common clinical

symptoms triggered by β-parvalbumins range from mild (oral allergy syndrome) to severe (angioedema, asthma, anaphylaxis) in fish-allergic individuals [24].

Arginine kinases

The arginine kinases belong to the ATP guanido phosphotransferases (also known as phosphagen kinases), which consists of a conserved family of functionally and structurally related enzymes that can reversibly catalyse the transfer of a phosphate between ATP and different phosphagens. Arginine kinases catalyse the phosphorylation of L-arginine residues [50] in crustaceans, which is a crucial reaction to the mechanism of cellular energy homeostasis [51].

Biochemically, these proteins have a molecular mass of 40-45 kDa with two polypeptides of 355-357 amino acids organised in an asymmetric monomeric structure (Table 1) [52]. The experimental determination of the crystal structure of natural arginine kinase evidences a fold with an α-helical N-terminal domain (composed by five α-helices) and an α-β C-terminal domain (containing seven α-helices and eight β-sheets). Moreover, different arginine kinases from distinct phyla/subphyla/classes (crustaceans, molluscs and arachnids) present high sequence identity, linear epitope similarity, as well as, conservation of spatial structure in the conformational epitope regions, thus confirming the reason for the frequent cross-reactivity of these allergenic proteins among species [52].

Arginine kinases have been described as allergens, not only in seafood (Pen m 2, Cra c 2, Lit v 2) [53-55] but also in cockroaches (Per a 9) [56] and mites (Der p 20) [57]. So far, eleven arginine kinases have been identified as allergenic proteins, with 6 of them being classified as food allergens (Bomb m 1, Cra c 2, Lit v 2, Pen m 2, Pro c 2 and Scy p 2 in silk moth, North sea shrimp, white shrimp, black tiger shrimp, crayfish and mud crab,

255 respectively) [7] and representing the third most relevant family of animal proteins [5,6].
256 Arginine kinases are classified as minor allergens, but clinically relevant ones, since
257 sensitisation to these allergens seems to be independent of tropomyosins, with allergic
258 patients experiencing systemic symptoms, or even anaphylaxis [58].
259
260 ## Caseins
261 In terms of animal food allergens, caseins rank the fourth position in the list of protein
262 families inducing allergic reactions by ingestion [5,6]. Caseins are a group of proteins
263 belonging to a large family of secretory calcium-binding phosphoproteins, present in milk
264 coagulum. As one of the most abundant proteins in milk (80% of the total protein
265 fraction), caseins are also considered as major allergens responsible for the development
266 of mild to severe allergic reactions in sensitised individuals [24,59].
267 The casein fraction (also known as Bos d 8) consists of four allergenic proteins, Bos d 9
268 (αS1-casein), Bos d 10 (αS2-casein), Bos d 11 (β-casein), and Bos d 12 (κ-casein), all
269 classified as important cow's milk allergens [7], and three γ-caseins deriving from the
270 hydrolysis of Bos d 11, which are considered not allergenic [60]. Caseins present primary
271 structures with 190-224 residues and small molecular size (20-30 kDa) (Table 1). In the
272 coagulum, caseins form ordered aggregates termed micelles, with a central hydrophobic
273 core (calcium-sensitive Bos d 9, Bos d 10 and Bos d 11) and a peripheral hydrophilic
274 layer (Bos d 12) containing major sites of phosphorylation mostly represented by
275 phosphoserine residues [61]. Caseins have a non-compact, flexible and greatly hydrated
276 structure, with a high hydrophobic surface due to the lack of a tertiary structure (Table
277 1).
278 The content and proportion of the four main casein fractions in milk vary according to
279 the animal species. Sheep's milk contains the highest concentration of caseins (4.18 g/100

g), followed by buffalo's milk. Almost half of this amount is present in cow's, goat's, and camel's milk. Human milk contains a low quantity of caseins (0.32 to 0.42 g/100 g), like mare's and donkey's milk [62]. Human milk is rich in Bos d 11, but it does not contain Bos d 9, which is very abundant in cow's and buffalo's milk, representing one of the most allergenic proteins in the milk of these species [63,64]. Bos d 9 is known to be the sensitising agent in about 60% of patients with cow's milk allergy. Goat's milk seems to be less allergenic than cow's milk due to a lower contribution of Bos d 9 in the elicitation of the adverse immunological reactions [62]. In the same way, camel's milk shows a high proportion of Bos d 11 and low proportion of Bos d 9 and Bos d 10 as in human milk [60,65], so camel's milk is often suggested as alternative source of nutrients for cow's milk allergic individuals [66]. Thus, these differences in the abundance of each casein, as well as the distinct degree of protein homology [67,68], are intrinsically related to their allergenic potential in different mammalian species.

Miscellaneous protein families

Miscellaneous families are defined as families containing only one or two important allergens, while most proteins are non-allergenic. This section describes some protein families containing important animal allergens.

Serum albumins

The serum albumins comprise a group of multifunctional proteins produced in the liver and secreted as a non-glycosylated protein into the plasma, presenting highly conserved sequential and conformational structures [69,70]. Serum albumins are abundant in the plasma of mammalian and avian species, displaying biological functions that include the transport of different molecules (water, cations - Ca^{2+}/Na^{+}/K^{+}, fatty acids, hormones,

bilirubin and drugs) and the regulation of the colloid osmotic pressure in blood [6,24]. They are relatively large molecules with a molecular weight of 60-69 kDa and immature primary sequences of 607-608 amino acids (Table 1), being present in dander, skin, saliva, milk, and meat of different animal species. Structurally, serum albumins present a very flexible α-helical conformation (to accommodate different ligands) composed of three domains and stabilised by several disulphide bridges [24,70].

So far, different serum albumins have been registered in the WHO/IUIS allergen database, although only three are classified as food allergens, namely Bos d 6 (bovine serum albumin - BSA), Gal d 5 (chicken serum albumin - CSA) and Sus s 1 (pig serum albumin - PSA) [7].

Bos d 6 is the serum albumin identified in cow's milk and meat, sharing high sequence identity (75.6%) and similarity (85.5%) with human serum albumin [24,69]. Mature Bos d 6 has 583 amino acids folded in an α-helical structure composed of three structurally similar domains (I, II, and III) organised in a heart-shaped molecule and stabilised by 17 disulphide bonds. Bos d 6 conformation is known to change to accommodate ligands, being able to coordinate the binding of three Ca^{2+}, all of them located at domain I [70].

Bos d 6 is classified as a minor respiratory allergen, being associated with cases of occupational asthma and rhinitis, and inducing mild to moderate clinical symptoms, such as rhinorrhoea, nasal itching, nasal obstruction, and chest discomfort [24,71-73]. Besides, Bos d 6 acts as an important food allergen, being responsible for triggering mild to severe allergic reactions (including anaphylaxis), especially in the case of consumption of unprocessed cow's milk or meat. Bos d 6 belongs to the whey fraction and represents 1% of total milk protein. More than 90% of meat-allergic patients are also allergic to cow's milk, due to the fact of being sensitised to Bos d 6, suggesting that this protein might be a good diagnostic marker for cow's meat and milk allergies [24,74]. Additionally, Bos d

6 has several biotechnological applications, such as vaccines and culture medium of spermatozoids for artificial insemination, which poses new health risks for the allergic individuals [73].

Sus s 1 is the serum albumin identified in pork's meat, it has a smaller molecular weight (60 kDa) than the rest of serum albumin family of proteins, but it presents high sequence identity with Bos d 6 (69.7%) and with human serum albumin (72.0%) [69]. This allergen is the cause of the pork-cat syndrome, due to its high cross-reactivity with cat dander allergen (Fel d 2). Patients sensitised to Fel d 2 are at risk of developing mild to severe allergic reactions, including anaphylaxis, angioedema, rhinitis, urticaria, itching eczema, when consuming food products containing pork meat [75,76].

Gal d 5 (also called α-livetin) is the serum albumin in chicken (including egg yolk, serum, meat, and feathers), presenting 69 kDa and a mature primary sequence of 592 amino acids. Gal d 5 exhibits less sequence identity (46.1%) and similarity (61.1%) with human serum albumin [7,69] compared to other serum albumins, particularly with mammalian ones. Gal d 5 is classified as a respiratory allergen causing asthma, conjunctivitis and rhinitis associated symptoms, and as a food allergen (bird-egg syndrome) capable of triggering OAS, angioedema and anaphylaxis [77].

Glycoside hydrolase family 22

The glycoside hydroxylases encompass a large group of enzymes that catalyse the hydrolysis of a glycosidic bond between two or more carbohydrates, or between a carbohydrate and a non-carbohydrate moiety [78], which are divided into families and some families into clans. Two important food allergens belong to the glycoside hydrolase family 22, namely the Gal d 4 (lysozyme C) from hen's egg and the Bos d 4 (α-lactalbumin) from cow's milk.

Gal d 4 is expressed in the egg white (tissue-specific), representing about 3.4% of the total protein fraction [79]. It hydrolyses specific polysaccharides within bacteria cell walls, thus functioning as a bacteriolytic enzyme [80]. The mature protein of 129 amino acids in a single polypeptide chain is composed of two domains, one mostly formed by antiparallel β-sheets and one by α-helices. It has a monomeric conformation of approximately 14 kDa (four disulphide bonds with no free thiol groups), (Table 1) [80-82], with a theoretical pI of 9.3. Gal d 4 has been recently reported as presenting two potential N-glycosylation sites, N^{39} and N^{44}, both localized at a nonconsensus sequon [83,84]. Gal d 4 is classified as an important allergen, which can cause allergic sensitisation via inhalation, being associated with Baker's asthma [85]. Clinical symptoms, such as angioedema and urticaria, have also been reported for egg-allergic patients, upon consumption of raw or minimally processed egg white [86]. Gal d 4 shares 35 to 40% of the sequence identity with Bos d 4, as well as, the positions of the four disulphide bonds [87].

Bos d 4 intervenes in milk production (regulatory subunit of lactose synthetase), being classified as a monomeric globular calcium-binding metalloprotein with 123 amino acids and 14 kDa (Table 1), and reported as having 3 genetic variants [88]. It possesses a high-affinity binding site for calcium and four disulphide bridges, which helps to stabilise its secondary (2D) structure. Bos d 4 has a compact and spherical conformation, with two structural domains: a large α-helical domain at the N-terminal and a short β-sheet domain at the C-terminal, flanking the calcium-binding loop [89]. Bos d 4 exhibits high sequence homology with α-lactalbumins of several species, including humans [90] and it has been identified as a major allergen in cow's milk, being commonly responsible for eliciting respiratory, cutaneous and gastrointestinal symptoms, and often anaphylaxis in milk-allergic individuals [24,91].

Transferrins

Transferrins are sulphur-rich iron-binding glycoproteins that function *in vivo* to control the level of free iron [92,93]. These proteins are accountable for the transport of iron, both from sites of absorption and heme degradation to those of storage and utilisation. Members of this family include hen´s egg white Gal d 3 (ovotransferrin or conalbumin) or and cow's milk Bos d LF (lactotransferrin or lactoferrin).

Gal d 3 represents 12% of egg white protein fraction, it has a primary structure of 686 residues with 78 kDa and a pI of 6.0 (Table 1). Gal d 3 binds two Fe^{3+} (one per each lobe) in tandem with two bicarbonate anions [94]. It has thirty cysteine residues, all involved in disulphide bonds (n=15), nine and six of them located at the C-terminal or the N-terminal lobes, respectively. Structurally, Gal d 3 is a glycoprotein with a compact and asymmetric monomeric conformation [83,95,96]. Besides regulating iron transport, this protein is also known to exhibit antibacterial activity in their iron-free form [79,93]. Gal d 3 is classified as a minor allergen, with clinical symptoms being mostly associated with urticaria and angioedema. Egg allergic patients sensitised to Gal d 3 are at higher risk of suffering from an adverse immunological response when consuming raw or slightly processed eggs [24,97].

Bos d LF is composed by a single polypeptide chain of approximately 690 residues with a molecular weight of 80 kDa, folded into two globular lobes, each of them having high-affinity iron-binding sites, connected by a 3-turn helix (Table 1). It has an asymmetric monomeric conformation, but it can exist in polymeric structures (tetramers) [24], which is analogous among mammal species (65-100% of sequence identity). Lactoferrins from ruminant species, like cow, buffalo, goat or sheep, share more than 90% of sequence identity, forming a particularly closely related cluster [98]. Bos d LF can be distinguished

405 from other members of the transferrin family by its greater pI (8.0-9.0) and its higher iron-
406 binding affinity [93,98,99]. Although, being present at very low concentrations in cow's
407 milk, as well as, in the milk of other species (<1%), Bos d LF is considered to be an
408 important allergen (41% of IgE-response, in co-sensitisation with major cow's milk
409 allergens) [100].
410
411 ***Lipocalins***
412 Lipocalins represent a cluster of diverse proteins with biological functions focused, not
413 only on the transport of small hydrophobic molecules (retinol, odorants, lipids, and
414 pheromones) [101] but also in the regulation of several immunological, metabolic and
415 developmental processes [102], that participate in the immune response mechanisms,
416 enzymatic activity, tissue development and allergic reaction initiation [103]. Lipocalins
417 are small extracellular proteins with 150-250 residues and 17-25 kDa (Table 1) [104,105].
418 They can be N- and/or O-glycosylated [103], and it is predicted that they can be
419 phosphorylated by regulation processes [106].
420 Sequence identity among lipocalins is generally low (20 to 30%, although it may reach
421 higher values) [103,105,107,108], but they share a common 3D structure made of a well-
422 conserved eight-stranded anti-parallel β-barrel (accommodating a ligand-binding pocket)
423 and an α-helix [109,110]. The ligand-binding pocket has a central location where small
424 molecules, such as lipids, steroids, hormones, bilins and retinoids can bind [103,111]. The
425 β-barrel structure is stabilised by two disulphide bonds and depending on the pH, it can
426 form monomers, dimers or higher-order oligomers [112,113]. Presently, several animal
427 lipocalins have been identified as allergenic proteins (*n*=25), nineteen of those being
428 registered in the WHO/IUIS allergen database [5-7]. Bla g 4 (cockroach), Mus m 1
429 (mouse urine), Rat n 1 (rat), Can f 1 and Can f 2 (dog), Equ c 1 and Equ c 2 (horse) and

Bos d 2 (cow) are some examples of allergenic lipocalins. These proteins are highly abundant in epithelial mucosa and skin, especially in body fluids and secretions [112], being widely spread in indoor environments as aeroallergens [109,111].

Among this family, only Bos d 5 (cow´s milk β-lactoglobulin) was classified as a food allergen, although very recently (dated May 2020) [7], Bos d 2 has also received the same classification. Bos d 5 is a major whey protein and a major allergen, corresponding to 10% of the total protein content of cow's milk and participates in several molecular transport processes [59]. Clinical symptoms induced by IgE-binding to Bos d 5 are quite similar to the ones triggered by Bos d 4, which involve cutaneous, gastrointestinal and respiratory manifestations (or even anaphylaxis) [91]. Additionally, Bos d 5 is reported as a potential molecular marker for persistent cow's milk allergy in adults [114].

Ovomucoids

Kazal-type serine protease inhibitors are a family of proteins (MEROPS inhibitor family I1, clan IA) [115] with main biological functions associated with the inhibition of several serine proteases, which includes avian ovomucoid, pancreatic secretory trypsin inhibitor, acrosin inhibitor, and elastase inhibitor [116,117]. Included in this family, the Gal d 1 (ovomucoid) functions as a trypsin inhibitor and it has been identified as an important allergen in hen's egg white. Representing almost 11% of its protein fraction, Gal d 1 primary sequence has 186 residues (containing 20-25% of carbohydrate moieties), a pI of 4.1 and a molecular weight of 28 kDa (Table 1). Structurally, this protein comprises three independent domains (I-III), each of them behaving like a native globular protein, which are linked by intradomain disulphide bonds. Each domain is homologous to pancreatic secretory trypsin inhibitor (Kazal) and presents an actual or putative reactive site for inhibition of serine proteinases [118].

Gal d 1 has nine asparagine residues with covalently attached glycan groups (nine glycosylation sites), mainly encompassing the oligosaccharides N-acetylglucosamine, mannose, galactose and N-acetylneuramic acid [83,119]. However, the carbohydrate chain attached to the third domain of Gal d 1 seems to perform a critical role in its IgE-binding capacity [119,120]. High IgE levels to Gal d 1 seems to be well correlated with persistent hen's egg allergy [121], suggesting that this protein might be a good molecular marker for egg allergy prediction [122]. Allergic patients sensitised to Gal d 1 are at risk of suffering adverse immunological responses towards all forms of hen's egg (raw, slight or highly processed egg white), exhibiting clinical symptoms like atopic eczema, urticaria or vomiting [24].

Serpins

Serpins compose a superfamily of proteins with related, but functionally diverse structures, belonging to the MEROPS inhibitor family I4, clan ID [115]. Serpins are widespread among nature, except in fungi [117,123] and they play biological roles mainly related to protease inhibitory activity and control of proteolytic cascades. Other non-inhibitory functions have also been attributed to serpins, namely hormone transporters, molecular chaperones and tumour suppressors [124]. Serpins are relatively large molecules, presenting primary structures ranging from 330 to 500 residues.

So far, Gal d 2 (ovalbumin) is the only food allergen identified within this family [5-7], whose biological function is non-inhibitory (main role as storage protein). Gal d 2 is composed of 386 residues, with a molecular weight of 44 kDa and a pI of 4.5 (Table 1) [79,123,125]. It is glycosylated at residue Asn292, with a second potential glycosylation site at residue Asn311, and N-linked glycans consisting of hybrid-type and high-mannose-type oligosaccharides [125,126]. Gal d 2 polypeptide chain is involved in a

480 defined secondary structure, with three β-sheets (A to C) and nine α-helices (A to H and
481 helix R) [127]. Its structural conformation corresponds to a cyclic homodimer (Table 1),
482 suggesting a quaternary organisation.
483 Gal d 2 is a major protein component of hen's egg white (almost 54%), but it is considered
484 as a minor allergen. Allergic individuals (most often children of small age, <3 years)
485 sensitised to Gal d 2 are at risk of experiencing allergic reactions upon consumption of
486 raw or slightly processed egg white, exhibiting clinical manifestations, such as atopic
487 dermatitis [128]. An additional risk factor concerns the use of Gal d 2 in vaccine
488 formulations, which can lead to severe and systemic allergic reactions (anaphylaxis) in
489 hen's egg-allergic patients within minutes upon administration of Gal d 2-containing
490 vaccines [121].
491

492 Physicochemical Properties Affecting Allergenicity

493 An extensive literature search was performed to evaluate the impact of different
494 physicochemical characteristics on the allergenicity of proteins from distinct families of
495 animal allergens. Accordingly, the list of parameters includes several PTM, which are
496 most commonly associated with allergens, namely glycosylation, phosphorylation,
497 acetylation and hydroxylation. The structural integrity and the organisational level of
498 allergens, their stability towards heat, pressure, light (radiation), mechanical and chemical
499 activities resulting from different food processing methods (Fig. 1), as well as, their
500 behaviour towards glycation and aggregation phenomena were also assessed. In addition,
501 ligand binding, potential food component interactions (with lipids), resistance to
502 gastrointestinal digestion and the ability to cross the epithelial barrier in altered states
503 (e.g. aggregates) finalise the list of parameters analysed in this review.

Concerning each animal protein family, data from an extensive literature search covering the impact of all these physicochemical parameters on the allergenicity of their protein members were collected and provided in detail as supplementary material. Summarised data resulting from this extensive analysis are presented in Tables 2-4.

Measuring the effect on allergenicity

The pathophysiology of food allergy involves two stages: the sensitisation and the elicitation phases that are also designated as induction and effector phases, respectively. The sensitisation phase can be defined as the interaction of an allergen with an antigen-presenting cell, T-cell, and B-cell leading to the production of allergen-specific IgE, while elicitation phase relates to the interaction of the allergen with the allergen-specific IgE on the surface of the mast cell or basophils, resulting in the release of mediators which are responsible for the clinical symptoms [24,129]. The sensitisation phase is not always followed by elicitation, thus hampering the prediction of a clinical food allergy by measuring alone the allergen-specific IgE. Still, most of the available approaches to assess the allergenic potential of a protein rely on IgE-mediated assays, which can be performed under different conditions and formats [130].

The evaluation of the impact of different physicochemical characteristics on the allergenicity of animal proteins depends on the data compilation from different assays (Table 3). Immunoblotting, ELISA (enzyme-linked immunosorbent assay) and radioallergosorbent test (RAST)/enzyme allergosorbent test (EAST)/ImmunoCap using human sera/plasma of sensitised or allergic patients provide an overall assessment of the IgE-binding capacity (either qualitative and/or quantitative) of allergens from almost all families of animal proteins under study (Table 3) [54,131-151]. Although representing a great portion of data on the IgE-binding capacity of animal allergens, their interpretation

needs to be carefully conducted, considering all the pitfalls associated with these assays (the use of different sources of sera/plasma from food sensitised/allergic patients, different analytical conditions, different target analytes, indirect/poor correlation with clinical outcomes) [152].

Another strategy lies on the use of *in vitro* biological assays (cellular models), which provide a functional analysis of the specific effector cell activation by allergen-mediated specific IgE crosslinking (measured by mediator release or upregulation of cellular surface molecules). Such strategies present advantages related to high clinical specificity and sensitivity [152,153]. Although being more laborious and expensive than the previous approaches, the human basophil activation tests (BAT), the humanised rat basophilic leukaemia (RBL) mediator release assay and the mast cell models can be considered as *in vitro* surrogate of the allergic reaction that happens *in vivo* in allergic patients [154-156]. Therefore, these tests can be used to explore the immune mechanisms of effector cell response to allergens [154], being also broadly applied to evaluate the allergenic potential of most families of animal proteins (Table 3) [135,138,157-167].

Presently, the *in vivo* models are the only methods able to assess the potential sensitising capacities of food proteins [168]. The skin prick tests (SPT) and food challenges, either as open food challenges (OFC) or as double-blind placebo-controlled food challenges (DBPCFC), are used for allergy diagnosis, but with very limited application to evaluate the allergic response to specific proteins or protein extracts as affected by different physicochemical properties (Table 3) [11,77,133,159,164,169,170]. However, carrying clinical trials in humans (OFC and DBPCFC) is time-consuming, expensive, and are not easy to perform, besides involving ethical issues.

To overcome this problem, animal models have been used as surrogates for the identification and characterisation of food allergens, representing potential valuable tools

554 for safety assessment [171]. Nonetheless, the use of animal models to mimic food allergy
555 in humans carries some concerns, such as how well they simulate the human disorder and
556 what are their main strengths and limitations [172]. Still, they can provide some insights
557 about the sensitising and eliciting capacities of specific allergens, representing the current
558 closest physiological *in vivo* model of human immunological events. Therefore, animal
559 allergy models have also been used to measure the influence of physicochemical
560 properties on the allergenicity of molecules from some families of animal proteins (Table
561 3) [166,173-194].
562 For this review, some general definitions and terminology were used to standardise an
563 approach to deal with all different aspects of the data collected. Therefore, the definitions
564 on the allergenicity/allergenic potential, immunoreactivity and IgG/IgE-binding capacity
565 were adopted from Verhoeckx et al. [195]. By allergenicity/allergenic potential we mean
566 "the potential of a material to cause sensitisation and allergic reactions, frequently
567 associated with IgE antibody", immunoreactivity describes "the ability of a material to
568 elicit an immune response" and with IgG/IgE-binding capacity we mean "an altered
569 ability of IgG (also antigenic integrity) or IgE (also allergenic integrity) to bind to
570 epitopes, respectively".
571 In practical terms, the data collected from immunoblotting, ELISA, and
572 RAST/EAST/immunoCAP assays with the sera of food allergic/sensitised patients were
573 classified as "IgE-binding capacity", while data from similar immunoassays using animal
574 antibodies were defined as "immunoreactivity". The terms allergenicity/allergenic
575 potential were applied to classify results simulating the elicitation of an allergic reaction,
576 namely the *in vitro* functional assays (RBL, BAT), *in vivo* assays (SPT, OFC and
577 DBPCFC) and animal allergy models (mice physiological responses, mice anaphylaxis).
578 It is also important to stress that despite the defined strategy of classifying the results from

579 different analytical methods within the terms defined above, it was difficult to separate
580 results from the events of sensitisation and elicitation. Therefore, the classification of IgE-
581 binding capacity or allergenicity was determined in terms of weight of evidence (WOE).
582 Highest WOE was concluded from animal models and functional biological assays that
583 mimic main events of allergic reactions, acceptable WOE was seen in IgE-binding
584 capacity, and modest WOE was seen in immunoreactivity studies (bearing in mind the
585 extensive explanations above).
586

587 **Abundance**

588 Proteins, including allergens, play specific biological roles within organisms, whose
589 expression is regulated by their physiological demands. In animals, most of the allergenic
590 proteins perform structural, regulatory and transport functions, except for Gal d 2
591 (serpins) that has nutritional storage function (Table 1). However, the correlation between
592 the abundance of certain proteins and their allergenic impact is still a matter of debate.
593 Within the four most relevant families of food allergens from animal origin, caseins are
594 by far the most abundant proteins [59,89]. In this case, their high abundance seems to be
595 well correlated with the increased risk for adverse immunological reactions in individuals
596 sensitised/allergic to milk.
597 Compared to caseins, tropomyosins and β-parvalbumins are minor protein components,
598 representing only up to 1% or 1-3% of muscle mass in invertebrates (e.g. crustaceans,
599 molluscs, insects) or fish, respectively [13,29]. Nonetheless, despite their relatively low
600 abundance, tropomyosins and β-parvalbumins are classified as important major allergens
601 of animal origin. In the specific case of β-parvalbumins, their greater abundance in certain
602 flesh tissues (e.g. white vs. dark muscle) and their location (e.g. rostral vs. caudal part of

603 the white muscle) has been positively correlated with their increased allergenic potential
604 [196-198].
605 Among the miscellaneous families of proteins, the serum albumins are present in
606 moderate/low amount (approximately 5%) in the plasma of mammals, namely in bovine
607 (Bos d 6), pork (Sus s 1), lamb, and deer meats [199] and also in hen's egg yolk (Gal d 5)
608 [77]. Their relative moderate/low amount seems to be well correlated with their ability to
609 induce allergic responses in sensitised individuals [24,170], especially due to the high
610 cross-reactivity among serum albumins (Bos d 6, Sus s 1) from different meats (bovine,
611 pork), epithelia and milk [199]. However, in cow's milk, Bos d 6 is a minor component
612 of whey (about 1% of total protein fraction) but is considered as a major food allergen
613 with high clinical relevance [24,60].
614 The two representative members of the glycoside hydrolase family 22 are the Gal d 4 and
615 the Bos d 4, which account for 3.4% of egg white and 5% of milk protein fractions,
616 respectively [59,79]. Regardless of their relative moderate/low abundance, Gal d 4 and
617 Bos d 4 have been classified as highly immunogenic [200,201]. Likewise, the Gal d 1 of
618 the ovomucoid family represents less than 11% of egg white protein fraction, but it is
619 considered the immunodominant allergen in egg, being often related to severe cases of
620 anaphylaxis [142]. Gal d 1 exhibits higher IgE-binding capacity than other allergens,
621 following this specific order: Gal d 1 (11%)>Gal d 2 (54%)>Gal d 4 (3.4%), despite their
622 different proportions in egg [175].
623 In the transferrin family of proteins, the two representative allergens are Bos d LF and
624 Gal d 3, which have different proportions in their respective matrices, namely <1% in
625 milk (variable according to the species) and 12% in egg white. Contrarily to other
626 allergens, the abundance of these proteins is not well interconnected with their allergenic
627 potential. In this case, the abundance seems to be inversely correlated with protein

allergenic potential since the Gal d 3 (12% of egg white) is described as presenting very limited clinical relevance [200], while Bos d LF (often less than 1% of milk protein) has strong IgE-binding response [100].

The Bos d 5 from lipocalin family represents 10% of the total protein fraction in milk, and it is classified as a major allergen. In the case of Bos d 5, its abundance seems to be well correlated with a higher risk to trigger allergic reactions in milk-allergic patients. This is most likely related to the fact that Bos d 5 is absent in human milk, as well as in milk from other mammalian species (e.g. camel), which have been demonstrated to be less allergenic than cow's milk [202].

Gal d 2 from serpin family accounts for more than 54% of egg white protein fraction, but despite its great abundance, Gal d 2 is not an immunodominant allergen in egg's white [203]. Nonetheless, it has been shown that there is a strong correlation between the amount of egg ingested by women that are breastfeeding and the concentration of Gal d 2 in breast milk, which is considered to be responsible for eliciting egg-allergic reactions in infants [204].

Concluding remarks:

- The high abundance of caseins, serum albumins (meats and egg yolk), lipocalins (Bos d 5), and ovomucoids (Gal d 1) seems to be related to increased allergenic risk.
- The high abundance of other allergens (Bos d LF, Gal d 3, Gal d 2) does not always represent an additional risk for allergic reactions.
- The limited quantity of specific allergens (tropomyosins, parvalbumins, glycoside hydrolase family 22, serum albumin – cow's milk Bos d 6) often imply added hazard of eliciting severe immunological responses.

- Within the families of animal allergens, it is not possible to establish a correlation between abundance and an increased risk for triggering allergic reactions in sensitised individuals since different patterns are observed.

Protein structure

Food allergens are typically defined as molecules of small size and/or with compact globular structure (monomeric conformation), which is the case of some families of animal proteins, namely parvalbumins, arginine kinases, serum albumins, glycoside hydrolase family 22 and transferrins (Table 1). However, like in plant food allergens [4], there are several examples of animal allergens that present structures with a high level of organisation (quaternary structures), such as tropomyosins, lipocalins, ovomucoids, and serpins (Table 1).

In opposition, caseins are intrinsically unstructured proteins, exhibiting very little secondary/tertiary structures. In milk, the four variants of caseins have structural differences, with Bos d 9 and Bos d 10 being unfolded proteins with extended coil-like conformations, and Bos d 11 and Bos d 12 presenting molten globule-like structures [205]. In the absence of calcium, caseins have no regular structures, but in response to calcium-phosphate binding, they form micelles that correspond to particles of colloidal size designated as supramolecules. In those cases, casein micelles are defined as complex molecules with quaternary structures, showing great conformational flexibility because they are easily adapted to different environments [206,207].

In most families of animal proteins, the loss of high level of spatial organisation (tertiary and quaternary conformations) leads to a reduction in the IgE-binding capacity of their members, which are the cases of parvalbumins, arginine kinases, glycoside hydrolase family 22 and serpins (Table 2). The reasons behind this accentuated reduction are

normally the damage of structural integrity (globular monomer), through Ca^{2+} depletion or by modification of different residues in the Ca^{2+} binding region in parvalbumins [35,208] or by the disruption of conformational epitopes in arginine kinases, glycoside hydrolase family 22 and serpins [139,209-211].

The loss of structural stability of tropomyosins, caseins, serum albumins, lipocalins and ovomucoids has limited impact on their IgE-binding capacity, mostly due to the presence of important sequential epitopes that become accessible upon disruption of native conformation [114,132,212-214]. However, the disruption of disulphide bonds and loss of secondary structure contribute to a small decrease in the IgE-binding capacity of caseins, serum albumins, lipocalins and ovomucoids [114,214,215]. For proteins of the transferrin family, the loss of their monomeric conformation seems to have a dual character. By one side, the exposure of hydrophobic groups and the partial unfolding of transferrin structure reavels hidden linear epitopes with increasing IgE-binding capacity, while the destruction of conformational epitopes (loss of secondary structure by the destruction of disulphide bonds), upon severe protein unfolding, reduces the immunoreactivity of these proteins [139,216,217].

The use of denaturing agents, such as urea, can disrupt the conformational structure of proteins, leading to a molten globule state with increased IgE-binding capacity (partially denatured protein but retaining native-like structure), which seems to be the case of glycoside hydrolase family 22 and transferrins [142].

Concluding remarks:

- The IgE-binding capacity of parvalbumins, arginine kinases, glycoside hydrolase family 22 and serpins is reduced by the loss of 3D/4D conformations (destruction of conformational epitopes).

- The IgE-binding capacity of glycoside hydrolase family 22 and transferrins is increased by the destruction of native structures caused by denaturing agents (e.g. urea).
- The IgE-binding capacity of tropomyosins, caseins, serum albumins, lipocalins and ovomucoids is hardly changed by the loss of native structural integrity (presence of linear epitopes).
- The disruption of disulphide bonds and loss of secondary structure contribute to a slight decrease in the IgE-binding capacity of ovomucoids and lipocalins.
- The loss of 3D structures of transferrins presents a dual character - exposure of hidden linear epitopes increases and the destruction of conformational epitopes reduces the IgE-binding capacity, respectively.

Post-translational modifications

Post-translational modifications have been greatly described as affecting protein conformational structure, which has a substantial influence on its allergenic potential. Conversely, it is not clear yet to what extent PTM impact distinct food allergen families, or even different members within the same protein family. In the case of animal protein families, three specific PTM can be found among their members, namely glycosylation, acetylation and phosphorylation. All involve enzymatic processes, where glycosyl, phosphoryl or acetyl groups, respectively, are added to the side chains of amino acids of different proteins [218,219].

In opposition to plant food allergens, whose glycosylated proteins are mainly restricted to members of the vicilin family [4], glycosylation is the most common PTM among the families of animal allergens (tropomyosins, caseins, arginine kinases, glycoside hydrolase family 22, transferrins, lipocalins, ovomucoids and serpins). Despite the generalised

concept that glycosylation greatly contributes to increase the allergenic potential of proteins, this fact cannot be defined as a rule. Depending on the family of animal proteins, or even among different members of a specific family, glycosylation has been described as showing contradictory effects on the allergenic potential of a protein.

Enzymatic deglycosylation of tropomyosins (glycosylated proteins with N- and/or O-glycans) from crab or prawn retained or increased their IgE-binding capacity, respectively [157,220]. Gal d 1 (ovomucoid family) is a glycosylated protein with high carbohydrate content (20-25%), although the role of the carbohydrate in the IgE-binding capacity of this allergen is still ambiguous. Deglycosylated Gal d 1 has been reported to preserve or decrease its allergenicity, which is explained by the fact that the carbohydrates are not part of the IgE-binding epitope or by potential structural alterations of the protein (deglycosylated forms are more easily digested), respectively (Table 2) [165,221]. Caseins are glycosylated (e.g. Bos d 12), which difficult their subsequent digestion [222], thus increasing their potential allergenicity. N-glycosylation sites have also been advanced in crayfish Pro c 2 (arginine kinase family), although their role in the IgE-binding capacity of this protein is still unknown [223].

Phosphorylation is another PTM that occur among members of some animal protein families, namely in caseins and serpins (Table 2). Dephosphorylated variants of Bos d 10 and Bos d 11 are less IgE-reactive than their native counterparts, suggesting that the phosphorylation site(s) might be part of the IgE-binding epitope(s). Additionally, different casein variants contain a common phosphorylation site that is considered to be responsible for the cross-reactivity among caseins in milk-allergic individuals [169,224,225]. Phosphorylated caseins and serpins (Gal d 2) are more IgE-reactive than their dephosphorylated counterparts, suggesting that phosphorylation increases the IgE-binding capacity of these proteins (Table 2).

751 Acetylation occurs in members of animal food allergens, although at a smaller scale. Fish
752 parvalbumins can be modified by N-terminal acetylation, a PTM that makes
753 parvalbumins highly stable and more allergenic [226].
754 Concluding remarks:
755 • Glycosylation occurs in tropomyosins, caseins, arginine kinases, glycoside
756 hydrolase family 22, transferrins, lipocalins, ovomucoids and serpins.
757 Phosphorylation is common among caseins and serpins, while acetylation occurs in
758 parvalbumins.
759 • Glycosylation has contradictory effects on the IgE-binding capacity of different
760 families: tropomyosins (increase/maintain/decrease), arginine kinases (unknown
761 effect), ovomucoids (maintain/increase) and caseins (increase).
762 • Phosphorylation increases the IgE-binding capacity of caseins and serpins.
763 • Acetylation increases the IgE-binding capacity of parvalbumins.
764

765 **Ligand-binding**

766 Protein structure might be greatly influenced by the presence of specific ligands (metals,
767 ions) because they are often essential for protein folding. Some families of proteins can
768 bind ligands, although in different ways, which is the case for parvalbumins, caseins,
769 serum albumins, transferrins and lipocalins (Table 1). Structurally, parvalbumins are
770 calcium-binding proteins presenting two available sites (two domains) for binding Ca^{2+}
771 and Mg^{2+}. Metal-binding stabilises protein conformation and contributes to maintaining
772 their allergenicity as assessed by basophil histamine release assay when compared to their
773 apo-forms [35,135,208,227].
774 Caseins contain phosphoryl groups that can sequester Ca^{2+} and form thermodynamically
775 stable complexes (casein micelles), which prevents their aggregation into amyloid fibrils

(insoluble proteins) [228] and to conserve their IgE-binding capacity [224,225]. Transferrins and lipocalins are also able to accommodate and transport metal ions. In both cases, Gal d 3 (transferrin) and Bos d 5 (lipocalin) are less allergenic in their holo-forms (iron-bound) than in apo-forms (iron-free). Iron-binding seems to attenuate the immune responses by maintaining Th1/Th2 balance (holo-forms are more immunosuppressive than apo-forms), thus decreasing their allergenicity [163,177,229]. Besides iron, Bos d 5 is also able to transport other small molecules (e.g. retinoic acid) in its central core. Lipid-binding of Bos d 5 with retinoic acid (active vitamin A metabolite) can prevent an immune response by inducing profound inhibitory effects on different T-cell subsets and cytokine expression, therefore greatly reducing its allergenicity [230].

Concluding remarks:

- Parvalbumins, caseins, serum albumins, transferrins and lipocalins can bind ligands (Mg^{2+}, Ca^{2+}, Fe^{2+}, Na^{+} and retinoic acid).
- Ca^{2+}- and Mg^{2+}-binding stabilise the structural conformation of parvalbumins, which maintain their allergenicity.
- Caseins bind Ca^{2+} (by phosphoryl groups), forming casein micelles (stable macromolecules) and conserving their allergenic potential.
- Transferrins and lipocalins can bind iron, decreasing their allergenic potential. Bos d 5 binds other small molecules (e.g. retinoic acid), reducing its allergenicity.

Glycation and Aggregation

Glycation is a chemical reaction between the amino groups of proteins, lipids or nucleotides and the carbonyl groups of monosaccharides (typically reducing sugars), and it is called as Maillard reaction or non-enzymatic browning [231]. Glycation is responsible for changing colours, odours and flavours of foods, resulting from non-

enzymatic reactions during food processing under mild conditions. Although representing two distinct processes, glycation is frequently incorrectly designated as glycosylation (post-translational modification of proteins with the addition of carbohydrates during protein synthesis) [232]. In this section, we tried to include all manuscripts for the literature using the term glycosylation but meaning glycation (Maillard reaction).

Glycation is known to affect the allergenicity of specific proteins, although its effects are not yet fully clear. This process requires the application of heat treatments to thermodynamically favour the chemical reactions between amino and carbonyl groups, which often contributes to protein unfolding and formation of macrostructures, such as aggregates [233]. Protein aggregation can also result from other processes (e.g. mistakes in protein synthesis, mutations); although during food processing, it is most likely to occur as a consequence of Maillard reactions.

Protein behaviour towards glycation and aggregation processes can reflect their allergenic potential. Tropomyosin [131,157,173,234-237], parvalbumin [190,238-240], casein [241,242], lipocalin [143,243-250] and serpin [185,251-257] allergenicity is differently affect by glycation (Table 2). The behaviour of Gal d 2 (serpins) towards glycation is probably one of the best-studied, with several reports supporting the dual character of Gal d 2 IgE-binding capacity upon Maillard reactions. Accordingly, glycation of this protein with reducing sugars decreases the IgE-binding capacity of its glycated products [251-253], while advanced glycation end-products of Gal d 2 or glycation products in the presence of different concentrations of sodium carbonate-bicarbonate buffer contributed to increasing their allergenic potential, as assessed by *in vivo* mice allergy models and mediator release assays [185,254,256,257].

The allergenicity of glycoside hydrolase family 22 (Bos d 4) and arginine kinases (e.g. Scy p 2) are decreased by glycation, as determined by *in vivo* mice allergy models and

826 mediator release assays [173,258], while in ovomucoids (Gal d 1) its IgE-binding capacity
827 is increased (Table 2) [252]. In addition, Maillard reactions with different reducing sugars
828 (glucose, mannose, ribose) might also induce distinct alterations in conformational
829 structures, thus contributing to the contradictory effects in terms of protein IgE-binding
830 capacity (e.g. tropomyosins, parvalbumins) [235-239].
831 In all referred families, the formation of aggregates as a result of glycation is commonly
832 pointed out as the main factor for both increasing or decreasing the IgE-binding capacity
833 of most allergens [237]. As an example, the formation of aggregates contributes to a
834 decreasing effect on the IgE-binding capacity of arginine kinases and lipocalins, although
835 when neoepitopes are formed in the aggregates, their IgE-binding capacity can increase
836 [173,223,245,259]. Caseins naturally tend to form ordered aggregates, which contributes
837 to maintaining their IgE-binding capacity [260]. However, when caseins form aggregates
838 with other proteins, like the whey and wheat proteins, their IgE-binding capacity is
839 increased or reduced, respectively [261-263]. In the case of tropomyosins and
840 parvalbumins, aggregated proteins seem to have increased IgE-binding capacity
841 [134,237], while aggregated forms of serum albumins, glycoside hydrolase family 22,
842 ovomucoids and serpins are normally classified as less IgE-reactive
843 [145,176,179,180,264,265].
844 Concluding remarks:
845 • Chemical changes in tropomyosins, parvalbumins, caseins, lipocalins, and serpins
846 (as a consequence of glycation) can lead to decreased, increased, or maintained
847 allergenicity (depending on the allergen within a family, or even for the same
848 allergen).

- Chemical changes in tropomyosins and parvalbumins, as a consequence of glycation with different reducing sugars (glucose, mannose, ribose), can affect their IgE-binding capacity (maintain, decrease or increase).
- Structural changes (formation of aggregates with other molecules) in caseins can increase or decrease their IgE-binding capacity when aggregates are formed with whey or wheat proteins, respectively.
- Structural changes (formation of aggregates) in arginine kinases and lipocalins induce a decrease in their IgE-binding capacity (except when neo conformational epitopes are formed, leading to an increase in IgE-binding capacity).
- Structural changes (formation of aggregates) in tropomyosins and parvalbumins increased their IgE-binding capacity, while in serum albumins, glycoside hydrolase family 22, ovomucoids and serpins reduced their allergenicity.

Heat stability

Heat stability is generally considered as an important characteristic of allergenic proteins. For the evaluation of heat stability on the allergenicity of proteins, the influence of different thermal treatments used for food processing was extensively reviewed (Fig. 1). This is the case for tropomyosins [133,237,266-268], parvalbumins [190,197,269] and caseins [162,261,270,271], whose members are heat-stable proteins that conserve or increase their allergenicity (as determined by BAT, mediator release assays and *in vivo* mice allergy models), even after being submitted to extreme thermal conditions.

Moreover, treatments like pasteurisation, boiling, frying, and roasting can induce severe alterations on the secondary structures of tropomyosins with subsequent exposure of hidden epitopes, contributing to increasing their allergenicity. This feature seems to be common to several crustacea and mollusc tropomyosins, as confirmed by their increased

874 overall IgE-binding capacity, greater basophil activation, and larger wheal size in skin
875 prick tests compared to their raw counterparts [133,237,267,268]. Gal d 1 from
876 ovomucoid family is also considered a heat-stable protein, thus preserving its IgE-binding
877 capacity upon thermal processing [141,261], although when this protein is submitted to
878 temperatures above 90ºC and for several minutes (>15 min), its IgE-binding capacity is
879 significantly reduced [252,272]. Serum albumins have been described as partially heat-
880 labile (Gal d 5), but in fact, their behaviour is more likely to be heat-stable (Bos d 6 and
881 Sus s 1), since these proteins tend to preserve their IgE-binding capacity upon boiling,
882 broiling or even autoclave [151,170,214,273,274]. Therefore, depending on the family
883 member, serum albumins might be differently affected by distinct heat treatments.
884 Accordingly, Bos d 6 and Sus s 1 tend to conserve their IgE-binding capacity when
885 submitted to temperatures above 90ºC [151,170,214,273,274], probably due to the
886 presence of sequential epitopes, while the allergenicity of Gal d 5 is greatly reduced after
887 10 min at 90ºC, as assessed by skin prick tests and food challenges [77,275].
888 Proteins belonging to arginine kinase and other miscellaneous families (glycoside
889 hydrolase family 22, transferrin, lipocalins and serpins) are all heat-labile, which means
890 that most thermal treatments are efficient in reducing or even eliminating the IgE-binding
891 capacity of their members [136,139,141,223,252,261,270,276,277]. The loss of
892 tertiary/secondary structures and destruction of conformational epitopes, or the formation
893 of protein aggregates, as a consequence of heat treatments, are among the main reasons
894 justifying the decrease in the IgE-binding capacity of these heat-labile proteins
895 [139,216,261,270]. However, the application of mild heat treatments (55-60ºC) for short
896 periods (<10 min), causing an incomplete unfolding and subsequent exposure of
897 hydrophobic regions in proteins from transferrin and serpin families, might result in a

transient increased or preserved IgE-binding capacity of these members, respectively [141,216].

It is also important to highlight that the heat processing in the presence of proteins from other matrices, namely from wheat, might contribute to a great reduction (or even elimination) of the IgE-binding capacity of ovomucoids and serpins, probably due to aggregation through intermolecular disulphide bonds with wheat proteins [145,264].

Concluding remarks:

- Tropomyosins, parvalbumins, caseins and ovomucoids are heat-stable proteins. Serum albumins are partially heat-labile/stable proteins. Arginine kinases and other miscellaneous protein families (glycoside hydrolase family 22, transferrin, lipocalins and serpins) comprise heat-labile proteins.
- Heat stability (upon extreme heat conditions) contributes to increase the allergenicity of tropomyosins (exposure of hidden epitopes) and preserve the allergenic potential of parvalbumins, caseins and serum albumins (Bos d 6 and Sus s 1), but not for proteins of the ovomucoid family (e.g. Gal d 1 decreases its IgE-binding capacity).
- Structural changes (unfolding, exposure of hidden linear epitopes) increases the allergenic potential of tropomyosins and maintain the allergenicity of parvalbumins, caseins and serum albumins.
- Structural changes (unfolding, destruction of conformational epitopes, and formation of aggregates) reduce or even eliminate, the IgE-binding capacity of arginine kinases, glycoside hydrolase family 22, transferrins, lipocalins, and serpins.

Pressure stability

Regarding food allergens, the parameter of pressure stability has gained some relevance over the last few years, especially due to the increasing application of the novel food

923 processing technologies (Fig. 1). Despite their numerous advantages related to the
924 preservation of food quality (prolonging self-life, improving sensorial attributes) and
925 safety (eliminating microorganisms), the impact of these technologies on the allergenicity
926 of different proteins is still controversial (Table 2).
927 In the case of tropomyosins and parvalbumins (Table 2), the application of pressure
928 treatments seems to contribute to a generalised reduction in their IgE-binding capacities,
929 which is even more pronounced by the combination of pressure with heat [174,190,278].
930 Likewise, the use of high pressures also contributes to decreasing the immunoreactivity
931 of caseins by affecting the intermolecular forces in the micelles and by changing the
932 surface structure of these molecules [279]. The application of pressure at 600 MPa caused
933 casein aggregation (involving Bos d 5 with Bos d 12), shifting the balance of Th1/Th2
934 type cytokines towards Th1, thus diminishing the allergenic capacity of caseins [280].
935 However, when pressure is combined with high temperatures, for short bursts of time,
936 followed by instant pressure drop to vacuum, the IgE-binding capacity of caseins is
937 increased due to the dissociation of the casein micelles or to the aggregation of casein's
938 monomers [281]. In the case of serum albumins, the application of high-pressure
939 treatments (400 MPa) does not affect their immunoreactivity [282].
940 Treatments using high-pressures seem to have contradictory effects on the IgE-binding
941 capacity of members from the glycoside hydrolase family 22. By one side, high-pressure
942 treatments contribute to increasing the sensitising capacity of Gal d 4 (by inducing limited
943 denaturation), as assessed by *in vivo* mice allergy models [175], on the other side, it
944 maintains or even reduces the IgE-binding capacity of Bos d 4 [280,281]. In Bos d 5
945 (lipocalin family), the application of high-pressure treatments has a similar behaviour as
946 in the glycoside hydrolase family 22. Although keeping its internal core and
947 primary/secondary structures, Bos d 5 undergoes small rearrangements in its 3D

948 conformation when subjected to high-pressure treatments. These rearrangements are
949 reported as the main factor to increase or reduce its IgE-binding capacity
950 [144,280,281,283,284]. The combination of dynamic high-pressure treatments with the
951 glycation process seems to reduce the IgE-binding capacity of Bos d 5 conjugates in a
952 pressure-dependent-manner (greater reduction with higher pressures) [143]. The
953 application of high pressures (400 MPa) during enzymatic hydrolysis also reduces the
954 sensitising capacity of Bos d 5 in mice allergy model [178].
955 In ovomucoids (Gal d 1) and serpins (Gal d 2), there were no significant differences in
956 the levels of Gal d 1- or Gal d 2-specific IgE between the group of mice allergy model
957 sensitised with pressurised egg white (400 MPa for 10 min at 37 °C) and the native egg
958 white groups, suggesting that pressure treatments induce similar allergic sensitisation
959 capacity of Gal d 1 and Gal d 2 in mice, as their native counterparts [175]. Regarding
960 arginine kinase and transferrin families, no information on the effect of pressure stability
961 on the allergenicity of allergens could be retrieved from literature.
962 Concluding remarks:
963 • Tropomyosins and parvalbumins are pressure-labile proteins, while serum albumins
964 and ovomucoids and serpins seem pressure-stable. Caseins, glycoside hydrolase
965 family 22 and lipocalins have dual behaviour towards pressures (most likely
966 pressure-stable).
967 • Pressure treatments of ovomucoids (Gal d 1) and serpins (Gal d 2) induce similar
968 allergic sensitisation capacity of their native counterparts. Pressure treatments of
969 serum albumins do not affect their immunoreactivity.
970 • Structural changes induced by pressure (especially when combined with heat) reduce
971 the IgE-binding capacity of tropomyosins and parvalbumins.

- Structural changes induced by pressure (affecting the intermolecular forces in the micelles and changing the surface structure) reduce the immunoreactivity of caseins, but when combined with heat (dissociation of casein micelles or aggregation of caseins) increases their IgE-binding capacity (clinical impact unclear).
- Structural changes induced by pressure (limited unfolding) have contradictory effects on the IgE-binding capacity of glycoside hydrolase family 22. HP treatments increase the sensitising capacity of Gal d 4 (by inducing limited denaturation), but it maintains or even reduces the IgE-binding capacity of Bos d 4.
- Structural changes induced by pressure (with the conservation of the internal core and the 2D structure) have contradictory effects on the IgE-binding capacity of lipocalins. HP increase (clinical impact unclear) or reduce the IgE-binding capacity of lipocalins (Bos d 5). HP combined with glycation or with enzymatic hydrolysis reduce IgE-binding or sensitising capacities, respectively, of Bos d 5.
- Effect of pressure on the allergenicity of other protein families (arginine kinases and transferrins) is not known.

Light/radiation stability

Along with processing technologies using pressure, there are other novel non-thermal treatments of great interest in the food industry. Based on the application of light/radiation to increase the safety, quality and organoleptic characteristics of processed foods, treatments like gamma-radiation (γ-radiation), high voltage impulses, pulsed electric fields (PEF), pulsed UV light and microwave are widely used by industry [285-287]. However, the knowledge about the impact of this type of treatments on the allergenicity of proteins from animal origin is still very limited (Fig. 1, Table 2).

In general, the application of treatments with light/radiation (UV, pulsed UV, γ-radiation, microwave and PEF) seems to reduce the IgE-binding capacity of most proteins from different families [167,184,191,223,265,272,288-295], although some exceptions have also been described (Table 2). This is the case of tropomyosins, whose IgE-binding capacity has been reported to increase or decrease, depending on the dose of γ-radiation used (small dosages lead to a small increasing effect, while upper dosages contribute to a slight reduction) [296]. Similarly to tropomyosins, the IgE-binding capacity of Gal d 2 (serpin family) is negatively affected by increasing dosages of γ-radiation, ranging from an increase at low levels of radiation to a decrease at higher ones (>10 kGy) [147,276,293,297]. A decline in the secretion of IgE and cytokines (IL-4 and IL-5) associated with Th2 immune response is pointed out as the main cause for the reduction of Gal d 2 allergenicity [186,187].

In parvalbumins, treatments based on the application of UV light do not affect their IgE-binding capacity [190], while PEF induces contradictory outcomes in lipocalins and serpins [247,298,299]. The application of PEF (25 kV cm^{-1} for 60 μs) as a pre-treatment greatly increases the IgE-binding capacity of Bos d 5 (lipocalin family) by unfolding the structure to a certain degree. Conversely, when the treatment is followed by glycation with mannose, it expressively diminished Bos d 5 IgE-binding capacity, by masking the conformational epitopes through covalent binding with carbohydrate [247]. When submitted to radiation (≥10 kGy) the IgE-binding of Gal d 2 is greatly reduced (even abolished) [147,276,293,297], as well as its ability to induce sensitisation in mice allergy models [186,187]. The application of low electric field intensity (<25 kV/cm, for 180 μs) or for short time (<60 μs, at 35 kV/cm) to Gal d 2 induces gradual intensification in its IgG/IgE-binding capacities due to the partial unfolding of the protein and to an increase of free thiol content, surface hydrophobicity and UV absorption. However, when

increasing the exposure time or the intensity of the electric field, Gal d 2 IgE-binding capacity is significantly reduced due to aggregation [298].

Concluding remarks:

- Most protein families are light/radiation labile, with some exceptions (tropomyosins, parvalbumins, lipocalins, and serpins).
- Structural changes induced by light/radiation (unfolding) reduce the IgE-binding capacity of proteins from most of the investigated protein families, with some exceptions (tropomyosins, lipocalins, and serpins).
- Structural changes caused by high doses of radiations (unfolding) and long periods of exposure (formation of aggregates) contribute to reducing the IgE-binding capacities of tropomyosins, lipocalins, and serpins (only exception for Gal d 2, whose application of low-intensity electric fields increases its IgE-binding capacity).

Mechanical/chemical stability

The application of ultrasound or sonication treatments are among the most common mechanical processes used by industry, which might include drying, sterilization, enzyme inactivation, extraction, filtration, homogenisation, and meat tenderisation [300]. Ultrasound or sonication alone is not capable of altering the allergenic potential of animal proteins [164,190,223,290,301,302]. However, when combined with other treatments, especially thermal processes, like boiling or glycation, the IgE-binding capacity of certain proteins is reduced, as reported for tropomyosins, glycoside hydrolase family 22, lipocalins, and serpins [174,248,255,301].

In addition to the mechanical processes, there are several chemical or enzymatic treatments commonly used by the food industry that might include fermentation, acid or urea treatments, carboxymethylation, enzymatic hydrolysis, and crosslinking (Fig. 1).

Fermentation (chemical modification of sugars to other end-products by the metabolic activity of microorganisms, typically in anaerobic conditions) and enzymatic hydrolysis (enzymatic crosslinking of proteins using enzymes like transglutaminases, alcalase, among others) are the most effective in mitigating, or even eliminating, the allergenicity of most allergens from animal origin [138,181,182,189,303-309]. Such treatments are often combined with heat to reduce the allergenicity of different proteins, thus leading to the production of hypoallergenic foods [309-313]. Both processes, enzymatic hydrolysis, and fermentation of foods can induce severe protein modifications, causing the alteration or destruction of conformational and linear epitopes and converting highly IgE-reactive proteins into small and non-reactive peptides. However, it is important to highlight that the efficiency of these treatments is highly dependent on several factors, such as pH, temperature, time, the extent of hydrolysis, enzyme-substrate ratio, type of microorganism (specific strains), and substrate concentration [287].

Protein hydrolysis can be carried out with acids and alkali (chemical hydrolysis), but such reactions are normally difficult to control, leading to the formation of products with reduced nutritional qualities. Nevertheless, in some cases, they are used by industry for food processing. Chemical hydrolysis of tropomyosins has been reported to contribute to a great reduction (in some cases, up to 90%) of their IgE-binding capacity, which is independent of the type of acid used [267,314]. Treatments with acids also contribute to a strong reduction in the IgE-binding capacity of Gal d 3 (transferrin family) and Gal d 2 (serpin family), but in the case of Gal d 1 (ovomucoid family), its IgE-binding capacity was not significantly affected by boiling (10 min) followed by acidic treatment [139,146]. Some amino acids (mostly serine residues) of Gal d 2 can naturally suffer some conformational modifications during storage, converting Gal d 2 into a more stable protein (S-ovalbumin) and contributing to reducing its IgE-binding capacity. The same

effect can be obtained when treating Gal d 2 with high pH (~10) and heat (~55ºC) during several hours, thus allowing to decrease the IgE-binding capacity of Gal d 2 [315]. In the case of Gal d 2, its immunoreactive epitopes are destroyed by the application of heat and alkali treatments [211].

Concluding remarks:

- The integrity (intactness) of the proteins is affected by processes that destroy primary sequence (fragmentation due to hydrolysis), while mechanical, heat, pressure, and light change the protein conformational structure (e.g. unfolding).
- Changes in protein structure (by combining ultrasound and heat) are seen for members of tropomyosins, glycoside hydrolase family 22, lipocalins, and serpins, thus reducing their IgE-binding capacity.
- Changes in protein size (resulting in protein fragmentation, as a consequence of fermentation, enzymatic hydrolysis, or treatments with reducing agents) reduce or even mitigate the IgE-binding capacity of all animal protein families.

Digestibility and epithelial transport

The correlation between protein allergenicity and high resistance to pepsin digestion has been widely considered as an important parameter related to food allergens. Conversely, this correlation fails to explain why relatively well-digested allergens (e.g. some members of tropomyosins) are still able to trigger potent clinical symptoms in allergic individuals, while stable non-allergens remain non-immunoreactive [316]. When considering that digested peptides with an estimated size of 3-5 kDa can induce mast cell degranulation, the production of resistant allergen fragments represents an increased allergenic risk. Since the uptake of proteins/peptides via the mucosal-associated lymphoid tissue is highly

dependent on their shape, polarity, size, 3D structure, and aggregation status, the mechanisms mediating this crossing are of major allergological importance [317-319]. Several pathways enable the movement of molecules between the lumen and the mucosa, which consist of transport through the specialised microfold cells of Peyer's patches and isolated lymphoid follicles or across the epithelium, via transcellular (through cells) or paracellular (between cells) mechanisms. Therefore, the molecular form (allergen properties) and cellular processing of antigens are equally crucial in the elicitation of an allergic reaction [319,320].

In general, caseins are resistant to gastrointestinal digestion, thus preserving or even increasing their immunoreactivity, especially when digested peptides: i) present PTM, as phosphorylation and glycosylation, or ii) result from the formation of aggregates with whey proteins, whose structures are stabilised by disulphide bridges [241,242,262,263]. Parvalbumins, arginine kinases, and transferrins are quite resistant to trypsin/chymotrypsin activities, but they seem to be easily digested by pepsin, thus contributing to a significantly reduced IgE-binding capacity [223,316,321,322]. However, in the case of parvalbumins, the formation of amyloid fibres (polymeric structures of partially or completely unfolded protein chains) leads to a strong resistance to proteolytic activity at acidic and neutral conditions. The formation of such amyloid structures greatly facilitates their passage across the intestinal epithelial barrier, increasing their IgE-binding capacity [134,323].

After pepsin digestion, the allergenicity of tropomyosins is diminished, as assessed by skin prick tests and basophil activation tests, being greatly reduced or eliminated by subsequent intestinal digestion [159,188]. However, pepsin sensitivity does not seem to be a common trait of all tropomyosins, as it has been demonstrated for Pen m 1 and Lit v 1, which are rather resistant to pepsin activity [11,324]. Deglycosylated, glycated, or

crosslinked forms increase the susceptibility for gastrointestinal digestion, contributing to significantly decrease the allergenicity of tropomyosins [157,188,234,235,325]. Gal d 4 from the glycoside hydroxylase family 22 is resistant to trypsin/chymotrypsin activities, but it is partially degraded by pepsin at very low pH (<1.5) [139,326]. Bos d 4 is easily destroyed by pepsin [252,277,306,327,328], thus greatly reducing, or even abolishing, Bos d 4 IgE-binding capacity. Some IgE-binding and basophil activation capacities are maintained, being explained by the presence of high proportions of intact Gal d 4 that can cross the epithelial barrier in an activated state [140,161,326]. Additionally, Gal d 4 may contain some linear epitopes, previously hidden in its conformational structure, which become accessible after the digestion process, increasing its allergenic potential [161].

Bos d 6 (serum albumin family), Bos d 5 (lipocalin family), Gal d 1 (ovomucoid family) and Gal d 2 (serpin family) are in part resistant to pepsin activity but susceptible to trypsin/chymotrypsin digestion [148,151,329,330]. After complete digestion, the IgE-binding capacity of Bos d 6 and Bos d 5 is practically abolished [170,331], while Gal d 1 and Gal d 2 retain some allergenicity, most likely due to the presence of digested peptides containing linear IgE-binding epitopes [140,149,166,252,332,333]. The thermal processing of Gal d 1 and Gal d 2 induces small irreversible changes in their secondary structures, which facilitate their gastrointestinal digestibility, contributing to the reduced IgE-binding and mast cell degranulation capacities [166,252]. Differences in the immunogenic properties of heat-digested fragments seem to promote shifts from Th2 to Th1-type responses, leading to a significant reduction in allergenicity [183]. Additionally, thermal processing before gastrointestinal digestion of Gal d 1 and Gal d 2 prevent their transport across human intestinal epithelial cells in a state capable of inducing basophil or T-cell activation, thus reducing their allergenicity [166].

The formation of Bos d 5 aggregates during the glycation process enhances the resistance to proteolytic digestion, changing the mechanism of transport across the intestinal epithelium. On one side, Bos d 5 aggregates are more prone to endolysosomal degradation, inducing lower effector response, and reduced basophil activation. On the other side, these aggregates are redirected to Peyer's patches, promoting a significantly higher Th2 response than the native allergen, thus increasing its allergenicity [176,192,246].

Concluding remarks:

- Most animal allergens are pepsin-sensitive, while caseins, serum albumins, lipocalins, and serpins are considered pepsin-resistant.
- Most animal allergens present reduced IgE-binding capacity after complete digestion, with some exceptions:
  - In caseins, the presence of PTM or formation of aggregates in digested peptides preserved/increased immunoreactivity.
  - In parvalbumins, the formation of amyloid fibres (facilitate crossing epithelium barrier) increase their IgE-binding capacity.
  - In transferrins, the partial protective effect of matrix components (facilitate crossing epithelium barrier in intact forms) preserve their IgE-binding capacity.
  - In lipocalins, the formation of aggregates hampers digestion, changing the mechanism of transport across the epithelium barrier, increasing its allergenicity.

Lipid interactions

Since food allergens are not likely to be presented to the human immune system in their natural state (native molecules), it is important to consider the immunomodulatory effects of the surrounding components (e.g. lipids) within the protein source (e.g. food matrix)

[334,335]. Although the association between allergens and lipids is not yet clearly understood, some studies seem to indicate that lipids intervene in the early stages of allergic sensitisation by interacting with numerous components of the innate immune system. Additionally, lipids are also known to protect allergens from the enzymatic activity during digestion and to facilitate allergen passage through the epithelial barrier [334].

The effect of the interaction of lipids on the allergenicity of proteins was evaluated for some members of specific families, namely tropomyosins, parvalbumins, glycoside hydrolase family 22, lipocalins, and serpins (Table 2). In general, the presence of lipids contributes to preserving the IgE-binding capacity of proteins from parvalbumins, glycoside hydrolase family 22, lipocalins, and serpins [140,148,161,326,336]. In most cases, lipids increase the resistance of proteins towards proteolytic activity during digestion (often protecting the allergen native structure) [161,326,336] and facilitate their passage through the intestinal lumen as intact molecules [337,338]. Even when lipids enhance the proteolysis during digestion, as seems to be the case of Gal d 4 (glycoside hydrolase family 22) and Gal d 2 (serpin family), the IgE-binding capacity of these allergens remain unaltered [140,148].

Contrarily to the referred proteins, tropomyosins can be oxidised by acrolein and malondialdehyde (compounds resulting from lipid peroxidation during shrimp conservation), modifying their digestibility, as well as their IgE-binding properties. Met e 1 (tropomyosin) oxidation by malondialdehyde can enhance the resistance to pepsin digestion, while oxidation by acrolein produces structural changes, which in both cases significantly reduce the IgE-binding capacity of tropomyosins [325,339]. The release of inflammatory cytokines and mediators from activated RBL-2H3 cells was also strongly

1193 influenced by Met e 1 crosslinked with malondialdehyde in a dose-dependent manner,
1194 thus confirming a reduction in its allergenicity [158].
1195 Concluding remarks:
1196 • Lipids have a protective effect on the allergen stability during digestion for
1197 parvalbumins, glycoside hydrolase family 22, lipocalins, and serpins, preserving
1198 their IgE-binding capacity.
1199 • Lipid oxidation (by acrolein and malondialdehyde) of tropomyosins during
1200 conservation, increased their susceptibility to proteolytic digestion and reduced their
1201 allergenicity.
1202

1203 Can Physicochemical Properties Shape Allergenicity?

1204 After evaluating the effect of the selected set of physicochemical parameters on the
1205 allergenicity of distinct animal protein families, it has become clear that the importance
1206 of each parameter is quite different depending on the protein family or even on the
1207 allergen itself (Table 2-4). Independently on the effect that each parameter has on the IgE-
1208 binding capacity/allergenic potential of a specific protein (Table 4), they all converge to
1209 a common outcome, which concerns protein integrity.
1210 Within the studied animal protein families, PTM during protein synthesis occurs with
1211 high frequency. Glycosylation is the most common PTM, followed by phosphorylation
1212 and acetylation. However, not every glycosylated protein seems to be correlated with
1213 increased allergenicity. In fact, among the families of animal proteins, glycosylation is
1214 common (e.g. tropomyosins, arginine kinases, caseins, serpins), but it cannot be
1215 considered as an important parameter for allergenicity, since glycosylated proteins are
1216 often described as less IgE-reactive than their deglycosylated counterparts (e.g.
1217 tropomyosins).

Phosphorylation is well correlated with increased IgE-binding capacity of caseins and serpins, but it is not important or described for other animal protein families. Therefore, PTM could be involved in allergenicity but it is not necessary to induce an allergic reaction, meaning that not all allergens have PTM (phosphorylation or glycosylation). Contradicting the generalised concept that allergens have globular and compact structures, there is a huge number of potent animal allergens (tropomyosins, lipocalins, ovomucoids, and serpins) presenting a high level of structural organisation (quaternary conformations). The decrease in the IgE-binding capacity of several allergens can be correlated with the loss of high ordered structures (3D and 4D structures), specifically because most of the conformational epitopes are destroyed. However, there are several examples of allergenic proteins that preserve or even increase their IgE-binding capacity upon loss of 2D structures or rupture of disulphide bonds (e.g. tropomyosins, caseins), as a consequence of unmasking hidden linear epitopes. This means that there is no straightforward correlation between the loss of 2D structures/disruptions of disulphide bonds and the allergenic potential of different proteins, due to conflicting effects for different animal protein families, or even for the same allergen.

Protein stability towards heat is normally associated with potent allergens since they tend to return to native states upon cooling to lower temperatures. However, like in the case of plant allergens, this physicochemical property also fails to explain potent heat-labile animal allergens (e.g. arginine kinases, lipocalins). Protein stability towards light/radiation is similar to heat stability, normally because radiation often results in the raising of temperature, contributing to increasing the degree of protein unfolding.

Most of the available literature considers pressure-treated proteins as of lower allergenicity, an interpretation that is based on data from IgE-binding studies. With no clinical studies available and only a few studies based on mice allergy models, indicating

a slight reduction of allergenicity in proteins (tropomyosins, parvalbumins, and serpins) combining pressure and heat [174,175,190], the impact of pressure in allergenicity might be overestimated. The formation of aggregates has also a conflicting effect on allergen IgE-binding capacity, since in their aggregated forms new conformational epitopes may become accessible (e.g. tropomyosins, parvalbumins, glycoside hydrolase family 22, arginine kinases and lipocalins).

Protein stability towards chemical and enzymatic processes is well correlated with a significant decrease, or even mitigation, of the IgE-binding capacity of practically all animal allergens [138,189], mostly due to the extensive fragmentation of protein primary structure, with subsequent destruction of IgE-binding epitopes. The high resistance of allergens towards the digestion process is also a generalised concept, but it cannot be interpreted straightforward. In fact, it fails to explain several potent pepsin-sensitive animal allergens, such are the cases of some members of tropomyosins and parvalbumins. The protective effects of lipids towards allergen digestion are well correlated with the preservation of the IgE-binding capacity of animal proteins, as demonstrated for glycoside hydrolase family 22, lipocalins, and serpins.

Conclusions

Some families encompass many proteins, but with only one or two acting as potent allergens, while others are comprised of a large number of important allergens, which confirms the existence of unknown factors that render protein to be allergenic. By the end of this analysis, it was possible to conclude that there are still several gaps concerning the impact of different physicochemical parameters on animal allergens. One of those is related to the fact that numerous allergens have not yet been the target of intensive

research, which hampers to determine the real effect of different properties on protein allergenicity.

At this point, there is a great number of techniques (mostly by indirect means) that can be used to test the influence of those physicochemical properties, but it is also true that most of those are highly dependent on the use of sera from sensitised/allergic patients. Along with the difficulties of most research groups to have access to sera, it is also crucial to refer that the quality/composition of sera can be highly variable according to several factors (e.g. geographical origin, age, patients' sex, among others). Data from interlaboratory analysis (considering that similar allergens would be analysed using similar conditions) is practically inexistent, but which could help clarify if most of the contradictory effects observed for specific allergens are real or if they result from cumulative differences in protocols used by distinct research teams. Another aspect that has not been investigated is the comparison of the behaviour of non-allergens with allergens towards the same physicochemical parameters (only very few exceptions [11]). Comparing data retrieved from methods simulating sensitisation (IgE-binding capacity) with elicitation (clinical symptoms) phases is not ideal. However, considering the limited information for different allergens within the same family or across families, this comparison was performed to provide a more holistic picture of the impact of different physicochemical properties on animal protein allergenicity.

Despite the gaps herein identified, we were able to draw some important conclusions regarding specific physicochemical properties and to demystify some preconceived concepts. Glycosylation is not a universal trait of allergens, as well as heat stability and proteolytic resistance are not always a synonym of increased protein allergenicity. Like in the case of plant allergens, the body of evidence confirms that several physicochemical properties may shape the allergenicity of animal proteins, although at different extents.

Moreover, the level at which each parameter may impact protein allergenicity is not the same among plant or animal allergens.

Properties affecting protein integrity and composition can be correlated with the elicitation capacity of certain allergens, but what renders a protein to be allergenic in the first place and which properties might impact sensitisation are still quite unclear. The integration of all the factors (proprieties) that link large protein families containing numerous allergenic proteins with protein families with only one or two allergens (data integration by multivariate models), could give a broader picture of how the complete set of properties impact protein allergenicity (Fig. 2), instead of looking at individual proteins or events. It would also clarify why a protein behaves as an allergen in some people, while for others is innocuous, thus possibly paving the way for novel therapeutic concepts.

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Authors Contributions

All authors contributed to the study conception, design, data collection, and analysis. All authors have taken part in the discussions and writing of the article. All authors read and approved the final manuscript.

Supplementary material

Data from the extensive literature review on each protein family and their allergenic members is fully described/summarised in the supplementary material section, as an excel file. This excel file is divided into 10 pages, each dedicated to a single protein family (tropomyosins, parvalbumins, arginine kinases, caseins, serum albumins, glycoside hydrolase family 22, transferrins, lipocalins, ovomucoids, and serpins). All the abbreviations used in the excel file are presented in this manuscript as an abbreviation list. Based on the information gathered in this excel file, Tables 2 and 3 were constructed, thus summarising the data presented in the supplementary material section.

Compliance with Ethical Standards

Declaration of conflicts

All authors declare no conflict of interest.

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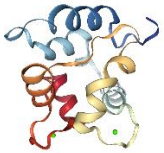
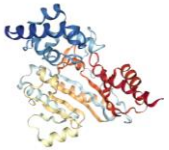
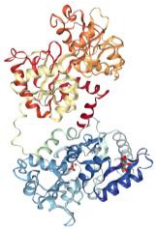
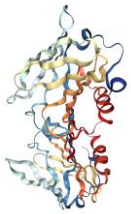
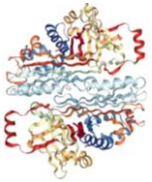
Table 1. Data on the composition and structure of proteins from the most important animal allergen families.

	Tropomyosins	**Parvalbumins**	**Arginine kinases**	**Caseins**	**Serum albumins**	**Glycoside hydrolase family 22**	**Transferrins**	**Lipocalins**	**Ovomucoids**	**Serpins**
Size (aa)	~284	~109	355-357	190-224	607-608	129 (Gal d 4) 123 (Bos d 4)	686-690	172	210	386
MW (kDa)	34-38	11-12	40-45	20-30	60-69	~14	78-80	17-25	22.5	44
Biological function	Structural	Structural	Enzymatic/ Regulatory	Regulatory	Transport	Defence (Gal d 4) Structural (Bos d 4)	Transport	Transport	Regulatory	Regulatory Reserve
Protein structure	4D Homo-/heterodimer (coiled-coil)	3D Globular monomer	3D Monomer	Typically 2D Casein micelles (4D)	3D Globular monomer	3D Monomer	3D Monomer	4D Globular homodimer	4D (3 globular native configurations)	4D Homodimer
Crystal structures (Method: X-ray diffraction)	There are structures experimentally determined at PDB (e.g. chicken tropomyosin), but none is classified as an allergen.			 Partial structure					There are structures experimentally determined at PDB (e.g. turkey ovomucoid), but none is classified as an allergen.	
Example of an allergen (source)	Shrimp Pen m 1	Cod Gad m 1	Shrimp Lit v 2	Cow's Bos d 10	Cow's Bos d 6	Chicken Gal d 4	Chicken Gal d 3	Cow's Bos d 5	Chicken Gal d 1	Chicken Gal d 2
PDB accession number	NR	2MBX	4AM1	6FS4	3V03	2LYM	1N04	2Q2M	NR	1OVA

MW, molecular weight; aa, amino acid; NR, not reported; LMW, low molecular weight; HMW, high molecular weight; PDB, Protein Data Bank, https://www.rcsb.org/.

2361 Table 2. Summary of the physicochemical parameters and their effect on the allergenicity of different animal protein families

	Tropomyosins	**Parvalbumins**	**Arginine Kinase**	**Caseins**	**Serum albumins**	**Glycoside hydrolase family 22**	**Transferrin**	**Lipocalins**	**Ovomucoids**	**Serpins**
Protein structure	→loss of 4D	↓,→loss of 3D, →molten globule state, ↓Ca^{2+}/Mg^{2+} depletion	↓loss of 3D	→loss 2D, →urea treatment, ↓modification by crosslinking	→loss of 3D/2D, ↓β-ME treatment, ↓loss of S-S, ↓modification by crosslinking	↓loss of 3D, ↓loss of S-S, ↑urea treatment, ↑molten globule state	↑exposure of hydrophobic groups ↑molten globule state, ↑urea treatment, ↓loss of S-S	↑partial unfolding, ↓loss of 3D/2D	↓loss of S-S	↓loss of 4D/3D
PTM	→,↓,↑ glycosylation	↑acetylation	→glycosylation	↑phosphorylation ↑glycosylation	NR	NR	NR	NR	→,↑glycosylation	↑phosphorylation
Ligand-binding	NR	↑ Ca^{2+} or Mg^{2+}	NR	→Ca^{2+}	NR	NR	→,↓iron	↓iron, retinoic acid	NR	NR
Glycation	↑,↓	↑,↓	↓	↑,↓	NR	↓	NR	↑,↓	↑	↑,↓
Aggregation	↑	↑	↑,↓	↑,↓	↓	↑,↓	NR	↑,↓	↓	↓
Heat stability	Heat stable: ↑boiling, frying, roasting	Heat-stable: → boiling, → autoclaving, ↓canning	Heat-labile: ↓ boiling, ↓ pasteurisation	Heat-stable: →boiling, baking	Heat-labile/ stable?: ↓,→boiling, ↓,→broiling, ↓autoclaving,	Heat-labile: ↓boiling, frying, baking, →pasteurisation	Heat-labile: ↑low T, ↓heat (T>80 ºC, boiling, frying, baking) →pasteurisation	Heat-labile: ↓boiling, →,↑heating (T 50-90ºC), →pasteurisation	Heat-stable: →,↓boiling or T>90ºC, →boiling + acid, ↓heat + wheat proteins	Heat-labile: ↓heat (T>90ºC), →heat (T>90ºC for <30 min), ↓heat + wheat proteins, ↓heat +acid
Pressure stability	Pressure-labile: ↓HPP, HPS ↓HHP + heat	Pressure-labile: →,↓pressure + heat	NR	Pressure-labile/stable? ↓HHP, HPP ↑ICPD (pressure + heat	Pressure stable: →HPP	Pressure-labile/stable? →,↑HHP, HPP, →HP + ultrasound, ↓ICPD (pressure + heat), ↓HP+ heat+ultrasound	NR	Pressure-labile/stable? ↓,↑HPP, HHP, ↑HPP + heat (T 40-50ºC), ↓HPP + heat (T>60ºC), ↓HP + glycation, ↓HP + enzymatic hydrolysis	Pressure-stable? ↑HPP	Pressure-stable? →HPP
Light/ radiation stability	Light-labile/stable? ↓PUV, ↑low radiation dose,	Light-stable: →UV light,	Light-labile: ↓microwave,	Light-labile: ↓UV-C, far-IR ↑low radiation dose,	Light-labile: ↓γ-radiation (> 3 kGy), ↓microwave	Light-labile: ↓UV treatment, ↓γ-radiation	NR	Light-labile/stable? ↑PEF, ↓PEF + glycation, ↓microwave,	Light-labile: ↓γ-radiation (>10 kGy), ↓γ-radiation + heat	Light-labile/stable? ↓γ-radiation (>10 kGy) ↑low EF, ↓high EF

	↓high radiation dose			↓high radiation dose)				↑low radiation dose, ↓high radiation dose		
Mechanical/ Chemical stability	↓ultrasound + boiling, ↓chemical hydrolysis, ↓fermentation, ↓enzymatic crosslinking, but preserving reactive epitopes	→ultrasound, →sonication, ↓enzymatic hydrolysis/ crosslinking	→ultrasound, ↓enzymatic hydrolysis/ crosslinking	→ultrasound, ↓enzymatic hydrolysis/ crosslinking, ↓fermentation	→sonication, ↓enzymatic hydrolysis/ crosslinking	→ultrasound, ↓ultrasound + heat, ↓carboxymethylation, ↓enzymatic hydrolysis, ↓fermentation ↓fermentation + heat	↓ carboxymethylation, ↓chemical hydrolysis, ↓fermentation	→ultrasound, ↓ultrasound + glycation, →,↓fermentation, ↓fermentation + heat, ↓enzymatic hydrolysis, an effect dependent on the enzyme	↓carboxymethylation, →urea-treatment	↑ultrasound, →carboxymethylation, →urea-treatment, ↓ultrasound + glycation, ↓enzymatic hydrolysis/ crosslinking
Digestibility	Pepsin-sensitive? →,↓after pepsin, →,↓after trypsin, ↓after digestion deglycosylated TM, ↓after digestion of glycated TM ↓after digestion of lipid-peroxidised TM	Pepsin-sensitive: ↓after pepsin, →after pancreatic digestion, →,↓after digestion of glycated PV	Pepsin-sensitive: ↓after pepsin, →resistant to trypsin/ pancreatic digestion	Pepsin-resistant: →,↓after pepsin of glycated products →,↓after trypsin or chymotrypsin, ↓complete digestion, ↑peptides with PTM	Pepsin-resistant: →after pepsin 60 min, ↓after complete digestion, more evident for irradiated Sus s 1	Pepsin resistant: (Gal d 4) ↓after pepsin pH 1.5, some proteins remain intact, →partially resistant to trypsin/chymotrypsin, Pepsin-sensitive: (Bos d 4) ↓after pepsin	Pepsin-sensitive: ↓after pepsin, →protective effect of matrix components	Pepsin-resistant: →after pepsin, ↓after complete digestion, but preserving reactive digested peptides, ↓after trypsin digestion of fermented Bos d 5	Pepsin-sensitive: ↓after pepsin until pH 2.5, ↓after complete digestion, some peptides remain reactive, →protective effect of matrix components	Pepsin-resistant: →after pepsin, ↓after complete digestion, some immunoreactive peptides, ↓after pepsin at HPP, some digested peptides conserve reactivity, →protective effect of matrix components
Epithelial transport	NR	↑amyloid structures, aggregates	NR	NR	NR	→intact proteins	→intact proteins	↓,↑aggregates	↓heated protein	↓heated protein
Lipid interaction	↓	↑	NR	NR	NR	→	NR	→	NR	→

β-ME, β-mercaptoethanol; HPP, high-pressure processing; HPS, high-pressure steaming, ICPD, instant controlled pressure drop; → maintain IgE-binding capacity; ↑ increase IgE-binding capacity; ↓ decrease IgE-binding capacity; ↑↓ contradictory data about the effect on IgE-binding capacity; NR, not reported; PEF, pulsed electric fields; PUV, pulsed ultraviolet, PV, parvalbumins; S-S, disulphide bond; 2D, secondary structure; 3D, tertiary structure; 4D, quaternary structure; TM, tropomyosin, T, temperature.

Table 3. Summary of the assays used to assess the effect of physicochemical parameters on the allergenicity of proteins from animal food families.

	Specific serum screening			**Cellular *in vitro* or *ex vivo* assays**			***In vivo* assays**			
Families	**Immunoblot/ dot blot**	**ELISA**	**RAST/EAST/ ImmunoCAP**	**Basophil activation test**	**RBL mediator release assay**	**T-cell proliferation**	**Murine IgE response**	**Murine anaphylaxis**	**Human Skin prick tests***	**Human Food challenges****
Tropomyosins	√	√	NR	√	√	√	√	√	√	NR
Parvalbumins	√	√	√	√	√	NR	√	NR	√	NR
Arginine kinase	√	√	NR	NR	√	√	√	√	NR	NR
Caseins	√	√	√	√	√	√	NR	NR	√	√
Serum albumins	√	√	√	NR	NR	√	NR	√	√	√
Glycoside Hydrolase	√	√	NR	√	√	√	√	√	√	√
Transferrins	√	√	NR	NR	NR	√	√	√	NR	NR
Lipocalins	√	√	NR	√	√	√	√	√	√	√
Ovomucoids	√	√	NR	√	√	√	√	√	NR	√
Serpins	√	√	NR	√	√	√	√	√	NR	√

IL, interleukins; IFN, Interferons; RAST, radioallergosorbent test; EAST, enzyme allergosorbent test; RBL, rat basophilic leukaemia; ELISA, enzyme-linked immunosorbent assay; √, confirmation of tests performed as reported on literature; NR, no evidence found in the literature; *, Human SPT were performed mainly with pure protein, although pure food extracts were also used. **, Food challenges are normally performed using pure food extracts or entire food (either alone or hidden within a prepared matrix), respectively.

Table 4. Main conclusions about the adequacy of each physicochemical property as potentially shaping allergenicity.

	Impact on IgE-binding capacity	**Supporting evidence/Main concerns**
ABUNDANCE	Low	Low abundant as well as high abundant proteins are known as potent allergens, e.g. tropomyosins (low abundant), caseins (high abundant).
BIOLOGICAL FUNCTION	High	Potent allergens display biological functions as storage, regulation, transport, and defence.
PTM		
Glycosylation	Low	Contradictory effects are found for potent allergens. Information is limited to tropomyosins, arginine kinases, caseins, and ovomucoids.
Acetylation	Limited	Increase the IgE-binding capacity of parvalbumins. Information limited to parvalbumins
Phosphorylation	Limited	Phosphorylation increases IgE-binding capacity. Information limited to caseins and serpins
LIPID-BINDING	Limited	Reduces allergenicity. Information is limited to Bos d 5 (lipocalins).
LIGAND-BINDING	Low	Contradictory effects are found for different potent allergens. Information is limited to parvalbumins, caseins, transferrins, and lipocalins.
PROTEIN STRUCTURE		
Loss of 2D	Low	Contradictory effects. Loss of structural stability decrease (destruction of conformational epitopes) or maintain/increase (unmasking hidden linear epitopes) IgE-binding capacity.
Loss of S-S bonds	Low	Contradictory effects. Loss of structural stability decrease (destruction of conformational epitopes) or maintain/increase (unmasking hidden linear epitopes) IgE-binding capacity.
GLYCATION	Low or inconclusive	Chemical changes (formation of advanced glycation products) can decrease, maintain, or increase IgE-binding capacity (depending on protein family or within the same family). Data missing for transferrins and serum albumins.
AGGREGATION	Low or inconclusive	Structural changes (formation of aggregates and potentially new conformational epitopes) can decrease, maintain, or increase IgE-binding capacity. Data missing for transferrins.
HEAT STABILITY	Low	Heat stable allergens are potent allergens. Fails to explain potent heat-labile allergens (e.g. arginine kinase, lipocalins)
PRESSURE STABILITY	Low	Pressure alone has a limited effect on allergens, but *in vivo* evidence is needed. Maintain protein integrity. Data missing for arginine kinases and transferrins.
LIGHT/RADIATION STABILITY	High	Light/radiation stable proteins are potent allergens. High doses of radiation decrease IgE-binding capacity (promotes unfolding). Data missing for transferrins.
MECHANICAL STABILITY	Low	Most allergens are stable to mechanical processing, preserving their IgE-binding capacity. Maintain protein integrity. Data missing for caseins, transferrins, and ovomucoids.
CHEMICAL STABILITY		
Changes in protein structure	High	Reduce the IgE-binding capacity.
Changes in protein integrity (fragmentation)	High	Reduce/mitigate the IgE-binding capacity. Loss of protein primary structure.
DIGESTIBILITY		
Pepsin resistance	Low or inconclusive	Fails to explain potent pepsin-labile allergens (e.g. some members of tropomyosins)

Trypsin/chymotrypsin resistance	High	Most allergens are labile to trypsin/chymotrypsin activities.
Lipid interaction	High	Presence of lipids protects allergens from proteolysis. Maintain protein integrity.

Figure captions

Fig. 1. List of food processing technologies analysed for each parameter.

Fig. 2. Decision tree interconnecting different physicochemical parameters and their influence for evaluating a protein allergenicity