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## Allergenic Milk Proteins. Friend or Foe Nutritional Proteins?

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Additional information is available at the end of the chapter

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### Abstract

Allergies are complex inflammatory diseases with a not fully understood etiology. Several factors, including genetic, environmental, age of exposition, diet, etc., are associated with the induction of these diseases. The incidence of allergies has increased during the last decades and constitutes the most common immune-based disease worldwide. According to the hygiene hypothesis, a lower exposure to pathogens and commensal microbes that reside in the intestinal lumen is responsible for the rapid rise of the prevalence of atopic and allergic disorders, specifically food allergy. To overcome this tendency, the immunological mechanisms underlying this pathology should be better understood, which will undoubtedly impact the development of novel therapies. A large body of evidence demonstrates that immunotherapies constitute corrective treatments of the impaired regulation of the immune system in allergic patients.

The aim of this chapter is to present an overview about allergic diseases and food allergies, mechanisms involved, differences with toxic reactions and food intolerances, a brief description of the main milk allergenic proteins, and the new therapeutic strategies derived from the old immunotherapies to restore oral tolerance, and finally, to describe different pathologies associated with milk allergy.

**Keywords:** milk allergens, food allergy, IgE, mucosal immunology, immunotherapy

## 1. Introduction to allergic diseases

### 1.1. Overview of allergic responses

The significant contribution of milk to the nutritional intake and health is well recognized. With a unique nutrient profile, it constitutes a relevant source of essential nutrients (minerals, vitamins, riboflavin, amino acids, proteins) and energy. This makes milk and dairy products essential components in the diet and is considered a protective food. For this reason, it has been included as an integral component of different programs in several countries. In the United States, there is a federal child nutrition program, which includes hundreds of millions of half-pints of milk. In particular, the importance of cow's milk proteins (CMP) resides in its significant amount, but also in its exceptionally high quality. Fluid milks contain approximately 3.5% proteins, being caseins 82% of the total milk proteins and whey proteins the remaining 18%. Regarding the high quality of these components, it provides all of the amino acids required by humans, in a distribution that resembles that needed by the human body [1]. In addition, milk contains other biological factors that are active in humans: growth factors (the human active insulin growth factor-1), cytokines (transforming growth factor- $\beta$ ), and immunoglobulins (IgM, IgG, and IgA).

Although milk is one of the most nutritious foods with health-promoting properties, in a restricted proportion of the population, it may have adverse effects. It has been described that dairy intake may increase the risk of prostate and ovarian cancer, type 1 diabetes, multiple sclerosis, acne, increased cholesterol levels, and probably atherosclerosis, lactose intolerance in a high proportion of individuals, and finally, is the most common food allergen in the world [2–5].

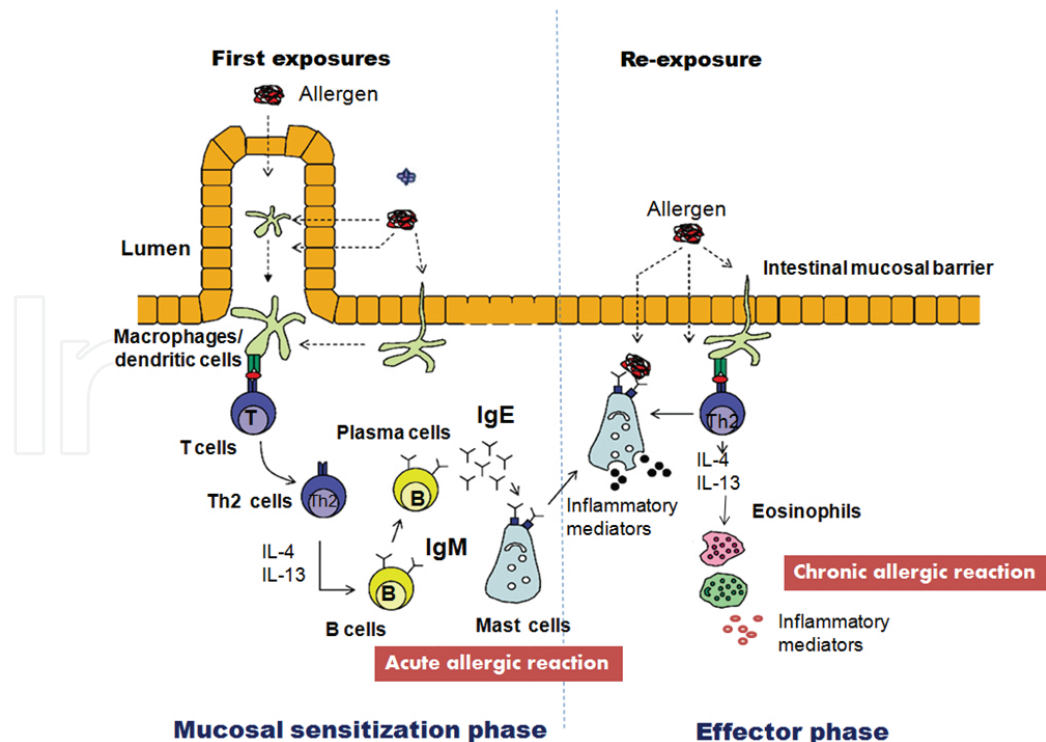
Cow's milk allergy (CMA) accounts for most diagnosed food allergies, mainly in the first year of life (6–8% at 1 year of age) [6], it is rare in adults (1–3%) and it can cause severe anaphylaxis [4, 7]. The natural history of food allergy indicates that it is lost over time. However, this process of outgrowing a food allergy does not mean a cure for the disease and is highly dependent on the food and individual susceptibility. For some foods, the intestinal mucosa “learns” how to manage dietary antigens during childhood, while for other foods sensitivity persists in adulthood.

Allergy is a chronic condition involving an abnormal reaction of the body to substances in the environment that are harmless for most people. These substances that act as immunogens are called *allergens* and were previously exposed to patients by inhalation, ingestion, injection, or skin contact. The immune system considers the allergen as a potential harmful antigen or threat, and triggers a misguided, inappropriate, and exaggerated reaction that comprises complex circuits of molecular and cellular components that promote inflammation. These mechanisms are called hypersensitivity mechanisms and any of the four types of hypersensitivity reactions described by Gell and Coombs [8] can be involved in an allergic disease. Since different organs can be targeted (nose, eyes, skin, lungs, gastrointestinal tract), allergic disorders are not a unique clinical entity. Allergy is actually a syndrome, with a spectrum of disorders with a heterogeneous and variable clinical presentation. It may include hay fever,

food allergies, atopic dermatitis, allergic asthma, and anaphylaxis; and symptoms may include itchy eyes, runny nose, difficult breathing, congestion, wheezing, skin rash, eczema, urticaria, bronchoconstriction, or diarrhea. Most allergic reactions are mild to moderate and may cause irritation and discomfort. However, a small number of people may experience a life-threatening reaction called anaphylaxis, which is a severe condition that requires immediate life-saving medication (epinephrine).

Another term that should be defined is *atopy*. It is the genetic predisposition to develop allergic diseases and is mainly mediated by IgE-dependent or immediate Type I mechanisms. The allergic reaction mediated by IgE antibodies is called atopic allergy and patients are atopic. The role of the Type 2 T helper (Th2) cell-mediated immune response against innocuous environmental antigens in the immune pathogenesis of allergic atopy is well documented by an extended body of experimental evidences. The critical role of Th2 cytokines, such as interleukin (IL) 4, IL-5, IL-9, and IL-13, in the initiation, maintenance, and amplification of human allergic inflammation has been thoroughly characterized, while the tissue damage produced by the sustained inflammation is mainly due to eosinophils, neutrophil, etc (type IV hypersensitivity).

Allergens are innocuous, environmental, and widely distributed antigens that upon frequent exposure to genetically predisposed individuals (atopic subjects) can lead to immunologic sensitization (sensitization phase of the allergic response) (**Figure 1**) that involves the production of IgE antibodies by plasma cells. Upon re-exposure to allergens, immediate and delayed (late-phase) responses may occur and tissue inflammation arises (effector phase). If



**Figure 1.** Mechanisms involved in an acute and chronic inflammation of an allergic reaction.

this mechanism is repeatedly triggered, different cells are attracted to the inflamed area and the condition may progress to a clinically detectable disease that is indicated by reversible or even irreversible tissue remodeling. In this state, a myriad of soluble mediators that are released by inflammatory cells under different specific or unspecific triggers (allergens, viruses, tobacco smoke, air pollutants, etc) exacerbate the severity of the disease.

During the sensitization phase, which occurs in the first exposures to environmental allergens, IgE antibodies are produced by plasma cells and secreted. Soluble antibodies are rapidly bound to high affinity surface receptors for IgE (FcεRI) on tissue mast cells, circulating basophils, and bone marrow-released eosinophils. Cells with surface-bound IgE antibodies are called sensitized cells and are prepared to rapidly respond to the specific antigen through the membrane-bound IgE antibodies. After activation, cells secrete numerous vasoactive and proinflammatory mediators, such as histamine, accumulated in cytoplasmic granules, and induce the synthesis of other pro-inflammatory mediators (leukotrienes, prostaglandins, growth factors, etc). Continuous re-exposure to allergen may occur, sensitized cells are activated, and these soluble mediators maintain tissue inflammation.

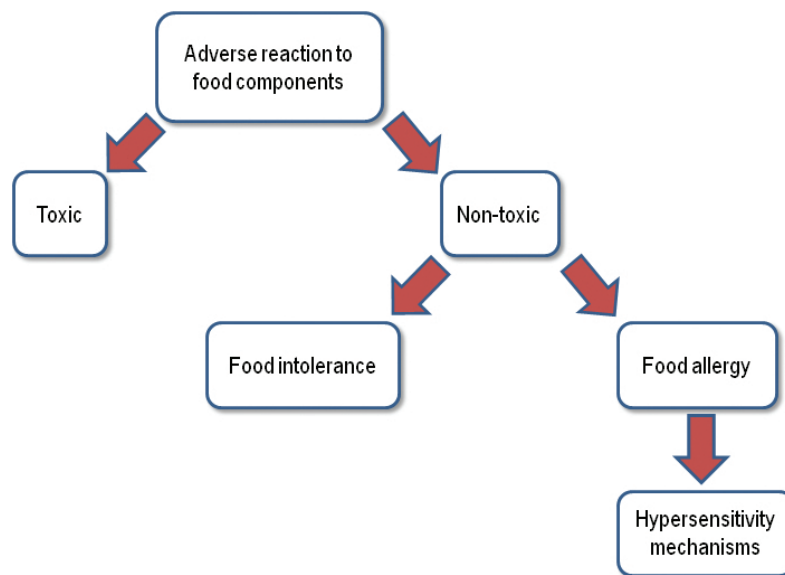
The term *allergen* refers to the immunogen (the substance that induce the immune response) or antigen (the substance that reacts with the induced immunological elements) involved in an allergenic reaction. Most of them are carbohydrates, proteins, and glycoproteins with common sequential, conformational, structural, and evolutionary features [9]. Most of the allergenic proteins are concentrated in 70 protein families, out of the 10,000 described (2–5% of all known structural protein families), and food allergens (500 allergens) are distributed in approximately 20 families and 4 super-families (prolamins, prophilins, cupins, and Bet v1 homologues).

The most common natural sources of allergens are comprised in household dust mites, pollens, animal dander, insect stings, moulds, some drugs (antibiotics), and certain foods. Despite the high number of food allergens described, a relatively small number of allergens cause a high proportion of food allergies. More than 170 foods have been reported to be allergenic. However, allergy to certain foods appears to be especially common. The “Big Eight” is referred to the eight foods (milk, egg, soy, fish, shellfish, peanuts, wheat, and tree nuts) that produce the 80–90% of food allergies, milk being the most allergenic food worldwide. In order of prevalence, and depending on the population studied, the most common food allergens are milk, egg, peanut, tree nuts, crustaceans, shellfish, fish, wheat, and soy.

## 1.2. Definition of the different adverse reactions caused by foods

To unify the terms used by the medical community and the common people, which is sometimes influenced by social and cultural perceptions creating confusion, and to avoid misdiagnosing, it is useful to define the different conditions that involve the exposure to foods and may produce symptoms (**Figure 2**). There are still too many situations where people, and even certain professionals, do not recognize that food allergy is a medical condition, not a food preference. The clinician should be aware that adverse reactions to food can be categorized based on whether the immune system is involved or not.

The food-related *adverse reactions* comprise any abnormal reaction that is produced by a food or food component. They include both toxic and non-toxic reactions. The former are only dependent on the food and maybe caused by the presence of histamine (chocolate, strawberry, etc), histamine-releasing factors (pineapple), or most commonly, contaminated foods (bacteria toxins from *Staphylococcus*, *Clostridium*, etc.). The latter is dependent on individual susceptibility and involves *food intolerances*, which are independent of immune activation, and the immune-mediated *food allergy*. In addition, allergic reactions to food components can be classified as IgE-mediated (immediate gastrointestinal hypersensitivity, eosinophilic esophagitis, anaphylaxis, etc) or non-IgE-mediated (food-protein-induced enteropathy, allergic colitis, and proctocolitis, etc.). Therefore, food intolerances (lactose intolerance, anatomical abnormalities of the gastrointestinal tract, etc.) and food poisoning are separate conditions, they are not food allergies. Similar clinical reactions can occur to some chemicals and food additives (urticaria); however, if they do not involve the immune system, they are known as adverse reactions rather than allergy (toxic adverse reactions).



**Figure 2.** Classification of adverse reactions to food components.

In conclusion, *food allergy* is defined as an adverse reaction elicited on exposure to a given food and is mediated by a specific immune response that occurs reproducibly on time, and might be either IgE-mediated and/or non-IgE-mediated.

The literature reflects that it is very common the overdiagnosis of food allergies due to the difference between food allergy and food intolerance, which is sometimes confusing [10, 11]. For diagnosis, once an adverse reaction is suspected, it should be demonstrated that the immune system is involved and the offending food should be identified to avoid unnecessary restriction diets that may affect growth, nutritional deficiencies, or impair the quality of life. Many children are unnecessarily placed on restrictive food diets on the basis of serum food-specific IgE testing or skin-prick testing, which are the most common complementary assays



to clinical history. The presence of specific IgE does not necessarily imply a clinical allergy [11]. Therefore, the gold standard assay for confirmation of food allergy, the oral food challenge, should be done. Nevertheless, in most places, it is complicated and rather impracticable to perform as a standardized challenge, and hence food allergy is commonly not confirmed, and overdiagnosis is possible.

### 1.3. Main causes of allergic diseases

It is widely accepted that although allergies can develop at any age, the risk of developing allergies is genetically determined. If neither parent is allergic, the chance for allergies is about 15%. If one parent is allergic, the risk increases to 30%, and if both are allergic, the risk is greater than 60%. Several polymorphic genes in almost all chromosomes have been identified in different allergic populations [12, 13]; however, all of them only determine susceptibility. Nevertheless, the expression of the allergic phenotype is dependent on the gene-environment interaction. A large body of experimental evidences has identified during the last 5–6 decades environmental factors as the main inducers of allergy in susceptible individuals. This makes allergy inheritance a complex polygenic disorder and is considered a multifactorial disease.

There are clear evidences that in Western societies, these disorders are increasing in frequency, with reports showing that prevalence has doubled over 10-year periods. It was initially seen in UK, other countries of Europe, and USA, that 30–35% of people are affected of asthma and rhinitis. But more recently, food allergy has emerged over the last 10–15 years as a “second wave” of the allergy epidemic, affecting 10% of infants in Australia. As changes in genes take many hundreds of years in humans, the genetic basis alone cannot account for the current increase in allergy seen over the past decades. New studies suggest that environmental factors are responsible for these changes. Epidemiological studies have clearly shown that modification of the pattern of microbial exposure of children represents a key factor to understand the changes in severity and prevalence of atopic diseases. The hygiene hypothesis, formerly expressed by Strachan [14] and later modified by Bach [15], is nowadays the most reasonable explanation for the striking increase observed from the twentieth century in the incidence of many chronic inflammatory disorders, including allergies and autoimmune disorders. The modern urbanization and lifestyle have generated immunoregulatory problems attributable to depletion of exposure to organisms that are present in the urban environment with which mammals co-evolved and shaped the immune system. The immune system needs to come into contact with a variety of microorganisms, while it is developing at the infant stage, in order that it responds appropriately later in life. We now live in an environment with less contact with pathogens and commensal microorganisms since we use cleaners containing anti-microbial agents, antibiotics, better vaccines, and compulsive vaccination programs, more hygienic food preparations, etc. While children living in farms were directly exposed to animals, and their environment contained a range of microbial agents and plant-derived agents, most of people in westernized societies now live in cities with minimal exposure to animals. The literature reflects that people with a rural lifestyle have a lower incidence of allergy. Inadequate exposure to environmental microorganisms may therefore result in the

immune system of atopic children developing a tendency toward allergy. The development of a new generation of antibiotics and vaccines have protected people from many infections that previously killed large numbers of the population and have proven to be essential to protect the health of the population. However, the cost of this may be the reduction in the pressure placed on the immune system to mount a strong immune response against infectious agents. This may be one of the factors to explain why the immune system in a restricted, but increasing, percentage of the population is now reacting to innocuous allergens. In other words, the initial interpretation by Strachan [14] proposed a missing immune deviation of allergen-specific responses from Th2 to Th1 immune profile, mainly as consequence of the reduced production of IL-12, interferon- $\alpha$ , and interferon- $\gamma$  by innate cells (mainly dendritic cells, NK cells, and innate lymphocytes), which are stimulated by prokaryotic products. The reduced contact with pathogens that prime Th1 responses in early life can result in a stronger and compensatory induction of the contra-regulatory Th2 response. Notwithstanding, this postulate could not explain the contemporary close rise in the prevalence observed for the Th1-mediated autoimmune diseases (type 1 diabetes, multiple sclerosis, and inflammatory bowel diseases) (**Figure 3B**) in developed countries, and the epidemiological observations reporting a low prevalence of allergy in geographical regions characterized by chronic helminth infections (stronger inducers of Th2 as well as suppressive cytokines). Therefore, an alternative view has emerged, which suggests the importance of a reduced immune suppression rather than missing immune deviation. The modified and unifying hypothesis later suggested by Bach postulated that a lower microbial burden may favor increased prevalence of allergy by inducing a lower activity of regulatory cells (dendritic and T cells). Epidemiological and experimental findings suggest that both mechanisms, missing immune deviation, and reduced immune suppression, may be independently involved in allergic and autoimmune disorders [16], and both, allergic diseases and autoimmunity, can independently and simultaneously increase. Therefore, the expanded hygiene hypothesis may provide a better understanding of these epidemiological changes observed in immune-mediated inflammatory disorders.

Since it has recently been demonstrated the importance of the intestinal microbiota in shaping the immune system, changes in the composition of the microbiota is suggested as a critical factor to generate impaired mucosal and systemic regulatory circuits. The gut is always in a state of controlled inflammation, and regulatory cells are abundant in this tissue. Defects in Tregs promoted by the “unhealthy microbiota” or “bad microbiota” undoubtedly impact in the regulation of several immune mechanisms, including those that protect from allergy and autoimmune diseases. In conclusion, the modern hygiene hypothesis postulates that the restricted exposure to pathogenic and commensal microorganisms may be implicated in the increased observed in several immunological disorders.

This hypothesis not only provides the theoretical framework to explain the rise in the prevalence of immune disorders but also has therapeutic implications, as it will be discussed later in this chapter. Allergen-specific immunotherapy has proven to be the unique disease-modifying therapy for allergy. It has been demonstrated in treated patient the suppressive role for the therapy-induced Tregs and secreted IL-10.

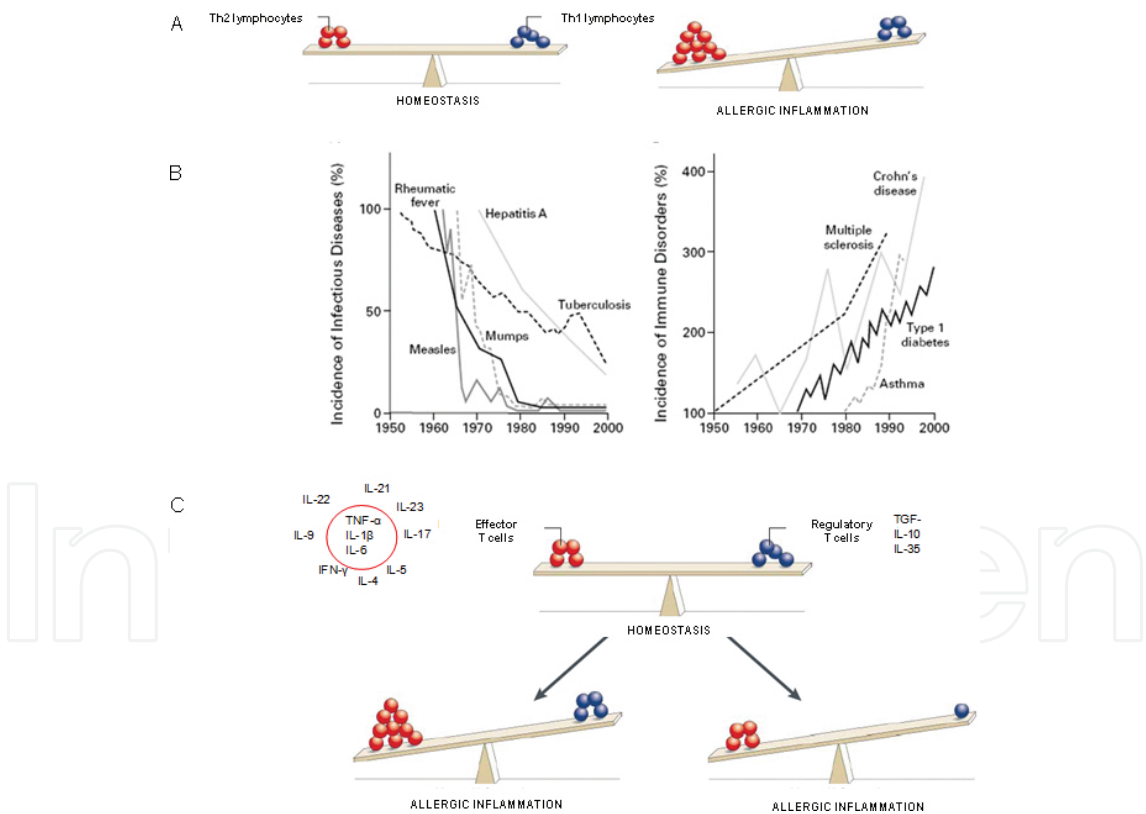


## 2. Allergenic proteins

### 2.1. Milk allergens

#### 2.1.1. Allergens of cow's milk

Acute reactions in CMA patients can be caused by several proteins in cow's milk. Cow's milk contains approximately 30–35 g/L of CMP. Milk protein fractions can easily be obtained using either chymosin (rennin) or acid precipitation (pH 4.6). The insoluble coagulum containing the whole casein fraction constitutes approximately 82% of the CMP, while lactoserum or whey proteins, which comprise approximately the remaining 18% of the CMP, constitute the soluble fraction. Both fractions contain allergens that could elicit allergic symptoms in susceptible individuals. The casein fraction or Bos d 8 (from *Bos domesticus*) that contains  $\alpha$ S1-,  $\alpha$ S2-,  $\beta$ -, and  $\kappa$ -casein (32%, 10%, 28%, and 10% of total CMP, respectively) constitutes the major allergens of milk. The whey major allergens are  $\alpha$ -lactalbumin (Bos d 4) and  $\beta$ -lactoglobulin (Bos d 5) (5% and 10% of total CMP, respectively), while less common allergens are serum albumin (Bos d 6) and immunoglobulins (Bos d 7) [17, 18].



**Figure 3.** The hygiene hypothesis originally defined by Strachan and re-formulated by Bach. *A*, Strachan postulated that the balance between Th1 and Th2 cells is impaired in allergy. *B*, Infectious diseases were controlled in the last decades while Th1- and Th2-immune-mediated diseases increased in prevalence; *C*, Bach postulated that an impaired balance between Treg and effector T cells promotes immune-mediated inflammatory disorders. Part of figure was taken with the permission from Bach [15], NEJM 2002.

#### 2.1.1.1. Caseins

The allergen Bos d 8 refers to a heterogeneous mixture of proteins. The casein fraction of milk proteins contains components that belong to two unrelated protein families, one family comprising  $\alpha$ S1-,  $\alpha$ S2-, and  $\beta$ -caseins (the  $\alpha$ S2-casein gene is not expressed in humans), while  $\kappa$ -casein constitutes the other family. Even within the  $\alpha$ -/ $\beta$ -casein family, sequence identities are below 15%. Hence, the entry Bos d 8 was demerged into four separate allergens: Bos d 9.0101 ( $\alpha$ S1-casein), Bos d 10.0101 ( $\alpha$ S2-casein), Bos d 11.0101 ( $\beta$ -casein), and Bos d 12.0101 ( $\kappa$ -casein) in a proportion of 37/37/13/13%, respectively. The name Bos d 8, which is widely established and has been used in numerous publications and names of commercial diagnostic tests, was kept and designates the whole casein fraction.

There is considerable similarity in the caseins from different mammalian milks used for human consumption, which explains their IgE cross-reactivity [19]. The analysis of sequences showed that bovine caseins had more than 80% of sequence homology with goat and sheep caseins, while bovine  $\beta$ -casein has 50% of sequence homology with human  $\beta$ -casein.

#### 2.1.1.2. Whey proteins

Whey contains essentially globular proteins. The major allergens of lactoserum are  $\beta$ -lactoglobulin and  $\alpha$ -lactalbumin. The  $\beta$ -lactoglobulin (Bos d 5) is the only lipocalin that acts as a food allergen and occurs naturally as a dimer. It has no homologous counterpart in human milk, which explains its high immunogenicity. The relative resistance of  $\beta$ -lactoglobulin to acid hydrolysis and gut proteases determines that this protein can be absorbed as a native antigen in the intestinal mucosa. Lipocalins have a high allergenic potential, and several allergens of animal origin belong to this protein family.

Alpha-lactalbumin (Bos d 4), the other major whey protein allergen, is a monomeric protein stabilized by four disulfide bonds with a calcium-binding domain. The complete amino acid sequence of bovine  $\alpha$ -lactalbumin shows extensive homology with hen's egg white lysozyme but also with human  $\alpha$ -lactalbumin; however, it has been described as a milk allergen.

Other minor whey allergens are bovine serum albumin, immunoglobulins, and lactoferrin.

#### 2.1.2. Milk allergens from other mammals

Since cow's milk is the most consumed milk, the incidence of CMA is higher than for other milk sources. Nevertheless, goat's milk and sheep's milk represent important sources of milk allergens. Due to the high degree of sequence homology and hence cross-reactivity with cow's milk, goat's and sheep's milk are potent allergens for most cow's milk allergic patients. Notwithstanding, and for unknown reasons, some patients with CMA can tolerate these alternative milks as dairy substitutes during treatment. As it can be observed in **Table 1**, bovine casein sequences showed more than 80% of sequence homology with goat and sheep caseins, and the major whey allergens showed a sequence homology higher than 90% [20]. In addition, milks of other mammals such as horse, donkey, or camel are also used as dairy substitutes.

Protein	Concentration g/L	Allergen name	Molecular weight kDa	pI	Sequence homology % amino acid identity
Whole casein fraction (80%)	29.5	Bos d 8			$\alpha$ S1 and $\alpha$ S2-CAS from cow milk: 22.5% $\alpha$ S1-CAS from cow milk and sheep/goat milk: 87/89% $\alpha$ S2-CAS from cow milk and sheep/goat milk: 87/89%
$\alpha$ S1-casein	12–15	Bos d 9.0101	32.4	4.9–5	
$\alpha$ S2-casein	3–4	Bos d 10.0101		5.2–5.4	
$\beta$ -casein	9–11	Bos d 11.0101	26.6	5.1–5.4	$\beta$ -CAS from cow milk and sheep/goat milk: 91% $\beta$ -CAS from cow milk and human milk: 50%
$\kappa$ -casein	3–4	Bos d 12.0101	19	5.4–5.6	$\kappa$ -CAS from cow milk and sheep/goat milk: 84%
Whey fraction (20%)	6.3				
$\alpha$ -lactalbumin	1–1.5	Bos d 4	14.2	4.8	$\alpha$ -LA from cow milk and water buffalo: 99% $\alpha$ -LA from cow milk and sheep/goat milk: 94%/95% $\alpha$ -LA from cow milk and human milk: 78%
$\beta$ -lactoglobulin	3–4	Bos d 5	18	5.3	$\beta$ -LG from cow milk and water buffalo/mouflon: 98%/95% $\beta$ -LG from cow milk and sheep/goat milk: 93%/94% cockroach allergen Bla g 4: about 20%
Bovine serum albumin	0.1–0.4	Bos d 6	67.0	4.7–4.95	BSA from sheep milk: 92% BSA from cow milk and pig, cat, human, rhesus macaque, horse milk: between 74 and 79%
Lactoferrin	0.09	Lactoferrin	80	8.7	Lactoferrin from cow milk and human: 69%
Immunoglobulins	0.6–1	Bos d 7	150	–	–

**Table 1.** Main characteristics of the major cow's milk proteins and sequence homology with other mammal milks.

## 2.2. Methods employed for characterization and prediction of allergenicity

### 2.2.1. Methods used for detection of allergens

Different methods can be used to study the allergenicity of milk proteins. Polypeptide masses usually range between 5 and 70 kDa; however, many allergens are oligomers with molecular masses greater than 200 kDa. Allergenicity assessment is generally performed using human IgE-containing sera, polyclonal antisera (rabbit, rat, mouse, goat, sheep, camelid, chicken, etc), or experimental animals (pig, mice, rabbits, etc). Protein-based methods employed to identify and characterize allergens usually involve immunochemical detection protocols such as the radio-allergosorbent test (RAST), enzyme allergosorbent test (EAST), dot blot, immunoblotting, and enzyme-linked immunosorbent assay (ELISA). These tests can be developed as qualitative, semi-quantitative, or quantitative assays using polyclonal antisera, human sera, or monoclonal antibodies. Furthermore, the ELISA technique is the most widely used assay to routinely detect and quantify allergens in foods due to its high precision, simple handling, and potential for standardization. Another emerging technology applied for allergens analysis is the use of biosensors. Biosensor instruments, based on the interaction between allergens and specific antibodies, can monitor the presence of a protein in real time. The immobilization of an antibody to a sensor chip surface allows the on-line recognition of a protein based on surface plasmon resonance. It has been applied for the detection of a few potentially allergenic foods like peanut hazelnut, egg, and milk in the food processing industry.

In addition, cell-based assays are used for characterization or quantification of allergens. The basophil activation and histamine release test can be used to confirm the allergenicity of a protein (two surface IgE epitopes on the allergen are needed to activate basophils). Sensitized basophils are used and cell activation can be assessed by ELISA (detection of histamine released in the supernatant), flow cytometry (translocation of CD63 and CD203c from the membrane of cytoplasmic granules to the plasmatic membrane), or electrochemically (modification of impedance in rat basophilic leukemia RBL-2H3 mast cells transfected with FC $\epsilon$ RI upon interaction of IgE with the allergen).

In order to characterize the presence of epitopes and further investigate its antigenicity (B epitopes) or allergenicity (IgE epitopes), epitope mapping is performed. This point is critical for developing protein- or peptide-based immunotherapies. Linear or conformational, B or T epitopes can be assessed using different strategies, and synthetic or recombinant peptides are commonly used. However, the main drawback in the development of these techniques is often the high cost and time-consuming. For this reason, functional proteomics or immunoproteomics constitute nowadays a high-throughput technology to identify epitopes. These methods employ mass spectrometry (MS) to study the interactions of peptides with the major histocompatibility complex or antibodies. Given the high sensitivity and high accuracy in determining molecular mass and high capacity for analysis, MS techniques currently figure prominently in identifying B-cell or T-cell epitope targets for vaccine and immunotherapy development [21].

Furthermore, epitope mapping is important in diagnosis (the so called “component-resolved diagnosis,” which is based on individual IgE pattern reactivity according to recombinant allergen recognition) and treatment (tailor-made immunotherapy and vaccine development).

### 2.2.2. *Methods used for prediction of allergenicity*

In addition to the different immunochemical techniques employed to assess the allergenicity of new proteins or to predict cross-reactive allergens, several online databases and computational methods have been developed to effectively determine potential allergens. Bioinformatics comparison of food proteins provides a mechanism to identify proteins that can lead an increased risk of food allergic reactions. The use of bioinformatics tools may help to identify new allergenic or similar proteins to an allergen that might induce cross-allergic reactions. This point will undoubtedly impact on allergy diagnosis, prognosis of potential reactions, and therapy. Several allergen databases and allergen prediction web tools are available on the Internet and can be easily operated by common users: IUIS Allergen Nomenclature Allergen, Database for Food Safety, Allergome, The Immune Epitope Database (IEDB), Structural Database of Allergenic Proteins (SDAP), Allermatch, AllerTool and AllergenPro, and others.

## 3. Clinical implications

### 3.1. Epidemiology and triggering factors

The perception of milk allergy is far more frequent than confirmed CMA, due to the few surveys of secular and geographical trends in food allergy in adults and children. Reports of CMA prevalence range between 1 and 17.5% among preschoolers, between 1 and 13.5 % at 5–16 years of age, and among 1–4% of adults [22, 23]. Clinical symptoms of CMA commonly appear during the first months of life, usually within days or weeks after feeding with CM-based formulas have been started or may sometimes be seen in exclusively breast-fed infants. With such an early age of onset, symptoms of an erythematous rash or hives shortly after intake of CM formula are suggestive of food allergy. Urticaria, exanthema, or both are more common in IgE-mediated CMA, whereas children with non-IgE-mediated CMA more often have atopic eczema and diarrhea. Tolerance to CM develops in children with non-IgE-mediated CMA significantly earlier than in the IgE-positive group (5 years old vs. 8 years old or more). At age of 8 years, children with IgE-positive CMA are more frequently sensitized to birch pollen, animal dander, and foods than those with IgE-negative CMA. Furthermore, it has been reported that CMA increased the risk for sensitization to inhalant allergens [24, 25].

Patients with IgE-mediated CMA develop gastrointestinal symptoms in 32–60% of cases, skin symptoms in 5–90%, and anaphylaxis in 0.8–9 % of cases. This frequency of anaphylaxis is the main concern pointed out in many CMA studies [22].

A variety of risk factors are proposed to influence food allergy or sensitization: sex (male sex in children), race/ethnicity (increased among Asian and black children compared with white



children), genetics (familial associations, HLA, and specific genes), atopy (co-morbid atopic dermatitis), vitamin D insufficiency, dietary fat (reduced consumption of omega-3-polyunsaturated fatty acids), reduced consumption of antioxidants, increased use of antacids (reducing digestion of allergens), breastfeeding, obesity (an inflammatory state), increased hygiene, and the timing and route of exposure for foods. Although it is not clear which factors are key in the increase in the incidence of food allergies observed in the last decades, the aforementioned hygiene hypothesis provides a convincing interpretation. Nevertheless, other non-considered factors should be taken into consideration. Changes in the diet might have a causal link with allergy development. A decreased intake of fruits and vegetables, changes in the type of fat included in the diet, the timing of feeding events during infancy, nutrients and micronutrients in the diet, such as long-chain polyunsaturated fatty acids, vitamin D, folic acid, etc, may likely affect food allergy development. However, a number of nutritional and dietary variables might be interfering food allergy manifestation in infants, instead of considering only one nutrient or dietary characteristic, which might be an oversimplification of the complex interactions taking place.

Although several theories have been proposed to explain the protective effect of breastfeeding, pros and cons are still controversial. It has been proposed that the effect of exclusive breastfeeding delays the introduction of cow's milk, thus preventing early sensitization. Nevertheless, it contradicts the fact that the administration of very small amounts of the allergen benefits tolerance induction and allergy control. Therefore, it is considered that the intake of CMP in the mother's diet during lactation could be relevant. It has been widely probed that mother's dietary native antigens are present in breast milk. Furthermore, and not less important, immunomodulatory components are present in breast milk, such as IgA, IgG, TGF- $\beta$ , macrophages, and dendritic cells.

The most striking component that may be affected by lifestyle and diet is the composition of the microbiota. It has been demonstrated that relatively harmless microorganisms (helminths, saprophytic mycobacteria, lactobacilli, etc) that have been present throughout mammalian evolution can drive maturation of regulatory immune cells and prime immunoregulation by release of IL-10 and TGF- $\beta$ . Developed countries with modern lifestyle have a diminished contact with these microbes, while they were kept intact in rural life. Additionally, it has been demonstrated that allergic patients have less frequency of lactobacilli and increased coliforms in the microbiota compared with microbiota of healthy individuals [26]. This means that a modification on the microbial community may induce a change in the background of the bystander suppression that is continuously induced in the gut. Compelling data in atopic individuals supports that different factors may induce a dysbiosis and be detrimental for the host [27].

In conclusion, a suboptimal microbiome depleted of beneficial bacteria can alter the host homeostasis in the gut, thereby increasing an aberrant immune response to innocuous allergens and autoantigens.

The evidence that link the composition of the microbioma and the immune system provides new insights into the causes of the increase in several immunopathologies and suggests potential new therapeutic targets.

### 3.2. Current treatments

CMA is a heterogeneous disorder with no single immunologic mechanism involved. Once food allergy to CMP is suspected, avoidance of the allergen is the only available treatment for infants. For 6-month-old or younger babies, milk should be substituted and the recommended formulae are currently extensively hydrolyzed proteins or amino acid-based formulae as the best choice for infants with high-risk of anaphylaxis [28]. As mentioned before, milks of alternative sources are also employed, with different consequences regarding its clinical tolerance. Mammalian milks, rice-, and soy-based formulae are the most used alternatives. As previously mentioned, most of the patients do not tolerate animal milks, although, and, for not fully understood reasons, a restricted proportion of patients can perfectly tolerate goat's milk and sheep's milk. Soy-based formulae are also frequently used in children older than 6 months old [29]. Nonetheless, soy intolerance is observed in some IgE-mediated or non-IgE-mediated milk allergic patients. This phenomenon can be explained by either co-sensitization or cross-allergenicity between soy and milk allergens. We have used a food allergy mouse model to CMP and characterized several soy allergens that cross-react with caseins. We identified B and T epitopes shared between the main soy allergens and bovine caseins [30–32]. Again, only a restricted proportion of milk allergic patients react against soy proteins during restriction diet. It probably depends on the individual pattern of reactivity, affinity of IgE antibodies, and on some unknown factor [33]. Therefore, this point adds a new restriction to the choice of a dairy substitute. In addition, the nutritional inadequacy of soy-based formulae makes them not recommendable for patients younger than 6 months old.

Therefore, even though a restriction diet seems to be the easiest and most efficient treatment for milk allergic patients, several drawbacks arise. Finally, patient and family education is crucial to prevent accidental reactions

### 3.3. Adverse reactions and related causes

Approximately 50% of patients with food allergy suffer accidental reactions upon contact with food and non-food containing the allergenic component during the restriction diet. This can be explained by several factors: contamination of food with the allergen (utensils at restaurant, cross-contamination during the food manufacturing or processing, during cooking, etc.), ignorance of the composition of the food (caseinate means the presence of milk proteins), mislabeling of food composition (omission), presence of cross-reactive components (soy, peanut, fish, nuts, etc), etc. Most of these accidental reactions are often severe and life-threatening for patients, who should be aware of self-use of pre-loaded pen injections (epinephrine) [34, 35]. In addition, and to make the situation even more complicated, there is an increasing tendency of CMA to persist with time.

Therefore, the current standard of care for the management of IgE-mediated food allergy involves the identification of causative foods and avoidance of the allergens while always having self-injectable epinephrine available [36]. The avoidance of the immunologic stimulus seems to be efficient; however, it is not a corrective procedure of the impaired immune response and accidental reactions are very common for patients. Considering all of these

complications, there is a need in the medical community to develop disease-modifying treatments.

### **3.4. New insights on immunotherapy and relevance of mouse models for experimental allergy**

Immunotherapy is nowadays a promising treatment for food allergies. It has been successfully used in different immunopathologies (cancer, autoimmunity, etc) to induce, enhance, or suppress an immune response. In allergy, it is nowadays accepted that it has the potential for disease modification [37]. Although allergen-specific immunotherapy has been used for the treatment of IgE-mediated allergy longer than a hundred years, the first randomized clinical trial of oral immunotherapy for food allergy was done in 2008 [38]. The controlled and step-wise administration of milk to patients with IgE-mediated CMA rendered a clinical tolerance to 200-fold higher amounts of milk than placebo-treated patients. Nevertheless, the development of long-term tolerance was unlikely. It should be mentioned that all clinical trials report the presence of adverse reactions during immunotherapy. For this reason, there are no approved therapies for food allergy in the clinical practice, and still remains as an experimental therapy.

Tolerance is the state in which a person can consume a food without any allergic symptom in weeks, months, or years after cessation of regular and therapeutic exposure to the food antigen. In this state, long-term clinical unresponsiveness is maintained [39, 40]. This mechanism contrasts with desensitization, which depends on the regular ingestion or exposure to the food allergen, once tolerance has been achieved, to sustain unresponsiveness. The immunologic mechanisms underlying the development of tolerance are yet not fully understood, but regulatory T cells are likely involved.

In recent years, the controlled oral or sublingual administration of the allergen has gained increasing attention. It has been demonstrated that a substantial number of allergic patients can tolerate gradually increasing amounts of the food allergen, which can probably assure that patients are protected against accidental natural exposure to the allergen-containing food. However, most of these clinical trials showed limitations [41, 42].

The development of animal models for food allergy holds great potential as powerful biological tools to investigate the underlying mechanisms involved in the allergic pathway and for developing and testing novel treatments to restore tolerance. Due to the ethical concerns and the chance of fatal anaphylactic reactions in humans, great interest has arisen in the use of animal models that resemble the pathology in man [43].

Mice are the predominant laboratory animals used to study many diseases for several reasons. However, the main limitation is that allergy is not spontaneously elicited in mice, due to its genetic background. Therefore, it should be artificially induced using pro-Th2 adjuvants (hydroxide aluminum, cholera toxin, Staphylococcus enterotoxin B, etc.) [44–46]. Since the first models developed, there has been significant progress in their optimization. The use of mucosal adjuvants, such as cholera toxin, promotes the induction of the inflammatory process

in the intestinal mucosa. These models closely resemble the pathology in man. For this reason, food allergy mouse models have become a useful tool as a pre-clinical assay.

The characterization of new mucosal adjuvant (molecular patterns from microorganisms) and new routes of administration (oral, nasal, or sublingual) will undoubtedly impact in the development of novel disease-modifying therapies using the offending allergen in a controlled and step-wise administration strategy. Immunomodulatory and tolerogenic immunotherapies that restore the immunoregulatory networks are promising treatments for food allergy.

### **3.5. Other pathologies associated to food allergies**

Many studies in the last decades linked the exposure to foods with disorders non-related with food allergy and might be considered food intolerances. The appearance of some disturbances in the central nervous system such as migraine, epilepsy, and hyperkinetic syndrome has been reported, or schizophrenia in patients undergoing a gluten-free diet. However, in this case, genetic and immunological components might be involved in symptoms associated with psychiatric disturbances [47]. It has been hypothesized that defects in the intestinal barrier allow the passage of neuroactive peptides of food origin and interfere with the central nervous system. This hypothesis has been proven in CMA children, in whom a slight improvement in autistic symptoms was achieved after milk restriction diet. This observation, along with the presence of high levels of IgA antibodies specific for the incriminated foods, was interpreted as a consequence of the increased absorption of protein fragments by the intestinal mucosa due to a peptidase defect detected in autistic patients. High prevalence of immune-mediated conditions, including asthma, allergic rhinitis, atopic dermatitis, urticaria, type 1 diabetes, and inflammatory bowel disease, has been reported in children with autism spectrum disorders [48–51]. In addition, peptides derived from casein gastrointestinal digestion may have an opiate-like effect on brain cells. It has been demonstrated in experimental animals that the slow digestion of caseins promotes natural morphine-like substances known as casomorphins. These peptides enter the bloodstream and reach the brain where they act through specific neuronal receptors and cause addictions to dairy products.

## **4. Conclusions**

Milk and dairy products are excellent sources of high-quality proteins, peptides, and amino acids with a range of potential health benefits and functional properties. Hence, they have attracted the interest of researchers and the food industry. Since no severe detrimental properties have been described in its components, symptoms attributed to milk intake are mainly associated to individual susceptibility. These reactions are sorted into food intolerances and food allergies, being the latter immune-mediated disorders which can be caused by any of the four types of hypersensitivity mechanisms. Strikingly, these inflammatory diseases have shown a rise in the last decades which was attributable to lifestyle changes in modern societies of developed countries. Nevertheless, the controlled administration of the “offend-

ing” proteins to food allergic patients can reverse the impaired immune response. Nowadays, immunotherapy is the only disease-modifying treatment for allergic patients.

In conclusion, the evolutive and traditional culture of milk consumption by man through thousands of years indicates its importance as a natural nutrient.

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