

Associations between Milk and Egg Allergens and the HLA-DRB1/DQ Polymorphism: A Bioinformatics Approach

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Key Words

Food allergy · Egg white allergens · Egg yolk allergens · Milk allergens · HLA-DRB1 · HLA-DQ

Abstract

Background: Little is known about the associations between human leukocyte antigens (HLAs) and food allergies. Our aim was to analyze the associations between the HLA class II polymorphism and food allergy using bioinformatics. **Methods:** A two-step algorithm was developed which mimics the food allergen processing in the human body. In the first step, the allergen is digested by pepsin, trypsin and chymotrypsin. In the second step, the digested fragments bind to the most frequent 12 HLA-DRB1 and 5 HLA-DQ alleles, and the binding affinities are predicted. **Results:** The algorithm was applied to 13 well-known milk and egg allergens. The predicted HLA binders were compared to known T-cell and IgE epitopes originating from the same allergens, and 77% of them were found to overlap. We found that the peptides generated from milk allergens bind to DRB1*01:01, DQ7 and DQ8 but not to DRB1*03:01, DRB1*04:04, DRB1*12:01 and DRB1*15:01. The peptides generated from egg allergens bind to DRB1*01:01, DQ4, DQ7 and DQ8 but not to DRB1*03:01, DRB1*04:04 and DRB1*12:01. They bind to all the DQs studied. The alleles that bind to allergen peptides could be considered as susceptible to the particular allergy and the

nonbinding alleles as protective. **Conclusions:** The alleles DRB1*01:01, DQ7 and DQ8 are considered as susceptible to cow's milk allergy and DRB1*03:01, DRB1*04:04, DRB1*12:01 and DRB1*15:01 as protective. The alleles DRB1*01:01, DQ4, DQ7 and DQ8 are considered as susceptible to egg allergy and DRB1*03:01, DRB1*04:04 and DRB1*12:01 as protective. Protective DQs against egg allergy were not revealed in this study.

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Introduction

The major histocompatibility complex (MHC) class II proteins are involved in allergen processing in the specialized antigen-presenting cells located at mucosal surfaces. In humans, the MHC proteins are known as human leukocyte antigen (HLA) proteins. The HLA class II proteins involved in the presentation of allergen fragments to the Th2 cells are extremely polymorphic and polygenic [1]. They consist of 2 protein chains, α and β , encoded by 3 HLA loci: DR, DQ and DP. The binding cleft on HLA class II proteins is occupied by nonamer peptides but is open-ended, allowing much longer peptides than nonamers to bind. The HLA polymorphism is concentrated in the binding cleft. Each cleft consists of several binding pockets which have a different shape

and polarity, and hence the ability to bind different amino acid residues [2–4].

The HLA class II polymorphism is associated with susceptibility to and protection against some allergies. Knapp et al. [5] found an association between HLA-DR1 and the mugwort pollen allergen, Art v 1. Rajagopalan et al. [6] reported that HLA-DR3 is associated with childhood asthma, while other studies found no association between atopic asthma and HLA [7, 8]. Kusano et al. [9] analyzed the association between HLA-DP5 and the Japanese cedar pollen allergen, Cry j 1. Associations between several DR and DQ alleles and the cow dander allergen, Bos d 2, have been reported [10]. The known HLA class II restrictions of allergens are reviewed by Jahn-Schmid et al. [11].

As peanut allergy is the most common, severe, usually permanent and increasingly prevalent food allergy, and is also associated with substantial morbidity and mortality [12], most studies looking for associations between the HLA polymorphism and food allergy focus on peanut allergy [13–17]. Less is known about the association between the HLA polymorphism and milk allergy. It was found that the sequence in α_{S1} -casein most immunogenic to T cells from children with cow's milk allergy (CMA) contained T-cell epitopes restricted to DQB1*0201, DPB1*0401 and DRB1*1501 [18]. In another study, the DR15-DQB1*06:02 haplotype was associated with high levels of β -lactoglobulin-specific and α -casein-specific IgG, while the DR1/10-DQB1*05:01 haplotype was associated with lower levels of β -lactoglobulin-specific IgG [19]. In the same study, one association between HLA polymorphism and egg allergy was found: the DR1/10-DQB1*05:01 haplotype was associated with lower levels of ovalbumin-specific IgG.

In this study, we apply a bioinformatics approach to analyze the associations between the 12 most frequent HLA-DRB1 and 5 most frequent HLA-DQ alleles and 13 known milk and egg allergens. The *in silico* processing mimicked the allergen processing in the human body. Initially, food allergens were degraded by 3 digestive enzymes, trypsin, chymotrypsin and pepsin. The digested fragments then bound to DR and/or DQ molecules with a different affinity. The stable peptide/HLA complexes were presented at the cell surface for Th cell inspection. The final set of peptides was compared to known allergen fragments (epitopes). Several associations between some HLA class II alleles and tested allergens were detected.

Datasets and Methods

HLA Class II Proteins Used in the Study

The 12 most frequent HLA-DRB1 and 5 most frequent HLA-DQ alleles were used in the study [20]. The DRB1s are: *01:01, *03:01, *04:01, *04:04, *04:05, *07:01, *08:02, *09:01, *11:01, *12:01, *13:02 and *15:01. The DQs are: DQ4 (DQA1*04:01/DQB1*04:02), DQ5 (DQA1*01:01/DQB1*05:01), DQ6 (DQA1*01:02/DQB1*06:02), DQ7 (DQA1*05:01/DQB1*03:01) and DQ8 (DQA1*03:01/DQB1*03:02).

Milk and Egg Allergens Used in the Study

The allergenic proteins from milk and egg were selected from the Allergome database [21] and checked for existence on protein level versus UniProt knowledgebase [22]. Thirteen known allergenic proteins from milk, egg white and egg yolk were used in the study. The milk proteins are: α -lactalbumin (Bos d 4), β -lactoglobulin (Bos d 5), bovine serum albumin (Bos d 6), α_{S1} -casein (Bos d 9), α_{S2} -casein (Bos d 10), β -casein (Bos d 11) and κ -casein (Bos d 12). The egg white proteins are: ovomucoid (Gal d 1), ovalbumin (Gal d 2), ovotransferrin (Gal d 3) and lysozym C (Gal d 4). The egg yolk proteins are α -livetin (Gal d 5) and vitellogenin (Gal d 6). The full-length protein sequences were downloaded from UniProt in FASTA format.

Both milk and egg are normally consumed after thermal processing. Whey milk proteins (Bos d 4, Bos d 5 and Bos d 6) denature progressively upon heat treatment [23]. However, the caseins (Bos d 9, Bos d 10, Bos d 11 and Bos d 12) are heat-stable because they lack secondary, tertiary and quaternary structures that can be destroyed by heating. Thus, heating milk can only partly reduce its allergenicity [23].

Egg is one of the foods which has its allergenicity most altered by heating [24]. The thermal stability of egg allergens ranges from the heat-resistant Gal d 1, Gal d 5 and Gal d 6 to the partially heat-labile Gal d 4 and the heat-sensitive Gal d 2 and Gal d 3.

Despite the thermal instability of some milk and egg allergens, all of them were considered in this study.

Known T-Cell and IgE Epitopes from Milk and Egg Allergens

Information about T-cell epitopes was available for only 4 milk allergens, Bos d 4, Bos d 5, Bos d 6 and Bos d 9, and for 2 egg allergens, Gal d 1 and Gal d 2. The information contained neither IC₅₀ values nor HLA restriction.

Information about IgE epitopes originating from the studied allergenic proteins was available for 7 milk allergens, Bos d 4, Bos d 5, Bos d 6, Bos d 9, Bos d 10, Bos d 11 and Bos d 12, and for 3 egg allergens, Gal d 1, Gal d 2 and Gal d 4. In the case of multiple overlapping epitopes, the longest one was selected.

*PeptideCutter: Server for *in silico* Protein Digestion*

The server PeptideCutter predicts potential cleavage sites cleaved by proteases or chemicals in a given protein sequence [25]. The server accepts the test protein as a 1-letter amino acid sequence or in FASTA format. The user selects the proteolytic enzymes from a wide range of enzymes. Our study used the enzymes trypsin and chymotrypsin with high and low specificity, pepsin at pH 1.3 and pepsin at pH >2. The server returns the query sequence with the possible cleavage sites mapped on it and/or a table of cleavage site positions. After the *in silico* digestion, each protein is presented as a set of peptides with different lengths and even single

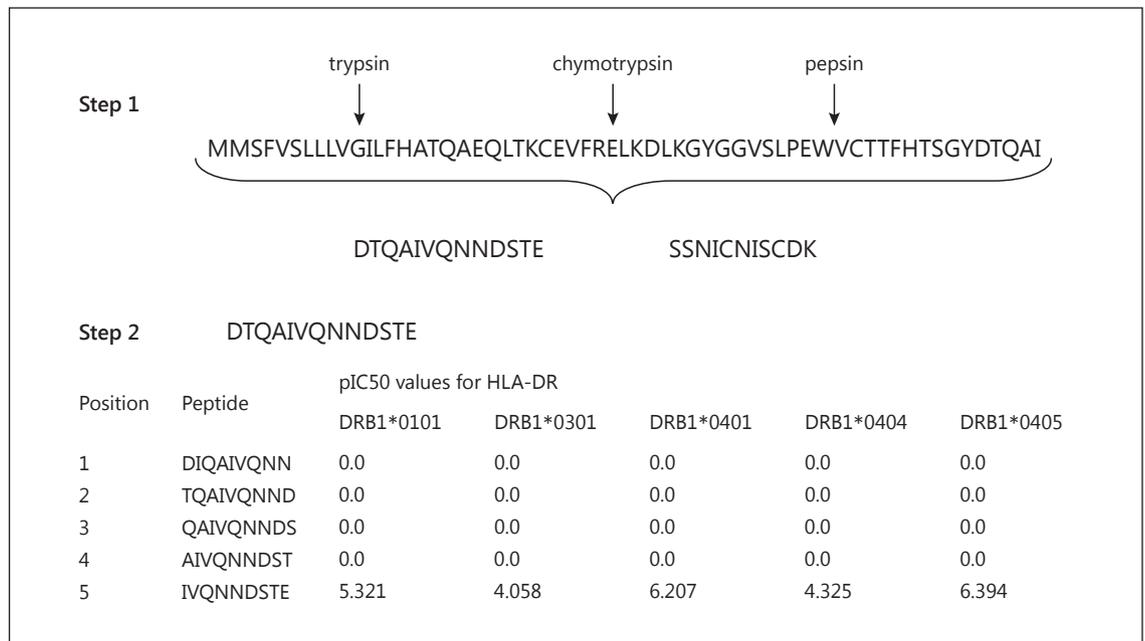


Fig. 1. Two-step algorithm for in silico food allergen processing in the human body. At the first step, the allergen is digested by pepsin, trypsin and chymotrypsin. At the second step, the binding affinities of the digested fragments to the most frequent HLA-DRB1 and HLA-DQ alleles are predicted.

amino acids. As the peptide-binding groove of MHC class II proteins accepts 9 residues, only peptides equal to or longer than 9 amino acids are selected and used in the next step, i.e. HLA class II binding prediction.

EpiTOP: Server for HLA Class II Binding Prediction

EpiTOP is a server for HLA class II binding prediction using proteochemometric models [26, 27]. It is a web-based application written in Python and HTML and integrating the MySQL database environment. EpiTOP v2 identifies peptides binding to 12 HLA-DRB1 and 5 HLA-DQ proteins within protein sequences, with options to vary HLA allele and cut-off. EpiTOP v2 is freely accessible at <http://www.ddg-pharmfac.net/epitop>.

Results

In order to analyze the associations between the DRB1 and DQ alleles and the known milk and egg allergens, the allergen processing in the human body was mimicked in silico. The algorithm is presented in figure 1. Initially, the food allergen digestion in the gastrointestinal tract was predicted by PeptideCutter. Three main digestive enzymes were considered: pepsin, trypsin and chymotrypsin. The peptide fragments gained after the digestion were endocytosed by antigen-presenting cells where they are processed and bind to HLA class II proteins with a differ-

ent affinity. The IC₅₀ values of the peptide/HLA complexes were predicted by EpiTOP. Only peptides with a pIC₅₀ >6.3 (IC₅₀ = 500 nM) were considered as binders. These peptides were compared to known allergen epitopes.

The in silico Digestion of Allergens

The ability of food allergens to be stable to digestion by the gastrointestinal tract is one of the prerequisites for allergenicity [28]. The in silico enzymatic treatment cuts the proteins into short peptides. Most of the resultant fragments consist of <9 residues. In order to interact with the immune system, the allergenic proteins or a part of them need to survive the digestion and to reach the antigen-presenting cells with an appropriate length. In our study, only peptides equal to or longer than 9 residues were considered. The initial lengths of the studied allergens and the number of peptides generated after the in silico digestion are shown in online supplementary table 1 (see www.karger.com/doi/10.1159/000444172 for all online suppl. material). The effectiveness of the digestion process is presented by percent of reduction: (initial protein length – total lengths of the resulted peptides)/initial protein length (online suppl. table 1). For example, the allergen Bos d 4 consists of 142 residues. After the digestion, it cleaved into 2 peptides equal to or longer than

9 residues (online suppl. table 2). The total length of the 2 fragments was 24 residues, i.e. 17% of the initial length. Therefore, the reduction of Bos d 4 after *in silico* digestion was 83%. The effect of the *in silico* enzyme digestion varies from a significant reduction (Bos d 5 and Bos d 6 with 92%) to a moderate reduction (Bos d 12 with 64%) with an average value of 81%. The peptides generated after the *in silico* digestion and their corresponding known epitopes are given in online supplementary table 3.

The protein fragments generated after the *in silico* digestion were compared to the sets of known T-cell and/or IgE epitopes from the same allergens. If a 9-residue overlap between them existed, the predicted epitope was considered as a true positive. Sensitivity is defined as the ratio between true positive epitopes to all protein fragments generated after the *in silico* digestion. An average sensitivity of 77% was achieved, i.e. 77% of the peptides generated after the *in silico* digestion overlapped with at least 1 known T-cell or IgE epitope.

The in silico DRB1 and DQ Binding Prediction of Allergen Fragments

The protein fragments generated after the *in silico* digestion were entered into EpiTOP, where they were chopped into nonamers and their binding affinities to the most frequent DRB1 and DQ proteins were predicted (online suppl. table 2). In general, the nonamers binding to DRB1 are significantly fewer than those binding to DQ (online suppl. tables 4, 5). This is explained by the fact that the most important anchor for DRB1 binding, i.e. the peptide position 1 (p1), requires hydrophobic residues as Leu, Ile, Phe, Trp, Met, Val, Tyr and Ala [2]. Only peptides bearing such residues at p1 are able to bind to DRB1 proteins. In contrast, the peptides binding to DQ have quite diverse structures. Most of the surviving peptides were found to bind to the 5 studied DQ proteins.

The average predicted pIC_{50} values of the milk allergens indicated that Bos d 11 showed the highest affinity to DRB1 with an average pIC_{50} of 7.140 ($IC_{50} = 72$ nM), followed by Bos d 6 ($pIC_{50} = 7.114$, $IC_{50} = 77$ nM) and Bos d 12 ($pIC_{50} = 6.908$, $IC_{50} = 124$ nM; online suppl. table 4). Bos d 12 contains the most DRB1 binders, i.e. 39, while Bos d 4 has only 2. The highest affinity to DQ proteins was shown by Bos d 6 with an average pIC_{50} of 7.100 ($IC_{50} = 79$ nM), followed by Bos d 12 ($pIC_{50} = 6.979$, $IC_{50} = 105$ nM) and Bos d 11 ($pIC_{50} = 6.970$, $IC_{50} = 107$ nM; online suppl. table 5). The number of good binders to DQ was significantly higher than the number of binders to DRB1. Bos d 12 contained 101 binders to DQ, while Bos d 6 had only 13.

The egg allergens contained more binders to both DRB1 and DQ proteins, with a slightly lower affinity (online suppl. tables 4, 5). Gal d 2 and Gal d 6 showed the highest affinity to DRB1 ($pIC_{50} = 7.003$ and 6.977 , respectively), while Gal d 4 showed the highest average affinity to DQs ($pIC_{50} = 7.605$, $IC_{50} = 25$ nM) with only 5 good binders. As it has the biggest molecular mass, Gal d 6 contains the largest number of binders.

Milk and egg allergens bound best to DRB1*01:01, with an average pIC_{50} of 7.513 and 7.198, respectively. For milk allergens, the next most attractive were DRB1*07:01 (average $pIC_{50} = 6.871$, $IC_{50} = 135$ nM) and DRB1*08:02 (average $pIC_{50} = 6.835$, $IC_{50} = 146$ nM). The egg allergens preferred DRB1*15:01 (average $pIC_{50} = 6.954$, $IC_{50} = 110$ nM) and DRB1*07:01 (average $pIC_{50} = 6.944$, $IC_{50} = 114$ nM). In terms of the number of binders, DRB1*04:05, DRB1*01:01, DRB1*07:01 and DRB1*04:01 were preferred by milk and egg proteins over the rest of the DRB1 alleles. No binders were found for DRB1*03:01, DRB1*04:04 and DRB1*12:01. Only 2 egg binders (but with a high affinity) were found for DRB1*15:01.

Regarding the studied DQ proteins, milk allergens preferred binding to DQ7 (average $pIC_{50} = 7.028$, $IC_{50} = 94$ nM), DQ8 (average $pIC_{50} = 7.006$, $IC_{50} = 99$ nM) and DQ4 (average $pIC_{50} = 6.955$, $IC_{50} = 111$ nM). Ruiter et al. [18] have found that the DQB1*05:01 allele (part of DQ5) frequency is lower in children with CMA than in non-atopic children. The preferences of egg allergens for DQ proteins are in the same order: DQ7 (average $pIC_{50} = 7.176$, $IC_{50} = 67$ nM), DQ8 (average $pIC_{50} = 7.135$, $IC_{50} = 73$ nM) and DQ4 (average $pIC_{50} = 7.096$, $IC_{50} = 80$ nM). The most binders were found for DQ6 and DQ7. Gal d 4 did not bind to DQ5.

Discussion

In this study, we attempted to analyze the associations between HLA polymorphism and food allergy using bioinformatics. We developed a two-step algorithm mimicking the food allergen processing in the human body. In the first step, the allergen is digested by pepsin, trypsin and chymotrypsin. In the second step, the digested fragments bind to the 12 most frequent HLA-DRB1 and 5 most frequent HLA-DQ alleles and the binding affinities are predicted. We applied this algorithm to 13 known milk and egg allergens. The peptides derived after the *in silico* digestion were compared to known Tcell and IgE epitopes originating from the same allergens, and 77% of them overlapped. It should be mentioned that most of the

known T-cell and IgE epitopes have been derived by tests on overlapping peptide libraries spanning the primary sequences of whole, intact allergenic proteins. No digestion was considered, resulting in an overestimation of the number of both T-cell and IgE epitopes.

Bos d 4 contains several IgE epitopes (online suppl. table 2). Some of them overlap. These epitopes have been found in the intact protein. However, after the *in silico* digestion, only 2 peptide fragments (⁵⁶D-E⁶⁸ and ⁸⁸S-K⁹⁸) survived that were longer than 9 amino acids (online suppl. tables 2, 3). They belong to the known IgE epitopes ³⁶G-K⁷⁷ and ⁷⁹W-K⁹⁸, respectively. Bos d 5 is the most abundant whey protein in cow's milk. It has a specific tertiary structure resistant to pepsin digestion and this makes it highly allergenic [29]. The subsequent exposure to duodenal enzymes completely degraded the protein in 30 min [30]. Nevertheless, scanning the sequence of the intact protein by a library of overlapping peptides revealed many IgE and T-cell epitopes (online suppl. table 2). The single peptide found after the *in silico* digestion, ¹²⁴ENSAEPEQS¹³², is part of the T-cell epitope ¹¹³T-L¹³³, but it has no affinity to any of the DRB1 and DQ alleles considered in this study (online suppl. table 3). Bos d 6 is the major beef allergen. Testing the regions in which the amino acid sequences of Bos d 6 differ significantly from human serum albumin, Tanabe et al. [31] identified 3 T-cell epitopes and 4 IgE epitopes from the intact protein (online suppl. table 2). However, after the *in silico* digestion, none of them survived (online suppl. table 3).

The casein fraction of bovine milk consists of 4 proteins: α_{s1} -casein (Bos d 9), α_{s2} -casein (Bos d 10), β -casein (Bos d 11) and κ -casein (Bos d 12). Caseins are very susceptible to proteases and peptidases and undergo rapid breakdown during digestion [32]. Despite the excellent digestibility of caseins, they act as potent allergens [32, 33], which means that sufficient immunologically active fragments survive after the digestion [34]. After the *in silico* digestion of Bos d 9, 4 peptides were generated (online suppl. table 2). Two of them were present in the fragments derived after the *in silico* digestion (online suppl. table 3). Twelve IgE epitopes were identified in the intact protein (online suppl. table 1). Most of them are from the same regions as the T-cell epitopes. All 4 digested fragments are parts of the known IgE epitopes (online suppl. table 3). Three peptides were generated after the *in silico* digestion of Bos d 10. Two of them are present in the known IgE epitopes (online suppl. table 3). No T-cell epitopes have yet been identified for this allergen. Bos d 11 is the second most abundant protein in cow's milk with a high content of prolines and a lack of disulfide bonds.

Like other caseins, Bos d 11 is highly sensitive to proteolysis and it is totally digested in the gastrointestinal tract. Five peptides, equal to or longer than 9 amino acids, were generated after the *in silico* digestion. All of them are part of known IgE epitopes (online suppl. tables 2, 3). No T-cell epitopes have yet been identified for Bos d 11. Bos d 12 differs from other caseins in that it contains disulfide bonds and is more structured. No T-cell epitopes were identified but several IgE epitopes exist in the intact protein (online suppl. tables 2, 3). Four protein fragments remained after the *in silico* digestion. Two of them belong to the known IgE epitopes.

Gal d 1 is the dominant allergen of hens' egg white. It is relatively stable against heat [35] and proteinases [36]. Seven protein fragments survived after the *in silico* digestion. Four of them are part of 3 T-cell and 1 IgE epitopes (online suppl. table 3). The total number of T-cell epitopes identified was 10, while there were 8 known IgE epitopes (online suppl. table 2). Gal d 2 is one of the major allergens in patients with hen's egg allergy [37]. The IgE epitope mapping for the entire primary sequence of Gal d 2 performed by different authors has identified several epitopes covering almost the whole protein (online suppl. table 2). Five T-cell epitopes were found (online suppl. table 2). Six peptides survived after the *in silico* digestion of Gal d 2 (online suppl. table 3). One of them is part of a T-cell epitope, and the other 5 belong to the known IgE epitopes. Gal d 3 is an iron-binding protein, and is a heat-labile and digestible allergen [28]. Neither T-cell nor IgE epitopes have been reported for it. Gal d 4 is an enzyme with antimycobacterial activity. It is less allergenic than Gal d 1 and Gal d 2 [38]. Three IgE-binding peptides were identified (online suppl. table 2). The single peptide generated after the *in silico* digestion overlapped partially with the epitope ¹⁰⁸A-G¹²² (online suppl. table 3). Gal d 5 and Gal d 6 are hen's egg yolk allergens. Yolk proteins are less allergenic than egg white proteins. Both are heat-resistant and heat treatment does not affect their allergenicity [39, 40]. The simulated gastric acid digestion completely eliminates the IgE reactivity of Gal d 6 [41]. The *in silico* digestion generated 5 fragments for Gal d 5 and 33 fragments for Gal d 6 (online suppl. table 2). There are no known T-cell and IgE epitopes for these.

The predicted binding affinities of the protein fragments generated after the *in silico* digestion of milk and egg allergens suggest several clear associations between the susceptibility to/protection against milk/egg allergy and the HLA-DRB1/DQ polymorphism. The milk allergens showed the highest affinity (average pIC₅₀ >7, IC₅₀ <100 nM) to DRB1*01:01, DQ7 and DQ8. These alleles

could be considered as susceptible to CMA. The milk allergens did not bind to DRB1*03:01, DRB1*04:04, DRB1*12:01 and DRB1*15:01. These alleles could be considered as protective against CMA. The egg allergens showed the highest affinity to DRB1*01:01, DQ4, DQ7 and DQ8 and they did not bind to DRB1*03:01, DRB1*04:04 and DRB1*12:01. Protective DQ alleles against egg allergy could not be detected, as all of the allergens, except for Bos d 5 and Gal d 4, bound to the DQs studied.

The results that we derived were compared to the sparse information available in the literature regarding the associations between the HLA polymorphism and milk/egg allergen proteins. Ruiter et al. [18] used a set of overlapping peptides, spanning the Bos d 9 molecule to stimulate T-cell clones derived from children with and without CMA and from nonatopic children. They have found that Bos d 9 contains T cell epitopes restricted to DQB1*02:01, DPB1*04:01 and DRB1*15:01. Of the 3 alleles, only DRB1*15:01 was included in our study. We searched the whole, intact Bos d 9 sequence (UniProt P02662) for DRB1*15:01 binders using EpiTOP, and found 5 high binders ($pIC_{50} > 6.3$; data not shown). However, after the *in silico* digestion of Bos d 9, no DRB1*15:01 binders remained. Savilahti et al. [19] found that the DR15-DQB1*06:02 haplotype is associated with high levels of Bos d 5-specific- and Bos d 9/Bos d 10-specific total

IgG and IgG4 in children with CMA, but not among control subjects. In the same study, the DR1/10-DQB1*05:01 haplotype was associated with lower levels of Bos d 5-specific and Gal d 2-specific total IgG and IgG4, particularly in control subjects. In this study, no binders from Bos d 5 were found to DR15 or DQ5. The duodenal enzymes trypsin and chymotrypsin completely degraded the protein in 30 min [30]. The only peptide that survived after the *in silico* digestion was part of a known T cell epitope, but no binders to any of the DRB1 and DQ alleles considered in this study were found (online suppl. table 3). In good agreement with the clinical studies, we found 12 binders to DQ6 originating from Bos d 9 (average $pIC_{50} = 6.889$; online suppl. table 5). However, Gal d 2 was found to have 5 strong binders to DR1 (average $pIC_{50} = 7.807$) and 7 intermediate binders to DQ5 (average $pIC_{50} = 6.655$). The lower allergenicity of Gal d 2 that was found experimentally could be explained by its sensitivity to heat denaturation, a process that was not considered in this study.

Disclosure Statement

The authors are members of FA COST Action FA1402: Improving Allergy Risk Assessment Strategy (ImpARAS) for new food proteins.

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