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Storage molecules from tree nuts, seeds and legumes: relationships and amino acid identity among homologue molecules

E. Scala¹, D. Villalta², G. Meneguzzi¹, M. Giani¹, R. Asero³

The families of seed storage proteins, together with profilins, oil-bodies-associated oleosins, and pathogenesis-related (PR) proteins like PR-10 (Bet v 1-like), PR-12 (defensins) and PR-14 (non-specific lipid transfer protein), are the main causes of IgE sensitization to tree nuts, legumes and seeds. All these allergens, with the exclusion of profilins and of PR-10, are heat-stable and possibly responsible for fatal or almost fatal adverse reactions to such foods. In this short review, we will discuss the relationship and amino acid identities among some of the seed storage homologue molecules identified to date from tree nuts, seeds and legumes.

Key words
2s albumins; cupins; vicilins; legumins; nsLTP; PR-10; defensins; oleosins; seed storage proteins

Summary
The families of seed storage proteins, together with profilins, oil-bodies-associated oleosins, and pathogenesis-related (PR) proteins like PR-10 (Bet v 1-like), PR-12 (defensins) and PR-14 (non-specific lipid transfer protein), are the main causes of IgE sensitization to tree nuts, legumes and seeds. All these allergens, with the exclusion of profilins and of PR-10, are heat-stable and possibly responsible for fatal or almost fatal adverse reactions to such foods. In this short review, we will discuss the relationship and amino acid identities among some of the seed storage homologue molecules identified to date from tree nuts, seeds and legumes.

Tree nuts, seeds and legumes storage proteins

The families of seed storage proteins, together with profilins, oil-bodies-associated oleosins, and pathogenesis-related (PR) proteins like PR-10 (Bet v 1-like), PR-12 (defensins) and PR-14 (non-specific lipid transfer protein), are the main causes of IgE sensitization to tree nuts, legumes and seeds (1). All these allergens, with the exclusion of profilins and of PR-10, are heat-stable and possibly responsible for fatal or almost fatal adverse reaction to such foods.

In this short review, we will discuss the relationship and amino acid identities among some of the seed storage homologue molecules identified to date from tree nuts, seeds and legumes (figure 1), and choosing those registered in the WHO/International Union of Immunological Societies Allergen Nomenclature Subcommittee (http://www.allergen.org) database (2), belonging to the following biological sources:

1. tree nuts (coconut, Cocos nucifera; Brazilian walnut, Bertholletia excelsa; hazelnut, Corylus avellana; walnut, Juglans regia; black walnut, Juglans nigra; pecan nut, Carya illinoiensis; pine nut, Pinus pinea; almond, Prunus dulcis; cashew, Anacardium occidentale; pistachio, Pistacia vera);
2. seeds (sunflower seed, Helianthus annuus; mustard, Sinapis alba; turnip, Brassica rapa; rape seed, Brassica napus; Indian mustard, Brassica juncea; buckwheat, Fagopyrum esculentum; Siberian wheat, Fagopyrum tataricum; sesame, Sesamum indicum; ricinus, Ricinus communis); and
3. legumes (peanut, Arachis hypogaea; lentil, Lens culinaris; pea, Pisum sativum; bean, Phaseolus vulgaris; lupine, Lupinus angustifolius; soy, Glycine max; mung bean, Vigna radiata).
Seed storage proteins

Storage proteins in plant seeds represent the reserve of amino acids and ions utilized by the plant as source of nutrients during germination and seedling growth. Up to a quarter of the dry weight of any single seed encompasses such proteins. Seed storage proteins encompass prolamin and cupins (3).

The prolamine superfamily includes cereal prolamins and α-Amylase inhibitors, 2s albumins, and nsLTPs.

2s albumins are water-soluble molecules, whose MW is approximately 10-16 kDa. 2s albumins are firstly synthesized as single-chain proteins, and then cleaved into two subunits, linked by 4 to 5 disulfide bonds to form a stable and compact α-helical molecule (4). 2s albumins serve not only as storage proteins, but can also play a defensive role against fungal attack (5).

The cupin superfamily comprises a large family of proteins named after their common conserved β-barrel structure (cupa from the Latin term meaning small barrel) probably originated from a prokaryotic precursor (6). The cupin superfamily includes the 7S vicilin-type globulins and the 11S legumin-type globulins, classified on the basis of their sedimentation coefficient (7).

The vicilins (7s globulin) represent up to 80% of total proteins in seeds of leguminous and non-leguminous plants, include three subunits with a MW of about 40-80 kDa, and show a cumulative molecular mass of 150-190 kDa (8).

The legumins (11s globulins) are hexameric proteins comprising two associated trimers of around 40-50 kDa MW. Both 7S globulin and 11S globulin allergens share a similar cupin structure, but have distinct IgE-binding epitopes (9).

These globulins act both as important nutrients, providing amino acids during the germination process, but are also involved in the defense process against fungi and insects (10).

Plant pathogenesis-related (PR) proteins

Plants have inducible defense systems that are stimulated upon attack of multiple pathogens such as viruses, bacteria, fungi, etc. (5). Among such defense mechanisms there are the PR proteins, which include a large number of families with components differing in incidence, appearance and biological activities (11). Some of these families are also important allergens, possibly causing IgE sensitization in predisposed individuals. In tree nuts, seeds and legumes, clinically relevant PR proteins causing IgE sensitization are represented by PR-10 (Bet v 1-Like), PR-12 (defensin) and PR-14 (ns LTP) proteins.

PR-10s are cross-reactive molecules of 16-18 kDa, belonging to the Bet v 1 family (12), sharing a common tertiary structure with seven-stranded anti-parallel β-sheets and three α-helices (13, 14). The amount of these proteins in seeds, nuts, and legumes is influenced by both a-biotic and biotic stresses (15, 16). PR-10s homologs are widely distributed in the plant kingdom (17). Such thermo-labile molecules, localized homogenously throughout peel and pulp (18,19), can provoke oral allergy syndrome after ingestion of raw fruits or vegetables (20). PR-10s are cross-reactive molecules of 16-18 kDa, belonging to the Bet v 1 family (12), sharing a common tertiary structure with seven-stranded anti-parallel β-sheets and three α-helices (13, 14). The amount of these proteins in seeds, nuts, and legumes is influenced by both a-biotic and biotic stresses (15, 16). PR-10s homologs are widely distributed in the plant kingdom (17). Such thermo-labile molecules, localized homogenously throughout peel and pulp (18, 19), can provoke oral allergy syndrome after ingestion of raw fruits or vegetables (20).

Figure 1 - Phylogenetic tree showing the relationships of tree nuts, seeds and legumes sources containing the storage molecules identified as allergen to date.
proteins are found in tree nuts, seeds and legumes from *Fagales* order (Cora 1.0401 from hazelnut and Jug r 5 from walnut), and *Fabales* order (Ara h 8 from peanut, Gly m 4 from soybean, and Vig r 1 from mung bean) (table Ia and c). The steadiness to moderate heating of Gly m 4 gives reason for the reported severe reactions to cooked soybeans (21). An heterogeneous PR-10 food allergens IgE: recognition profile was recently found in an Italian Bet v 1 free area, and Ara h 8, Cor a 1.0401, and Gly m 4 IgE reactivity was significantly associated with a history of OAS occurrence (22).

PR-14 allergens, the small, highly conserved, non-specific lipid transfer proteins (nsLTP) (23), are mainly concentrated in the skin of *Rosaceae* fruits (24), and have a very high resistance to gastrointestinal proteolysis, and high temperature exposure (25). nsLTPs belong in the vast majority of cases to the nsLTP1 subfamily (9 kDa), but allergens from the nsLTP2 subfamily (7 kd), as Ara h 16 from peanut, have also been described (26). A broad degree of IgE cross-recognition between the individual nsLTP components has been observed (27), and sensitization to nsLTPs is frequently associated with systemic, even anaphylactic, reactions. Hence, nsLTPs are the most frequent sensitizer in Italian subjects with food-dependent exercise-induced anaphylaxis (28). The recognition at the same time in the same patient of PR-10, PR-14 and profilin allergens is associated with a significantly lower risk of severe adverse reaction to food (29,30).

Ara h 9 from peanut represents an important cause of adverse reaction to food in southern Europe (31,32), and Jug r 3 from walnut is the molecule most frequently recognized in patients scoring negative for Pru p 3 in Italy (30). PR-14 proteins are found in tree nuts, seeds and legumes from *Fagales* order (Cor a 8 from hazelnut (33), Ara h 9 from peanut (31,34,35), Jug r 3 from walnut (36)), *Rosales* order (Pru du 3 from almond (37)), *Brassicales* order (Sin a 3 from mustard (38) and Bra r 3 from turnip (39)), *Asterales* order (Hel a 3 from sunflower seed) (40), and *Fabales* order (Ara h 9, Ara h 16 and Ara h 17 from peanut, Len c 3 from lentil (41), Pis s 3 from pea (42), Pha v 3 from bean (43), and Gly m 1 from soybean (44)) (table I).

Defensins are low-molecular-weight amphiphilic cationic proteins belonging to the pathogenesis-related protein family 12, and sharing common structural characteristics (45). Such molecules can be found in lipophilic extracts of peanut (Ara h 12 and 13) (46) and soybean (Gly m 2), but associated, in this case, with asthmatic symptoms occurring in workers exposed to soy (47) (table IIc).

**Oleosins**

Oleosins are proteins of around 16-24 kDa of MW, forming the structure of the plant lipid storage bodies called “oil bodies” (OB) (48), involved in severe systemic adverse reactions (49). The main characteristic of oleosins is their extreme hydrophobicity and, therefore, their scarce solubility once placed outside of their natural oil environment. As a consequence, such proteins are virtually lacking in defatted diagnostic commercial products for both in vitro and in vivo testing, thus resulting in a poor detection of IgE antibodies to such relevant allergens (50). Oleosins from peanut (49,51), sesame seed (52) and hazelnuts (50) have been described to date. As shown in table IIId a likely cross-reactivity is recorded between oleosins from different sources (53), thus representing a possible cause of reactivity towards not botanically related sources. Nevertheless, the clinical relevance and IgE cross-reactivity of these allergens are not fully defined, therefore requiring more studies.

**Relationships and identity degree between homologue proteins**

In table I, the list of the allergenic molecules currently registered in WHO/IUIS database and expressed in tree nuts (a), seeds (b) and legumes (c) is shown. With regard to legumes, all the allergens belong to the same plant order: the *Fabales*. Observing the tables as a whole, there are molecules from some biological sources missing, but this observation, more than a real lack in the respective source, is probably due to the fact that many of these allergens have not been yet discovered.

In table II the amino acid identities expressed as percentages among 2s albumins (a), vicilins (b), legumins (c) and oleosins (d) are shown.

The primary sequence correspondence among different molecules from nuts, seeds and legumes can be described as amino acid identity percentage. The sequence identity found comparing the different allergen families expressed by nuts, seeds and legumes is mainly associated with their botanical relationship, as in the case of cashew and pistachio, both belonging to * Sapindales* order or the tree nuts walnut, hazelnut and pecan, all from *Fagales* order (9). Accordingly, comparing the sequences of the 2s allergens (Ana o 3 vs Pis v 1), 7s globulins (Ana o 1 vs Pis v 3) and 11s globulins (Ana o 2 vs Pis v 5) from cashew and pistachio, high degree of amino acid identities can be observed (table IIa-c). Similarly, in the case of *Fagales* order, the 2s albumins from hazelnut (Cor a 14), walnut (Jug r 1), black walnut (Jug n 1) and pecan nut (Car i 1) ranged between 62 and 86% of amino acid identities, the 11s globulins (Cor a 9, Jug r 4, Jug n 4 and Car i 4) had from 71 to 94% of identities, and the vicilins from walnut (Jug r 2), black walnut (Jug n 2) and pecan (Car i 2), but not the 7s globulin from hazelnut (Cor a 11), showed from 58 to 78% of identities.

Comparable observation can be achieved also comparing the 2s albumins from *Brassicales* (Sin a 1 vs mustard, Bra r 1 vs turnip, Bra n 1 vs rapeseed, and Bra j 1 vs Indian mustard) (table IIa). Interestingly, very high levels of sequence identity (90.1%) can be observed comparing the vicilin from pea (Pis s 1)
Table 1: a, tree nuts allergens (WHO/IUIS allergen nomenclature); b, seed allergens (WHO/IUIS allergen nomenclature); c, legume allergens (WHO/IUIS allergen nomenclature).

<table>
<thead>
<tr>
<th>a</th>
<th>Storage proteins</th>
<th>PR-proteins</th>
<th>Oleosin</th>
<th>Prolin</th>
<th>Order</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2s albums</td>
<td>Vicia</td>
<td>Legumin</td>
<td>PRO10</td>
<td>nsTP (PRO14)</td>
</tr>
<tr>
<td>Coconut</td>
<td>Coc n 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brazilian walnut</td>
<td>Ber e 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hazelnut</td>
<td>Cor a 14</td>
<td>Cor a 11</td>
<td>Cor a 9</td>
<td>Cor a 1</td>
<td>Cor a 8</td>
</tr>
<tr>
<td>Walnut</td>
<td>Jug r 1</td>
<td>Jug r 2</td>
<td>Jug r 6</td>
<td>Jug r 4</td>
<td>Jug r 5</td>
</tr>
<tr>
<td>Black Walnut</td>
<td>Jug n 1</td>
<td>Jug n 2</td>
<td>Jug n 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pecan nut</td>
<td>Car i 1</td>
<td>Car i 2</td>
<td>Car i 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pine nut</td>
<td>Pin p 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Almond</td>
<td>Prunus dulcis</td>
<td>Pru du 6</td>
<td>Pru du 3</td>
<td>Pru du 4</td>
<td></td>
</tr>
<tr>
<td>Cashew</td>
<td>Anacardium occidentale</td>
<td>Ana o 3</td>
<td>Ana o 1</td>
<td>Ana o 2</td>
<td></td>
</tr>
<tr>
<td>Pistachio</td>
<td>Pistacia vera</td>
<td>Pis v 1</td>
<td>Pis v 3</td>
<td>Pis v 2</td>
<td>Pis v 5</td>
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Bold font indicates molecules currently available in the market.
Table II - Amino acid identities among 2s a, 2s albumin; b, vicilins (7s Globulin); c, legumins (11s Globulin); and d, oleosin, expressed as percentages. Bold font indicates values above 50% of sequence identity.

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and lentil (Len c 1), indicating that such molecules can be considered virtually identical (table IIb). Likewise, also the 2s albumins from buckwheat (Fag e 2) and Siberian wheat (Fag g 2) show a high degree of sequence identity (82.4%) (table IIa).

High primary sequence identity levels can be associated with an IgE co-recognition, but this cannot be considered as a definitive proof of cross-reactivity. Clearly, the higher the identity between two molecules, the higher is the possibility that a cross-reactivity

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Conflict of interest

R. Asero and E. Scala declare funds received by Italian Ministry of Health and honoraria by Thermo Fisher. Other authors declare no conflict of interest.

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Allergy to LTP: to eat or not to eat sensitizing foods? A follow-up study

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Summary

Background. Follow-up data about the onset of novel food allergies in patients allergic to lipid transfer protein (LTP) are missing. We investigated the occurrence of novel allergies over time in LTP hypersensitive patients. Methods. Sixty-seven LTP-allergic patients recommended to avoid foods responsible for systemic reactions and encouraged to eat other sensitizing foods avoiding the association with known co-factors, were re-evaluated after ≥ 1 year to assess the occurrence of allergy to novel foods. IgE to rPru p 3, rBet v 1, and r Phl p 12 were measured. Results. At baseline, the most frequent offending foods were Rosaceae / Prunoideae, tree nuts, and peanut. Most patients reacted to > 1 food, and 77% experienced systemic allergic reactions. Those monosensitized to LTP showed a higher prevalence of food-induced systemic reactions than patients co-sensitized to profilin and/or PR-10 (p < 0.01). Baseline Pru p 3 IgE levels did not differ between patients with local symptoms or systemic symptoms. 1-16 years after the baseline evaluation 18/67 (27%) patients had experienced new food allergies; 8 and 10 reported local or systemic symptoms following the ingestion of previously tolerated foods. Again, most new allergies were caused by Rosaceae / Prunoideae, tree nuts, and peanut. The clinical evolution did not depend on baseline total IgE, co-sensitization to PR-10 and/or profilin, or Pru p 3 IgE levels. Conclusions. Rosaceae / Prunoideae, nuts and peanut are the most frequent cause of new food allergies in the long term. Their exclusion from patient's diets at baseline should be considered on an individual basis.

Introduction

Nonspecific lipid transfer protein (LTP) is by far the main cause of primary food allergy in Italian adults (1) and generally in the Mediterranean European Countries, and is responsible for the largest number of food-induced anaphylactic reactions as well (2). Due to the widespread distribution of LTP in the plant kingdom, and to the cross-reacting nature of this protein, hypersensitive patients are potentially at risk of experiencing allergic reactions following the ingestion of an array of botanically unrelated fruits and vegetables. However, the clinical expression of LTP hypersensitivity is extremely variable, with many patients that tolerate foods they are strongly sensitized to (3-5), others that react only in the presence of co-factors such as exercise, NSAIDs, or alcoholic beverages, and subjects experiencing severe allergic reactions despite low specific IgE levels. This poses serious ethical problems to doctors dealing with LTP-allergic patients. In fact, whether patients should be advised to avoid all foods to which they are sensitized albeit tolerant, or be recommended to pursue their ingestion, is still undefined. To the best of our knowledge, no follow-up data exist in LTP-allergic patients, in terms of frequency and clinical presentation of novel food allergies over time. The aim of the present study was to investigate in the long term the occurrence of novel plant food
Allergies in LTP-allergic patients, in response to the recommendation to avoid only known offending foods and to continue to eat other plant foods irrespective of sensitization status.

**Patients and methods**

**Patients**

Sixty-seven subjects (m/f 31/36; mean age at the first visit 33.8 years; range 6-56 years) were included in the present study. All presented spontaneously at one of the two participating allergy centers during the last 16 years due to probable food allergy, and were diagnosed as having LTP allergy based on clinical history and reactivity to SPT with a commercial peach extract (ALK-Abellò) containing 30 mcg/ml of Pru p 3. Previous studies showed that commercial peach extracts lack the labile allergens Pru p 1 (PR-10) and Pru p 4 (profilin) (6). We also had an in-house purified peach LTP extract prepared by our lab, to use when commercially LTP enriched peach extract or rPru p 3 and other rLTPs were not yet available in ImmunoCAP (7).

At the baseline visit, patients underwent a thorough interview to ascertain all food-induced adverse reactions occurred before. Reported symptoms were classified as local (contact urticaria [CU], or oral allergy syndrome [OAS]) or systemic (urticaria / angioedema or anaphylaxis). OAS was defined as the occurrence of oral itching, with or without angioedema of the lips and/or tongue, a few minutes after the ingestion of an offending food on at least 2 distinct occasions. Patients co-sensitized to PR-10 proteins and/or profilin were also considered allergic to LTP when they had a history of systemic symptoms or, alternatively, of OAS following the ingestion of cooked or industrially processed foodstuff, as it is well known that in such foods labile proteins are no longer allergenic. Systemic reactions were considered as possibly food-induced if they occurred within 2 h after the ingestion. Hospital and emergency department recordings were analyzed as well, when available. All patients with a history of systemic reaction had already spontaneously withdrawn the putative offending food from their diets at the time of the first visit, whereas those with a history of local reactions were still on a free diet in most cases. At the end of the baseline visit, patients were recommended to go on avoiding the food(s) responsible for systemic reactions, and were encouraged to continue eating all the other sensitizing foods, with the only caveat to avoid the association with known co-factors such as exercise, nonsteroidal anti-inflammatory drugs, and alcoholics. Further, patients were recommended to peel the sensitizing fruits where possible. Patients were left free to stop eating foods causing local symptoms if they induced excessive discomfort.

The effects of such policy were assessed about every year during the follow-up visits, when patients were thoroughly interviewed about their compliance to the prescribed diet regimen and the occurrence of further, new allergic reactions (either local or systemic) to foods that were tolerated before. Hypersensitivity to novel, clinically offending foods was assessed by SPT with commercial extracts and/or fresh material as reported above, if not already detected at the baseline visit.

**In vivo-tests**

Along with SPT with the commercial peach extract, SPTs with a standard panel of commercial plant food extracts (ALK-Abellò 1/20 w/v) including apple, peanut, wheat, soybean, walnut, hazelnut, tomato, carrot, celery, and almond were carried out. Hypersensitivity to reportedly offending foods other than those included in the commercial panel was confirmed by SPT using fresh material by the prick-prick technique. SPTs were carried out on the volar side of the forearm with a sterile, 1 mm-tip lancet (ALK-Abellò) pricking through the drop of the extract. Readings were taken after 15 min, and results were assessed by established methods (8). A SPT with histamine 10 mg/ml was carried out as positive control.

**In vitro tests**

Serum specific IgE levels to rPru p 3 (the peach LTP), rBet v 1 (the major birch pollen allergen, representative of the cross-reactive PR-10 allergen family), and r Phl p 12 (grass profilin, as a representative of all homologous allergens) were measured by ImmunoCAP (ThermoFisher Scientific), immediately after the baseline visit or during the follow-up visit when they became commercially available. Values < 0.35 kU/l were considered negative. Total IgE were measured as well in 21 patients.

**Statistics**

Proportions were compared by chi-square test with Yates’ correction. Specific IgE levels were compared by two-tailed Student’s t-test. Probability values < 0.05 were considered statistically significant.

**Ethics**

Patients gave an informed consent to in vivo and in vitro investigations and to the use of their clinical data for research purposes in an anonymous form. In view of the purely observational nature of the study, along with the fact that all the interventions were part of routine clinical practice, a formal approval by the Ethical Committee was not needed and was not requested.
Results

Baseline data

The baseline clinical features of the study population, along with the offending foods and the clinical characteristics of allergic reactions, are summarized in Table I. Specific IgE to rPru p 3 were measured in 65/67 patients, and confirmed LTP sensitization in all cases. rPru p 3 IgE levels ranged from 0.39 kU/L to > 100 kU/L (mean 13.02 kU/L). Nineteen patients were co-sensitized to PR-10, as shown by rBet v 1 IgE levels ranging from 0.65 to > 100 kU/L, and 5 patients were co-sensitized to profilin (IgE to rPhl p 12 ranging from 0.37 to > 100 kU/L). Thirteen further patients were co-sensitized to both PR-10 and profilin. Total IgE were measured in 21 patients and ranged between 30 and 5000 kU/L.

At the baseline visit, the most frequently reported offending foods belonged to the Rosaceae / Prunoideae family (peach, apricot, plum, cherry, apple, pear, loquat, almond) and caused clinical symptoms in a total of 48 (71%) patients, specifically oral allergy syndrome in 34 subjects, urticaria in 12, contact urticaria in 8, gastrointestinal symptoms in 2, anaphylaxis in 7 cases, and FDEIA in 3. Tree nuts (n = 34 [51%]) and peanut (n = 13 [19%]) were also a frequent cause of food-induced symptoms, whereas other fruits and vegetables such as tomato, onion, citrus fruits, legumes, pesto sauce, beer, grapes, Brazil nut, lettuce, broccoli, and others were less frequently involved (Table I). Most patients experienced allergic reactions following the ingestion of more than one LTP-containing plant-derived food on different occasions. The peach was by far the most frequent cause of contact urticaria (n = 7), the other two cases being associated with apple and rice, respectively.

Fifty-two (77%) patients experienced systemic symptoms (urticaria / angioedema, anaphylaxis, or FDEIA) following the ingestion of offending foods. Again, Rosaceae / Prunoideae, tree nuts and peanut were by far the most frequent offenders. Other foods inducing systemic reactions in > 2 patients included tomato, lettuce, beer, onion, and grapes. Tree nuts (n = 9) and peanut (n = 8) were the foods most frequently causing anaphylactic reactions while, surprisingly enough, tomato was the food most frequently involved in FDEIA (n = 4). The LTP reactivity to tomato was confirmed by the prick-prick technique, using a commercially available triple concentrate tomato paste or by skin prick test with an in house tomato extract, obtained from triple concentrate commercially available tomatoes (9).

Table I - Main baseline features of 67 patients allergic to LTP

<table>
<thead>
<tr>
<th>Sensitization status</th>
<th>30</th>
</tr>
</thead>
<tbody>
<tr>
<td>monosensitized to LTP</td>
<td></td>
</tr>
<tr>
<td>co-sensitized to PR-10 only</td>
<td>19</td>
</tr>
<tr>
<td>co-sensitized to profilin only</td>
<td>5</td>
</tr>
<tr>
<td>co-sensitized to both PR-10 and profilin</td>
<td>13</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>Clinical food allergy</th>
<th>15</th>
</tr>
</thead>
<tbody>
<tr>
<td>local symptoms only</td>
<td></td>
</tr>
<tr>
<td>local + systemic symptoms</td>
<td>35</td>
</tr>
<tr>
<td>systemic symptoms only</td>
<td>17</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Offending foods</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rosaceae / Prunoideae</td>
</tr>
<tr>
<td>tree nuts</td>
</tr>
<tr>
<td>peanut</td>
</tr>
<tr>
<td>other legumes (pea, beans, lupine, soybean)</td>
</tr>
<tr>
<td>tomato</td>
</tr>
<tr>
<td>lettuce, chicory, rucola, etc.</td>
</tr>
<tr>
<td>fennel</td>
</tr>
<tr>
<td>kiwi</td>
</tr>
<tr>
<td>melon, watermelon</td>
</tr>
<tr>
<td>zucchini</td>
</tr>
<tr>
<td>rice</td>
</tr>
<tr>
<td>maize</td>
</tr>
<tr>
<td>wheat, barley</td>
</tr>
<tr>
<td>beer</td>
</tr>
<tr>
<td>onion</td>
</tr>
<tr>
<td>citrus fruits</td>
</tr>
<tr>
<td>spinach</td>
</tr>
<tr>
<td>sesame seed, poppy seed, sunflower seed</td>
</tr>
<tr>
<td>parsley</td>
</tr>
<tr>
<td>carrot</td>
</tr>
<tr>
<td>broccoli</td>
</tr>
<tr>
<td>banana</td>
</tr>
<tr>
<td>commercial pesto sauce</td>
</tr>
<tr>
<td>eggplant</td>
</tr>
<tr>
<td>grapes, wine</td>
</tr>
<tr>
<td>cashew</td>
</tr>
<tr>
<td>pineapple</td>
</tr>
<tr>
<td>bell pepper</td>
</tr>
<tr>
<td>fig</td>
</tr>
<tr>
<td>saffron</td>
</tr>
</tbody>
</table>
Baseline total IgE levels did not influence the prevalence of systemic or local food-induced symptoms. No correlation was found between total IgE and specific IgE levels for raw foods or specific recombinant molecules.

Follow-up data

The follow-up visits were performed every 12-18 months after the baseline evaluation. At that time point 47/67 (70%) patients were unchanged (i.e., did not experience any allergic reaction to foods they were sensitized to but tolerated when the baseline visit was performed). Two other patients experienced urticaria and urticaria + shortness of breath, respectively, following the inadvertent ingestion (in one case associated with exercise) of cookies containing tree nuts, but these foods had already been involved in systemic reactions before.

In total, 18/67 (27%) patients reported new food allergies (Table II). Of these, 9 (47%) were monosensitized to LTP and 9 co-sensitized to PR-10 and/or profilin. Eight patients reported local symptoms only (OAS in 7 cases, gastrointestinal in 1 case) following the ingestion of rice (3 cases), strawberry, walnut, kiwi, maize, zucchini, raw fennel, chestnut, lettuce, and string.

<table>
<thead>
<tr>
<th>Ph l p 12</th>
<th>Bet v 1</th>
<th>Follow-up (yrs)</th>
<th>Local</th>
<th>Systemic</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.06</td>
<td>neg</td>
<td>3</td>
<td>strawberry</td>
<td></td>
</tr>
<tr>
<td>3.39</td>
<td>6.75</td>
<td>6</td>
<td>peeled peach</td>
<td></td>
</tr>
<tr>
<td>8.54</td>
<td>4.67</td>
<td>3</td>
<td>saffron</td>
<td></td>
</tr>
<tr>
<td>neg</td>
<td>neg</td>
<td>4</td>
<td>tomato, fennel, apple</td>
<td>onions, pistachio</td>
</tr>
<tr>
<td>neg</td>
<td>neg</td>
<td>2</td>
<td>walnut, kiwi</td>
<td></td>
</tr>
<tr>
<td>neg</td>
<td>neg</td>
<td>5</td>
<td>apple juice</td>
<td></td>
</tr>
<tr>
<td>18.6</td>
<td>23.1</td>
<td>8</td>
<td>rice</td>
<td></td>
</tr>
<tr>
<td>neg</td>
<td>neg</td>
<td>7</td>
<td>popcorn</td>
<td>almond milk, rice + saffron</td>
</tr>
<tr>
<td>neg</td>
<td>neg</td>
<td>12</td>
<td>lettuce, walnut, pineapple, rice</td>
<td>plum, cherry</td>
</tr>
<tr>
<td>neg</td>
<td>neg</td>
<td>2</td>
<td>rice, maize</td>
<td></td>
</tr>
<tr>
<td>neg</td>
<td>2.70</td>
<td>3</td>
<td>rice, zucchini</td>
<td></td>
</tr>
<tr>
<td>neg</td>
<td>12.10</td>
<td>8</td>
<td>fennel</td>
<td></td>
</tr>
<tr>
<td>neg</td>
<td>21.70</td>
<td>7</td>
<td>lentil</td>
<td>pistachio</td>
</tr>
<tr>
<td>neg</td>
<td>neg</td>
<td>1</td>
<td></td>
<td>walnut</td>
</tr>
<tr>
<td>neg</td>
<td>64.30</td>
<td>10</td>
<td>chestnut</td>
<td></td>
</tr>
<tr>
<td>neg</td>
<td>neg</td>
<td>7</td>
<td>kiwi</td>
<td></td>
</tr>
<tr>
<td>0.50</td>
<td>7.41</td>
<td>8</td>
<td>broccoli, mandarin</td>
<td></td>
</tr>
<tr>
<td>neg</td>
<td>neg</td>
<td>10</td>
<td>lettuce, string beans</td>
<td></td>
</tr>
</tbody>
</table>
beans. Four patients reported OAS from more than 1 previously tolerated food. Ten patients experienced systemic symptoms following the ingestion of previously tolerated foods including peeled peach, saffron (2 cases), onion, pistachio (2 cases), apple juice, almond milk, rice, plum, cherry, walnut, kiwi, broccoli, and mandarin. These 10 patients reported also novel local symptoms following the ingestion of tomato, fennel, popcorn, lettuce, walnut, pineapple, rice, and lentil. Anaphylactic reactions to new foods occurred only in 2 cases, and in both of them were induced by pistachio nut, but, notably, one patient turned out to be co-sensitized to seed storage proteins.

The clinical evolution did not depend on the baseline total IgE level, and the presence / absence of co-sensitization to PR-10 and/or profilin did not influence the probability of allergic reactions to new foods, nor the severity of such reactions. Baseline rPru p 3 IgE levels, as well as IgE to rBet 1 or rPhl p 12, did not differ statistically between patients developing or not developing new food allergies (data not shown). Further, the two populations did not differ in terms of age and gender.

Discussion

In recent years, many studies of specific oral tolerance induction / oral immunotherapy (SOTI/OIT) have been carried out, mainly in patients with severe allergy to milk, egg, or peanut. Despite the substantial risk of adverse events, the results of these procedures suggest that the introduction of gradually increasing amounts of the relevant foods followed by their ongoing ingestion is able to push up significantly and steadily patients' tolerance threshold (at least until the food is eaten on a regular basis), thus reducing the risk associated with the inadvertent ingestion of limited amounts of the allergen protein(s) (10). In the case of allergy to lipid transfer protein, Spanish and Portuguese researchers have attempted to desensitize allergic patients by the administration of repeated, known amounts of Pru p 3 through sublingual route, apparently with good results (11-13). Both oral and sublingual immunotherapy have still to be considered experimental treatment strategies to reduce the food allergic status, and cannot be practiced on a routine basis in the clinic. However, based on all these observations and in view of the widespread diffusion of lipid transfer protein in the plant kingdom, and of the high degree of cross-reactivity within this protein family, we hypothesized that encouraging LTP allergic patients to go on eating all foods containing LTP they were sensitized to, and that had been tolerated until the first visit, might be a safer and more feasible policy than the strict avoidance of such foods, a policy that in some cases would have meant to exclude virtually all plant-derived foods from the diet with the possible exception of carrot and a few other items (14). In other words, we thought that this approach could work as a sort of “natural, attenuated oral immunotherapy” able to maintain a state of oral tolerance to specific foods by preventing a gradual decrease of the provocation threshold dose without the risks associated with an oral / sublingual immunotherapy with Pru p 3, the LTP that causes clinical allergy in virtually all patients. Of course, for safety reasons, patients were also recommended to avoid well known co-factors, particularly exercise (15), following the ingestion of all foods they were sensitized to.

At the follow-up visit, more than one fourth of the study population reported allergic reactions induced by foods that were previously tolerated, and in more than one half of such cases reactions were systemic and associated with an array of botanically unrelated foods. Notably, most new reactions were associated with Rosaceae / Prunoideae (peeled peach, apple juice, plum, cherry, and almond milk) and with tree nuts (walnut and 2 cases from pistachio nut, the latter inducing the only two anaphylactic reactions recorded, one of whom was eventually found to have become positive to storage protein as well). Regarding symptoms associated with peeled peach, we miss the information about how it was peeled; in fact, the use of a very sharp knife makes the association with Pru p 7 (the peach pama-clen) sensitization more likely, whereas a less accurate peeling could be in effect associated with Pru p 3 exposure. Similarly, regarding almond milk, we could hypothesize a contamination by almond peel, otherwise seed storage proteins become a more likely candidate as the cause of adverse reactions.

Interestingly, saffron appeared as an emergent cause of allergic reactions in LTP-hypersensitive subjects, possibly because in the Italian cuisine it is eaten in most cases in conjunction with rice, another potentially offending food for LTP sensitized patients (16,17), thus resulting in an additive provocation. In fact, these patients were able to tolerate the rice if it was eaten alone or with foods other than saffron (e.g., mushrooms).

Based on the findings of the present study it might seem reasonable to recommend LTP reactors to avoid strictly all members of the Rosaceae / Prunoideae family (with the possible exception of the pear as previous, unpublished studies from our group show that this fruit contains very small amounts of the protein), if the baseline in-vivo tests with fresh material suggest sensitization. Similarly, it might be the case to recommend the avoidance of tree nuts of different sorts, as well as peanuts, when the patient shows cross-sensitization, as these fruits are associated with particularly severe reactions. On the other hand, it has to be considered that only a minority of our patients developed new food allergies during the follow-up period, and that in the majority of cases these were represented by local reactions. Since we were not able to detect any predictive factor (neither specific IgE-level, nor monosensitivity or co-sensitization) it is likely that the decisions regarding the exclusion of certain foods have to be taken on an individual basis, taking into account the quality of life of the patient as well as the severity of prior reactions with other foods.
We are aware that our study shows some evident limitations. First, with such a study design the degree of exposure to LTPs contained in foods other than the offending ones cannot be assessed in individual patients and, even if the exposure occurred on a regular basis, its amount may have varied from one patient to another. Second, since a control group is missing, we are unable to state which the consequences would have been if the same individuals were advised to strictly avoid exposure. However, as noted before, it should be considered that the strict avoidance of all sensitizing foods would have heavily worsened the quality of life of a number of control patients on one side, and would have eventually led to the necessity to perform open oral food challenges with all the excluded (but previously tolerated) foods in the clinics and under medical supervision, in order to assess their tolerance after a long period of avoidance, on the other side. Alternatively, creating a different control group left at a free diet (i.e., not excluding known offending foods) without giving any clinical advice, would have exposed the members to severe risks of adverse events, which is unethical, and most probably patients in such a group would have eventually behaved as our current study group, that is avoid all offending foods and going on eating the tolerated ones. Third, the study is based on patients’ reports, and these were not confirmed by properly performed oral challenges. Bias may have been introduced for reactions that occurred long time before regarding the presence or absence of co-factors. Finally, the possibility that sensitization to other allergens (e.g., seed storage proteins such as 2S-albumins, vicilins or legumins, or alternatively pollen-associated allergens such as PR-10 molecules or profilin) was responsible for the novel allergies reported by the patients should be taken into consideration. However, although in effect one patient with a novel pistachio allergy was eventually found to be neo-sensitized to a seed storage protein, in most cases adverse reactions were associated with Rosaceae / Prunoideae, a group of fruits that lack seed storage proteins, which makes this hypothesis unlikely. Further, no patient who experienced allergic reactions to novel foods developed a new sensitization to birch pollen or profilin. The present study confirms the protective effect of co-sensitization to PR-10 and/or profilin against severe adverse reactions induced by offending foods in LTP-hypersensitive subjects (18). In fact, despite significantly higher levels of IgE to rPru p 3, co-sensitized patients showed a much lower propensity to experience systemic allergic reactions following the ingestion of offending foods. Previous studies found a direct association between high rPru p 3 IgE levels and the probability to react to a large array of botanically unrelated foods (19), but of course those studies were carried out on LTP-monosensitive patients, whereas the large majority of the patients in the present study were co-sensitized to PR-10 and/or profilin. This study also shows that allergy to lipid transfer protein probably represents the most difficult type of food allergy, in terms of preventive strategies. The widespread diffusion of the protein, along with its variable degree of cross-reactivity from one patient to another, make it virtually impossible to predict which foods the patients will react to, and which will be the clinical expression of such adverse events, with the exception of the peach, that is the primary sensitizer to LTP and represents the food most frequently responsible for allergic reactions (1,20).

In conclusion, this study shows that Rosaceae / Prunoideae (with the possible exception of pear and peeled apple), tree nuts and peanuts, are the most frequent cause of new allergic reactions in the long term in LTP-allergic patients that are sensitized but clinically tolerant to these foods. In view of the limited prevalence of new allergic reactions, and of the fact that these are frequently local, the decision regarding their preventive avoidance have to be taken on an individual basis until larger prospective studies are carried out. Regarding the other foods that induced adverse reactions, these events occurred in a minority of sensitized subjects, and such a low prevalence does not seem to justify their exclusion from patients’ diets.

Conflict of interest

The authors declare that they have no conflict of interest.

References


Histamine-release test in angioedema patients without urticaria - a retrospective cohort study of 404 patients

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2Department of Otorhinolaryngology, Head and Neck Surgery and Audiology, Rigshospitalet, University of Copenhagen, København, Denmark

Introduction

Angioedema (AE) is a non-pitting skin colored swelling of skin or mucosa, with a predilection for areas with loosely bound skin. It is caused by a temporary increase in vascular permeability due to vasoactive mediators.

Mostly, AE is accompanied by urticaria, which indicates activation of mast cells liberating histamine and other vasoactive mediators. A subset of patients with urticaria and AE has histamine-releasing autoantibodies. The basophil histamine release test (HR-test) is a remedy to identify activation of basophils or mast cells causing histamine release. It has been used as a tool in chronic urticaria (CU) to define the autoimmune subgroup (CU) and less frequently against IgE (1,2).

Functional histamine releasing autoantibodies have been identified by the HR-test in approximately 20-30% of patients with CU (2-6). In contrast to CU or AE accompanied with urticaria, where histamine releasing autoantibodies can be frequently detected, sparse data can be found on the prevalence of histamine releasing autoantibodies in recurrent idiopathic AE (7).

The objective of this study was to report the prevalence of positive histamine releasing autoantibodies in a cohort of patients with recurrent AE and evaluate the usefulness of this test in AE patients.

Patients and methods

This was a retrospective cohort study of 612 AE patients seen at the Department of Dermatology, Odense University Hos-
hospital, in the study period 1995-2013. All patients had been referred for specialized dermatologic evaluation, due to AE with or without urticaria. The study-population was identified by a search in the medical record system, using the International Classification of Disease version 10 (ICD-10) diagnostic codes T78.3 (angioneurotic edema / Quinke oedema / giant urticaria), L50.8 (urticaria, other), L50.8A (chronic urticaria) and L98.9 (disorder of the skin and subcutaneous tissue, unspecified). Patients were included in the cohort if the information in the medical records were in accordance with AE, with or without urticaria. Patients with complement C1 inhibitor deficiency and acquired complement C1 inhibitor deficiency, as well as patients with a history of angiotensin-converting enzyme inhibitor-induced angioedema, were excluded. Only AE patients who had a HR-test performed were included in this study (404 patients). Figure 1 demonstrates the process of inclusion and exclusion of patients. A consultant dermatologist or a resident reviewed the medical records. Relevant data on demographics, concomitant rash, co-morbidity, co-medication, treatment regimens and efficacy, hospital admissions and outcome were collected, as well as selected laboratory test results.

HR-test was analyzed at RefLab Aps Copenhagen (http://reflab.dk/). According to this laboratory, the threshold for a positive result was > 16.5 % histamine release.

Ethical considerations
This study was approved by the Danish Data Protection Agency (Journal number 2008-38-0035) and the Danish National Board of Health (Journal number S-20140165).

Statistical analysis
Patients with AE with or without urticaria were compared using Fisher’s exact test. Difference of proportion test and odds ratio calculations was employed when comparing treatment efficacy. P-values ≤ 0.05 were considered statistically significant. 95% confidence intervals (CI) were reported when appropriate.

Results
The study population comprised 612 patients. HR-test was performed in 404 patients meeting the study’s inclusion criteria. The male : female ratio was 0.73, with a mean age of 50.2 years (range 2-85 years). The cohort was almost exclusively Caucasian. The mean follow-up time was 66.4 weeks. Further details are shown in Table 1. In two patients the test results could not be retrieved, and in three patients it was uncertain from the medical record if they also had urticaria. These five patients were excluded (Figure 1). The HR-test was positive in 10 out of 231 patients with AE without urticaria (4.3%) and in 29 out of 168 patients with urticaria (17%).

Figure 1 - Flow-chart demonstrating the process of selecting patients with positive HR-test.
HR-test in angioedema patients without urticaria

Table I - Demographic and clinical data of patients with angioedema (AE) +/- urticaria.

<table>
<thead>
<tr>
<th>Total number of patients with HR-test performed</th>
<th>404</th>
</tr>
</thead>
<tbody>
<tr>
<td>males</td>
<td>171</td>
</tr>
<tr>
<td>females</td>
<td>233</td>
</tr>
<tr>
<td>m : f-ratio</td>
<td>0.73</td>
</tr>
<tr>
<td>Age, mean, median, [range], years</td>
<td>50.16, 51.39, [range 2.1-85.1 years]</td>
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</table>

<table>
<thead>
<tr>
<th>Ethnicity</th>
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<tbody>
<tr>
<td>Caucasian</td>
<td>393</td>
</tr>
<tr>
<td>Middle Eastern</td>
<td>3</td>
</tr>
<tr>
<td>Black</td>
<td>1</td>
</tr>
<tr>
<td>Asian</td>
<td>5</td>
</tr>
<tr>
<td>other</td>
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<table>
<thead>
<tr>
<th>Current tobacco use, n</th>
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</thead>
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<td>no</td>
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<table>
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<tr>
<th>Number of patients with a positive family history of AE</th>
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</thead>
<tbody>
<tr>
<td>Number of HR-tests, total</td>
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</tr>
<tr>
<td>positive HR-test performed</td>
<td>39 (9.7%)</td>
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<tr>
<td>negative HR-test performed</td>
<td>360 (89.1%)</td>
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<td>unknown result</td>
<td>5 (0.5%)</td>
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<table>
<thead>
<tr>
<th>Comorbidities</th>
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</thead>
<tbody>
<tr>
<td>diabetes mellitus</td>
<td>32</td>
</tr>
<tr>
<td>hypertension</td>
<td>115</td>
</tr>
<tr>
<td>ischemic heart disease</td>
<td>24</td>
</tr>
<tr>
<td>heart failure</td>
<td>7</td>
</tr>
<tr>
<td>atopic dermatitis</td>
<td>21</td>
</tr>
<tr>
<td>allergic rhinitis</td>
<td>56</td>
</tr>
<tr>
<td>asthma</td>
<td>42</td>
</tr>
<tr>
<td>other respiratory disease</td>
<td>4</td>
</tr>
</tbody>
</table>

| Follow-up time, mean ; [range], weeks          | 66.4; [0 - 675 weeks] |

<table>
<thead>
<tr>
<th>Number of reported efficacy of antihistamines</th>
<th>266</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of reported efficacy of corticosteroids</td>
<td>162</td>
</tr>
<tr>
<td>Number of patients hospitalized due to AE</td>
<td>138 (34.2%)</td>
</tr>
<tr>
<td>Number of patients with ER visits due to AE</td>
<td>144 (35.6%)</td>
</tr>
</tbody>
</table>

Table II - Different treatments and their efficacy on angioedema patients +/- urticaria.

<table>
<thead>
<tr>
<th>Medications</th>
<th>Number of treated individuals</th>
<th>Efficacy on angioedema</th>
</tr>
</thead>
<tbody>
<tr>
<td>positive HR-test and monosymptomatic angioedema (n = 10)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>anti-histamines</td>
<td>10</td>
<td>5 (50%)</td>
</tr>
<tr>
<td>corticosteroids</td>
<td>7</td>
<td>4 (92.9%)</td>
</tr>
<tr>
<td>adrenaline</td>
<td>3</td>
<td>2 (66.7%)</td>
</tr>
<tr>
<td>negative HR-test and monosymptomatic angioedema (n = 221)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>anti-histamines</td>
<td>202</td>
<td>132 (65.3%)</td>
</tr>
<tr>
<td>corticosteroids</td>
<td>134</td>
<td>80 (59.7%)</td>
</tr>
<tr>
<td>adrenaline</td>
<td>34</td>
<td>14 (41.2%)</td>
</tr>
<tr>
<td>positive HR-test and angioedema with urticaria (n = 29)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>anti-histamines</td>
<td>27</td>
<td>22 (81.5%)</td>
</tr>
<tr>
<td>corticosteroids</td>
<td>20</td>
<td>13 (65%)</td>
</tr>
<tr>
<td>adrenaline</td>
<td>3</td>
<td>1 (33.1%)</td>
</tr>
<tr>
<td>negative HR-test and angioedema with urticaria (n = 139)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>anti-histamines</td>
<td>135</td>
<td>107 (79.3%)</td>
</tr>
<tr>
<td>corticosteroids</td>
<td>86</td>
<td>65 (75.6%)</td>
</tr>
<tr>
<td>adrenaline</td>
<td>14</td>
<td>5 (35.7%)</td>
</tr>
</tbody>
</table>

AE and urticaria (17.3%) (p = 0.0005, 95% CI 5.64 to 20.14). Among 399 included patients, sufficient treatment data were available in 374 patients. Table II shows the different drugs used and their efficacy. When monosymptomatic AE patients with positive HR-test were compared with monosymptomatic AE having a negative HR-test, the odds ratio was 1.9 for a positive effect of antihistamines. This finding was not significant (p = 0.25, 95% CI 0.528 to 6.7352). Comparing the same groups, the odds ratio was 1.1 (p = 0.59, 95% CI 0.2391 to 5.1635) for having a positive effect of corticosteroids.

The treatment efficacy of antihistamines and corticosteroids was also studied among AE patients with concomitant urticaria. The subgroup of patients with positive HR-test was compared with patients having negative HR-test. The odds ratio was 0.86 (p = 0.51, 95% CI 0.302 - 2.498) for having a positive effect of antihistamines, and 1.67 (p = 0.24, 95% CI 0.5878 - 4.7261) for corticosteroids. The treatment response of adrenaline could also not be predicted by HR-test result, as those with monosymptomatic angioedema and positive versus negative HR-test had an odds ratio of 0.71 (p = 0.64, 95% CI 0.0589 - 8.6651). Patients with concomitant urticaria and positive versus negative HR-test had an odds ratio of 1.11 (p = 0.73, 95% CI 0.0795 - 15.5348).
Table III - HR-test results from studies of patients with urticaria (wheals) and angioedema (AE) also showing cut-off values for HR-test.

<table>
<thead>
<tr>
<th>Author</th>
<th>Basophil histamine release test (HR)</th>
<th>Number of patients with monosymptomatic AE</th>
<th>Number of patients with AE with wheals</th>
<th>Number of patients with only wheals</th>
<th>Number of healthy controls</th>
<th>Total positive HR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iqbal et al. 2012 (3)</td>
<td>&gt; 16.5% cut-off</td>
<td>398 (urticaria with and without AE)</td>
<td>105 positive HR</td>
<td></td>
<td></td>
<td>105</td>
</tr>
<tr>
<td>Grattan et al. 1991 (4)</td>
<td>&gt; 10% cut-off</td>
<td>14 positive HR</td>
<td>0 positive HR</td>
<td></td>
<td></td>
<td>14</td>
</tr>
<tr>
<td>Hide et al. 1993 (5)</td>
<td>&gt; 10% cut-off</td>
<td>26, 17 positive HR</td>
<td>17 positive HR</td>
<td></td>
<td></td>
<td>17</td>
</tr>
<tr>
<td>Tedeschi et al. 2012 (7)</td>
<td>5% cut-off</td>
<td>19, 2 positive HR</td>
<td>11 positive HR</td>
<td>20, 0 positive HR</td>
<td>31 (2 AE, 18 AE with wheals, 11 with wheals)</td>
<td>31</td>
</tr>
<tr>
<td>Grattan et al. 2000 (10)</td>
<td>&gt; 5% cut-off</td>
<td>27, 14 positive HR</td>
<td></td>
<td></td>
<td></td>
<td>14</td>
</tr>
<tr>
<td>Platzer et al. 2005 (11)</td>
<td>&gt; 16.5% cut-off</td>
<td>901, 323 positive HR</td>
<td>9, 0 positive HR</td>
<td></td>
<td></td>
<td>323</td>
</tr>
<tr>
<td>Szegedi et al. 2006 (12)</td>
<td>11.6% cut-off for atopic donor serum 7.3% cut-off for non-atopic serum</td>
<td>72, 37 positive atopic donor serum, 23 positive non-atopic donor serum</td>
<td>20, 0 positive HR</td>
<td></td>
<td></td>
<td>60</td>
</tr>
<tr>
<td>Zuberbier et al. 2000 (13)</td>
<td>&gt; 10% cut-off</td>
<td>13, 7 positive HR</td>
<td></td>
<td></td>
<td></td>
<td>7</td>
</tr>
<tr>
<td>Godse et al. 2010 (14)</td>
<td>&gt; 16.5% cut-off</td>
<td>20, 9 positive HR</td>
<td></td>
<td></td>
<td></td>
<td>9</td>
</tr>
<tr>
<td>Hyry et al. 2006 (15)</td>
<td>&gt; 12% cut-off</td>
<td>10, 4 positive HR</td>
<td></td>
<td></td>
<td></td>
<td>4</td>
</tr>
<tr>
<td>Sabroe et al. 1999 (16)</td>
<td>≥ 5% cut-off</td>
<td>155, 54 positive HR</td>
<td>40, 0 positive HR</td>
<td></td>
<td></td>
<td>54</td>
</tr>
<tr>
<td>Kaplan, Joseph. 2007 (17)</td>
<td>≥ 15% cut-off</td>
<td>104, 54 positive HR</td>
<td></td>
<td></td>
<td></td>
<td>54</td>
</tr>
<tr>
<td>Perez et al. 2010 (18)</td>
<td>&gt; 16.5% cut-off</td>
<td>6 (where HR test was performed), 2 positive HR with CU, 1 positive with urticarial vasculitis</td>
<td></td>
<td></td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>Berti et al. 2017 (19)</td>
<td>&gt; 16.5% cut-off</td>
<td>14</td>
<td>6</td>
<td></td>
<td></td>
<td>9</td>
</tr>
<tr>
<td>This study</td>
<td>&gt; 16.5% cut-off</td>
<td>231, 10 positive HR</td>
<td>168, 29 positive HR</td>
<td></td>
<td></td>
<td>39</td>
</tr>
</tbody>
</table>

1Atopic serum leads to a significantly higher histamine release. HR-test performed with blood from two donors.
Discussion

In this retrospective study of 404 patients, we analyzed the frequency of functional histamine releasing autoantibodies in a cohort of patients with AE with or without urticaria. We found a frequency of 4.3% with a positive HR-test in the subgroup of patients with mono-symptomatic AE, and 17.3% with a positive HR-test in the subgroup of patients with AE and urticaria. This makes sense, since the wheal and flare response is most often connected to histamine release, whereas the vasoactive mediators in angioedema are more dubious and may include other mediators such as bradykinin (8).

Comparing patients with mono-symptomatic AE with positive HR-test contra negative HR-test, the odds ratio was 1.9 for having a positive effect of antihistamines. This could be a signal to guide in an individualized therapy, but unfortunately the finding was not significant, possibly due to the number of included patients. In a large clinical survey, it has been shown that most patients (254 of 294) with monosymptomatic AE responded completely or partially to antihistamines, however no data on the HR-test was provided (9).

In the subgroup of AE patients with co-existing urticaria, neither the treatment efficacy of antihistamines, corticosteroids nor adrenaline differed significantly between HR-positive and HR-negative individuals. Unfortunately, we could not retrieve sufficient efficacy data of cyclosporine in this study. It is known from the literature that patients with antihistamine-unresponsive urticaria and a positive HR-test may respond better to cyclosporine than patients with a negative HR-test (3,10). No data on monosymptomatic angioedema, HR-test and treatment response to cyclosporine or other drugs could be found.

Sparse data could be found in the literature on HR-testing in patients with angioedema (7). Only the study by Tedeschi and coworkers investigated HR-testing in monosymptomatic angioedema patients. They divided their cohort into subgroups of patients with AE with or without urticaria, suggesting that a positive HR-test is linked to urticaria and not AE, which could be confirmed in this study. Higher rates of positive HR-tests were found in the literature, possibly explained by a lower cutoff value in the majority of these studies as seen in Table III (3,5,7,10-19).

The main limitations of the present study are the retrospective design with data collection from the patients’ medical records over a 20-year period. We cannot exclude possible bias in the study, as different colleagues have made the clinical observations and not all symptoms may be listed in the medical records. The HR-test became commercially available in our country in 2004, and has been used routinely in all patients with urticaria seen at our department between 2005 and 2013. A prospective study would be preferable, and should include a valid and reliable measure of disease activity; i.e. the Angioedema Activity Score (20).

We could not compare data with autologous serum or plasma skin tests (ASST and APST), as these are not routinely performed in our country. According to the findings of Berti et al., there does not seem to be any association between in vivo and in vitro tests in patients with CU (19), and the same could be true for AE. The performance of ASST and APST would be desirable to study in the future, to confirm these findings also in mono-symptomatic AE. In conclusion, we cannot see any diagnostic or therapeutic value of HR-test in mono-symptomatic AE.

Acknowledgments

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CSL Behring and Shire are thanked for their continuous contribution to angioedema research.

Conflicts of interest

Dr. Eva Rye Rasmussen has collaborated with Shire and CSL Behring regarding angioedema research. This study was not directly supported financially. Dr. Rasmussen has received honorariums for lectures at angioedema meetings from Shire and MSD Norway.

A. Bygum has received research grant support and/or speaker / consulting fees from CSL Behring, Shire/Jerini AG and Vi- roPharma; and participated in a clinical trial for BioCryst and Jerini AG. She is an advisor for the HAE Scandinavian Patient Organization.

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al. Development, validation, and initial results of the Angioedema
Omalizumab in chronic spontaneous and inducible urticaria: a 9 year retrospective study in Portugal

Abbreviations
CU, chronic urticaria; CSU, chronic spontaneous urticaria; CIndU, chronic inducible urticaria; QoL, quality of life; CU-QoL, chronic urticaria quality of life; ASST, autologous serum skin test; DLQI, dermatology life quality index; UAS, urticaria activity score; nsAH, non-sedating H1-antihistamines

Introduction
Chronic urticaria (CU) is a debilitating disease characterized by wheals, flares and/or angioedema that recur or last longer than 6 weeks. CU is subdivided into spontaneous (when lesions recur without the need of a stimulus) and inducible (when there is an identifiable trigger) (1).

Chronic spontaneous urticaria (CSU) affects 0.5 to 1% of the population, with a female predominance (2:1) and a peak incidence between the ages of 20 and 40 (2). This high prevalence makes the effects of CU in patients’ quality of life (QoL) relevant. O’Donnell et al. showed CSU patients suffer similar QoL impairment to patients with severe coronary artery disease awaiting for bypass surgery (3).

Current EAACI/GA2LEN/EDF/WAO guidelines therapy’s aim is complete symptom resolution, beginning with non-sedating H1-antihistamines (nsAH), which can be increased up to four-fold (1). However, less than 50% of patients respond to the standard dose, and 1/4 to 1/3 remain symptomatic after the fourfold increase (2). The high number of still symptomatic patients explains why third line therapies are so important.

Very thorough placebo-controlled clinical trials (ASTERIA I, II and GLACIAL) have shown clear improvement of CSU with omalizumab; but very little is known concerning its long-term
effects and efficacy (3-5). Omalizumab is currently approved in CSU, but has also been used with good results in many types of chronic inducible urticaria (CIndU) (6-16).

The main objective is to describe the clinical characteristics of CU patients treated with omalizumab at our Immunoallergology Department. Other objectives include evaluating the efficacy and long-term safety of omalizumab and the feasibility of stopping omalizumab.

Materials and methods

We performed a retrospective chart-review study, June 2006 - July 2015, of adults with CU treated with omalizumab at our Immunoallergology Department; with a minimum of 6 months of treatment. All patients who begin omalizumab for chronic urticaria, do so for a minimum period of 6 months. This time limit was implemented in our Immunoallergology Department so that a correct assessment of a patient’s response or lack of response to omalizumab could be made and, subsequently, the decision to continue or stop omalizumab. Because all patients take omalizumab for this 6-month “trial” period, no patient stopped omalizumab before this 6-month time limit due to lack of response. Therefore, no patient who could have been classified as non-responder was excluded from this study due to this time limit.

Patients were characterized according to demographic data (gender, presence of atopic comorbidities, age of CU onset and omalizumab’s initiation, CU duration), type of CU, previous failed therapies, current therapies, omalizumab initial dose, omalizumab therapy duration baseline total serum IgE, autologous serum skin test (ASST) and anti-thyroid antibodies.

Patients response to omalizumab was analyzed using validated patient reported questionnaires: urticaria activity score, UAS and UAS7, taken at baseline and at each visit for omalizumab administration; and Dermatology Life Quality Index, DLQI, taken at baseline and 6-12 months after beginning of omalizumab.

Stepping down of medication was mostly made as follows: all patients started omalizumab as an add-on therapy. When control was achieved (defined as UAS7 ≤ 6 at the time of omalizumab administration) stepping down occurred in the following order: oral corticosteroids; H2-antihistamines; leukotriene receptor antagonist (except for those taking it for atopic comorbidities); nsAH (4 tablets/day --> 2 tablets/day --> 1 tablet/day --> SOS [meaning as rescue medication for transient exacerbations]).

Patients were categorized (on July 2015) according to their response to omalizumab (classification adapted from Har et al) (17) as: 1, complete responders who only required SOS nsAH and omalizumab to maintain UAS7 ≤ 6 (includes patients on montelukast for atopic comorbidities); 2, partial responders who required daily medication other than omalizumab to maintain control; 3, non-responders who began omalizumab but had no improvement.

Complete responders were additionally categorized according to their response to tapering off omalizumab: 1, non-omalizumab-dependent who stopped omalizumab and maintained CU control; 2, omalizumab-dependent who had flares when tried to increase the time intervals between administrations or stop omalizumab.

Tapering off omalizumab was as follows: if UAS7 ≤ 6 was maintained between administrations, the time intervals between administrations was increased from every 4 weeks to every 5, then every 6 and then every 8. After 2 administrations 8 weeks apart with UAS7 ≤ 6, stopping omalizumab was tried.

Adverse reactions were monitored after each omalizumab administration for immediate reactions (150 minutes for the first 3 administrations and 60 minutes thereafter) and late reactions (by reviewing the patients’ urticaria diary).

Statistical analyses of the data were performed using SPSS software version 22.0 (IBM Corporation, New York, USA): descriptive statistics, Mann-Whitney tests, chi-square test, generalized linear model using a gamma distribution with log link function and a working correlation matrix with a autoregressive model of 1st order structure to evaluate the UAS and UAS7 scores. A p value < 0.05 was considered significant.

Consent from the Ethics Committee and Hospital Administration was obtained (Ref. 628/15, Comissão de Ética do Centro Académico de Medicina de Lisboa).

Results

Data is summed-up in table 1 and 2.

Twenty-six patients were included, 3 with CIndU (2 with delayed pressure urticaria and 1 with heat urticaria) and 23 with CSU. The age of urticaria onset was 38 ± 15 years in CSU and 57 ± 12 years in CIndU patients (p < 0.05).

Thyroid autoantibodies were found in 7 (30%) of the 23 CSU patients; but only 1 was symptomatic with hypothyroidism (due to Hashimoto’s thyroiditis). ASST was performed in 15 CSU patients and was positive in 9 (60%). The remaining could not stop anti-histamines to perform the test.

Atopic comorbidities in CSU patients were present in 12 patients (52%): 10 (43%) had asthma and/or allergic rhinitis, 3 (13%) had drug allergy and 2 (9%) had food allergy. In CIndU patients, 1 had asthma, allergic rhinitis and food allergy.

Previous failed therapies to control urticaria were: immunoglobulin IgG in 1 patient with CIndU and 4 with CSU; cyclosporine in 3 patients with CSU and azathioprine in 1 patient with CSU. Prior to omalizumab, all patients were medicated with montelukast, nsAH 4 times/day and systemic oral corticosteroids. The initial corticosteroid dose was 1mg/kg/day. The dose was subsequently tapered to the lowest dose possible, to maintain an equilibrium between maximum control of urticaria symptoms and the lowest
Urticaria activity was measured with the UAS and UAS7 scores during the first 12 months of therapy and evaluated using generalized linear models (figure 1 and 2). CIndU patients’ UAS/UAS7 score prior to omalizumab initiation averaged 4.7 ± 1.2 / 33.7 ± 1.9; after 12 months of omalizumab, the score averaged 0 ± 0 / 0 ± 0. CSU patients’ UAS/UAS7 score prior to omalizumab initiation averaged 4.7 ± 1.5 / 33.1 ± 9.1; after 12 months of omalizumab, the score averaged 0.8 ± 0.9 / 1.3 ± 2.2. The UAS and UAS7 values prior to omalizumab show urticaria activity whilst the patients were on montelukast, nsAH 4 times/day and systemic oral corticosteroids.

CIndU and CSU patients had a reduction of the UAS score per omalizumab administration of 16% (both with p < 0.001). Using the UAS 7 score, CIndU patients had a reduction of the score per omalizumab administration of 15% (p < 0.001) and CSU patients a 20% reduction (p < 0.001). The number of sessions of omalizumab needed to achieve UAS = 0 averaged 4 (CIndU) and 5 (CSU).

The QoL score at baseline in CIndU patients had a median of 18.5 (minimum 9, maximum 28) and in CSU patients had a median of 19 (minimum 6, maximum 28, interquartile range 8). After 6-12 months of omalizumab treatment, the QoL score in CIndU had a median of 0.0 (minimum 0, maximum 0) and in CSU had a median of 0.0 (minimum 0, maximum 6, inter-

### Table 1 - Comparison between CIndU and CSU patients' characteristics.

<table>
<thead>
<tr>
<th></th>
<th>CIndU</th>
<th>CSU</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>age of urticaria onset (years)</td>
<td>57 ± 11.9</td>
<td>38 ± 14.7</td>
<td>0.363</td>
</tr>
<tr>
<td>age of omalizumab start (years)</td>
<td>54 ± 10.6</td>
<td>43 ± 13.3</td>
<td>0.417</td>
</tr>
<tr>
<td>time between urticaria onset and omalizumab start (years)</td>
<td>2.7 ± 1.5</td>
<td>5.2 ± 5.7</td>
<td>1.000</td>
</tr>
<tr>
<td>duration of omalizumab treatment (months)</td>
<td>29.3 ± 15.4</td>
<td>29.7 ± 20.9</td>
<td>0.187</td>
</tr>
<tr>
<td>total serum IgE (kU/L)</td>
<td>76 ± 19</td>
<td>260 ± 446</td>
<td>0.0751</td>
</tr>
<tr>
<td>number of sessions of omalizumab administration until UAS = 0 was achieved</td>
<td>3.7 ± 0.6</td>
<td>4.7 ± 3.6</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>DLQI baseline score</td>
<td>18.5 ± 13.4</td>
<td>18.2 ± 6</td>
<td>0.058</td>
</tr>
<tr>
<td>DLQI final score</td>
<td>0 ± 0</td>
<td>0.7 ± 1.4</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>female gender</td>
<td>33% (n = 1)</td>
<td>87% (n = 20)</td>
<td></td>
</tr>
<tr>
<td>positive autologous serum skin test</td>
<td>-</td>
<td>60% (9 out of 15 patients)</td>
<td></td>
</tr>
<tr>
<td>anti-thyroid antibodies</td>
<td>-</td>
<td>30% (7 out of 23 patients)</td>
<td></td>
</tr>
</tbody>
</table>

CSU, Chronic Spontaneous Urticaria; CIndU, Chronic Induced Urticaria; A ± SD, Average ± Standard Deviation; M (min, max, IQR), median value (minimum, maximum, Interquartile range); M, Mann-Whitney test.
and, because of that, were classified by the end of this study as partial responders. The remaining 4, were complete responders. However, because they reinitiated urticaria after stopping omalizumab and required reinitiating omalizumab to control the urticaria, they were classified as omalizumab-dependent. It is important to emphasize the terms partial and complete responders are related to the need for medication other than omalizumab, not urticaria control (all the above mentioned patients had urticaria control defined as UAS7 < 6).

Seven (30%) CSU patients had stopped omalizumab by the end of this study. These patients achieved urticaria control and stopped omalizumab without relapsing. Therefore, they were classified as complete responders and non omalizumab-dependent.

**Table 2 - Data comparison between different studies.**

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>females</td>
<td>21/26 (81%)</td>
<td>6/7 (86%)</td>
<td>5/10 (50%)</td>
<td>36/46 (78%)</td>
<td>U</td>
</tr>
<tr>
<td>CU duration</td>
<td>CIndU 2.7 years</td>
<td>7 years</td>
<td>4 years</td>
<td>36% 1-5 years</td>
<td>U</td>
</tr>
<tr>
<td></td>
<td>CSU 5.2 years</td>
<td></td>
<td></td>
<td>38% 5-10 years</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>26% &gt; 10 years</td>
<td></td>
</tr>
<tr>
<td>average baseline IgE</td>
<td>CIndU 76 kU/L</td>
<td>162 kU/L</td>
<td>417 kU/L</td>
<td>U</td>
<td>U</td>
</tr>
<tr>
<td>responded to omalizumab</td>
<td>26/26 (100%)</td>
<td>7/7 (100%)</td>
<td>10/17 (59%)</td>
<td>27/36 (75%)</td>
<td>U</td>
</tr>
<tr>
<td>partial responders to omalizumab</td>
<td>CIndU 2/3 (66%)</td>
<td>U</td>
<td>2/10 (20%)</td>
<td>12/36 (75%)</td>
<td>U</td>
</tr>
<tr>
<td></td>
<td>CSU 12/23 (52%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>complete responders to omalizumab</td>
<td>CIndU 0 (0%)</td>
<td>7/7 (100%)</td>
<td>8/10 (80%)</td>
<td>15/36 (42%)</td>
<td>U</td>
</tr>
<tr>
<td></td>
<td>CSU 11/23 (48%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>omalizumab dependent</td>
<td>CIndU 1/3 (33%)</td>
<td>1/7 (14%)</td>
<td>9/10 (90%)</td>
<td>U</td>
<td>U</td>
</tr>
<tr>
<td></td>
<td>CSU 4/23 (17%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>non omalizumab dependent</td>
<td>CIndU 0 (0%)</td>
<td>6/7 (86%)</td>
<td>1/10 (10%)</td>
<td>U</td>
<td>U</td>
</tr>
<tr>
<td></td>
<td>CSU 7/23 (30%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>same efficacy when restarting omalizumab</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>U</td>
<td>yes</td>
</tr>
<tr>
<td>longest omalizumab therapy duration</td>
<td>73 months</td>
<td>40 months</td>
<td>112 months</td>
<td>U</td>
<td>U</td>
</tr>
<tr>
<td>adverse effects reported</td>
<td>none</td>
<td>none</td>
<td>none</td>
<td>36 events involving 37% of patients</td>
<td>U</td>
</tr>
<tr>
<td>DLQI baseline / after omalizumab</td>
<td>ClIndU 18.5 ± 13 / 0 ± 0</td>
<td>U</td>
<td>U</td>
<td>19.5 ± 5.2 / 3.2 ± 5.2</td>
<td>U</td>
</tr>
<tr>
<td></td>
<td>CSU 18.2 ± 6 / 0.7 ± 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1Described as patients who had symptom recurrence when spacing of administrations or discontinuation was tried. U, Unknown; DLQI, Dermatology Life Quality Index.

quartile range 1). The difference between initial e final median values was statistically significant (p < 0.001) in CSU. All ClIndU patients were still on omalizumab by the end of this study. Their average treatment duration was 29 ± 15 months (min 19, max 46). Two patients were classified as partial responders and 1 as a complete responder. The complete responder was classified as omalizumab-dependent, because he reinitiated urticaria after stopping omalizumab. Reinitiating omalizumab controlled the urticaria.

Sixteen CSU patients were still on omalizumab by the end of this study. Their average treatment duration was 30 ± 23 months (min 6, max 72). Of the 16, 12 had controlled urticaria, but were still on the stepping down process of other medication (as anti-histamines) and, because of that, were classified by the end of this study as partial responders. The remaining 4, were complete responders. However, because they reinitiated urticaria after stopping omalizumab and required reinitiating omalizumab to control the urticaria, they were classified as omalizumab-dependent. It is important to emphasize the terms partial and complete responders are related to the need for medication other than omalizumab, not urticaria control (all the above mentioned patients had urticaria control defined as UAS7 < 6). Seven (30%) CSU patients had stopped omalizumab by the end of this study. These patients achieved urticaria control and stopped omalizumab without relapsing. Therefore, they were classified as complete responders and non omalizumab-dependent. Their treat-
Omalizumab in chronic spontaneous and inducible urticaria: a 9 year retrospective study in Portugal

Indirectly down-regulate FcRI receptors, de-sensitize and raise the thresholds of mast cells and basophile degranulation and reduce the secretion of pro-inflammatory mediators (18,19). In 2014, omalizumab was licensed as add-on therapy for CSU in patients older than 12 years of age (20). Because it was only recently approved, little is known about the long-term effects of this therapy. Most data comes from small case reports (17,18,21). Even less is known about when and if omalizumab can be discontinued and there is no consensus regarding duration of omalizumab treatment in CSU. This is extremely important, as omalizumab is a very expensive therapy (17).

In our study, patients had severe urticaria. This can be observed by both CSU and CIndU patients showing an average baseline treatment duration averaged 30 ± 18 months (min 6, max 49). All patients who had to restart omalizumab or shorten back the administrations, had no lessening of effect. There were no non-responders in our population.

During the 73 months of omalizumab use, neither systemic immediate reactions were observed, nor late adverse side-effects attributed to omalizumab.

**Discussion**

Omalizumab is a recombinant humanized monoclonal anti-IgE IgG antibody. By sequestering free IgE, it prevents IgE binding to FcεRI on the surface of mast cells and basophils. It may also indirectly down-regulate FcRI receptors, de-sensitize and raise the thresholds of mast cells and basophile degranulation and reduce the secretion of pro-inflammatory mediators (18,19). In 2014, omalizumab was licensed as add-on therapy for CSU in patients older than 12 years of age (20). Because it was only recently approved, little is known about the long-term effects of this therapy. Most data comes from small case reports (17,18,21). Even less is known about when and if omalizumab can be discontinued and there is no consensus regarding duration of omalizumab treatment in CSU. This is extremely important, as omalizumab is a very expensive therapy (17). In our study, patients had severe urticaria. This can be observed by both CSU and CIndU patients showing an average baseline...
(before starting omalizumab) UAS7 score above 30. Some isolated patients had lower UAS7 scores, but were on high levels of oral corticosteroids (corticosteroid-dependent), which they could not sustain for long periods of time. Other patients, also with lower UAS7 scores, had very high DLQI scores and had these scores despite being medicated with montelukast, nsAH 4 times/day and systemic oral corticosteroids. Therefore, these patients were considered to have a severe enough urticaria to justify starting omalizumab.

In line with what is described in literature (1,2) and in a similar study (18), our CSU patients had a female predominance. In agreement with other studies, our patients tried immune-modulating therapies prior to omalizumab (immunoglobulin IgG in 1 patient with ClndU and 4 with CSU; cyclosporine in 3 patients with CSU and azathioprine in 1 patient with CSU), with limited or no effect (17,18,21-23). This lack of response did not predict lack of response to omalizumab. In addition, our patients were treated daily with montelukast, nsAH 4 times/day, H2-antihistamine (many of them) and systemic corticosteroids. This highlights the importance of omalizumab in weaning off other control therapies, especially corticosteroids, whose long-term use should be avoided.

Our CSU patients had a tendency for urticaria onset at an earlier age than ClndU patients. The reason for this finding is unknown. Our patients had a high baseline total serum IgE (table 1). Based on our data, Silva et al. (21) and Savic et al. (18), CU patients seem to have a tendency for elevated total serum IgE. As far as the authors are aware, no other publication has applied generalized linear models to study the progression of the urticaria activity score in response to omalizumab. This permitted to calculate, with statistical significance (p < 0.001), a decrease of the urticaria activity scores UAS and UAS7 between 15% and 20% with each omalizumab administration (figure 1 and 2). There was no significant difference between the CSU and ClndU groups. It is also interesting to observe that, with omalizumab, this decline in the urticaria activity scores occurred even though patients were simultaneously weaning off other control therapy. This may suggest that omalizumab is more important as a control therapy than other oral control therapies.

Given the impact in patients’ QoL, current guidelines propose the use of patient reported outcomes (1). Because the CU-QoL questionnaire is not validated for the Portuguese language, the authors used the validated DLQI. The QoL improvement was manifest in both ClndU and CSU patients. These results are similar to those reported by Savic et al. (18) who had a baseline score of 19.5 ± 2 (very similar to our patients) and who showed a 75% reduction in DLQI scores with omalizumab treatment (table 2).

The authors concur that the 6-month time limit is arbitrary and other time limits could be proposed to make the decision of response or lack of response to omalizumab. This practice was implemented to give enough time to evaluate the response of patients to omalizumab. Published data shows there are two types of response to omalizumab: fast responders and late responders. (24,25) Time is needed to identify these late responders and not incorrectly classify them as non-responders. Because all patients go through this 6-month “trial” phase, no patient who could have been classified as non-responder was excluded from this study due to this time limit.

All our patients responded to omalizumab (there were no non-responders). This response rate is similar to previous data from our center reported by Silva et al. (21), but higher than that reported by Har et al. (10 out of 17 patients) (17) and Savic et al. (27 out of 36 patients) (18). Overall, approximately 41-48% of patients in clinical trials (3-5) and 12-23% in “real-world clinical setting” (26-31) do not have a complete or significant response to omalizumab therapy.

The absence of non-responders is puzzling and was investigated. The argument these patients may have had less severe urticarias which might not have needed omalizumab is not in accordance with their clinical histories and the drugs the patients were taking and under which they still maintained high scores of UAS7 and DLQI. Another argument may be that it is due to the low number of CSU patients (23) included in this study. With time, as more patients start omalizumab, non-responders would appear. In addition to this, our department’s 6-month “trial” period may also have had influence. As already stated, reports suggest there are two kinds of response to omalizumab, fast responders and late responders (32,33). Which means, they only respond after several months of therapy. The percentage of non-responding patients in the clinical trials was calculated after 12 to 24 weeks of omalizumab. Therefore, some of the late responders could have been erroneously classified as non-responders. Consequently, because our 6-month time limit eliminates this bias, a lower rate of non-responders in our study could be expected. However, this is an important oddity of this study and further research is needed.

A key clinical feature is the type of response. Seventy-five percent of patients have a partial response; while 25% have a complete response. Of those, only 30% remain symptom free after omalizumab was discontinued; the others are omalizumab-dependent. This is very important, because no study has shown for how long this therapy can, safely, be maintained.

Some patients achieved remission and omalizumab could be stopped. Therefore, discontinuation should always be tried as successful remission is possible. Questions remain concerning how long omalizumab should be used until a successful discontinuation can be achieved. In our patients who achieved remission free of omalizumab, the average treatment duration was 30 ± 18 months.
Another important finding in our cohort, also shown by Metz et al. (32), is that there seems to be no loss of efficacy when reinitiating omalizumab after it has been discontinued.

In our study, patients were on omalizumab for up to 73 months with no apparent side-effects or loss of efficacy. Its good safety profile has been shown in the short term in various studies, with a recent meta-analysis of randomized clinical trials showing a similar rate of adverse events in the omalizumab and placebo groups (32). In a previous study by Silva et al. (21) and in our study, no adverse effects were reported with the long-term use. The authors believe that these results are comparable to the findings by Savic et al. (18) who reported 36 adverse events (involving 37% of the patients), but which were mostly skin reactions whose manifestation closely resembled CSU symptoms and 2 events (pregnancy) completely unrelated to omalizumab.

The limitations of this study are: it is a retrospective study, few patients were included and we grouped different types of CIndU in the same group. However, considering the statistical significant results obtained, our main objective appears to have been accomplished. Nonetheless, more studies with a greater number of patients are required before any generalization of these results can be made.

Conclusions

In our cohort, omalizumab was a safe and effective therapy, both in CIndU and CSU. This is evident by the lack of severe side effects and the significant improvement of the QoL and Urticaria Activity Scores.

In line with other publications, our CU patients were predominantly female and our CU patients had a high baseline total serum IgE.

Seven (30%) of our patients achieved urticaria control (UAS7 ≤ 6) and stopped omalizumab and all other medication without relapsing, showing disease remission. Therefore, an attempt to stop omalizumab should be considered, as remission is possible.

Patient consent

Obtained.

Conflict of interest

Ana Célia Costa M.D. is currently Principal investigator at Hospital de Santa Maria CHLN E.P.E., as part of the “AWARE” study, supported by Novartis Pharmaceuticals Corporation. Ana Célia Costa M.D. and Manuel Pereira-Barbosa M.D. are currently Principal investigators at Hospital de Santa Maria CHLN E.P.E., as part of the “Exploitation of the Basophil Activation Test (BAT) as a method for Xolair (omalizumab) treatment effectiveness in Chronic Spontaneous Urticaria (CSU)” study supported by Novartis Pharmaceuticals Corporation.

All authors consider that there are no other financial or personal relationship which could result in a conflict of interest with regard to the published article.

References


Reduction of the allergenicity of cow’s milk α-lactalbumin under heat-treatment and enzymatic hydrolysis in Moroccan population

Laboratory of Bioactive Molecules (LBM), Faculty of Sciences and Technology, Sidi Mohamed Ben Abdellah University, Fez, Morocco

Summary

The aim of the present study is to evaluate the effect of heat-treatment and enzymatic hydrolysis on the allergenicity of cow’s milk α-lactalbumin (α-LA) in a Moroccan population. A total of 557 patients were recruited from the University Hospital Complex and the Ibn El Khatib Hospital of Fez city. This population consented to realize a dosage of IgE levels to raw cow milk and then to α-LA native and treated with the studied treatments. The results revealed that 54.4% of the studied subjects presented positive values of serial IgE to raw cow milk. The effect of treatments on the allergenicity of α-LA showed that heat-treatment at 90°C and pepsin hydrolysis at 37°C, for 1 hour each, caused an important decrease in the IgE binding with an average of reduction of 59% and 74%, respectively.

Introduction

Cow’s milk is the first component introduced into the diet, and it is the most common cause of food allergy in the World. In Morocco, cow’s milk allergy is about 6.9% in schoolchildren (1) and between 2% to 3.6% in general population (2,3,4). Several studies have identified casein as a major cow milk allergen that induces strong immediate allergic reactions (5,6,7). β-Lactoglobulin represents another important cow milk allergen that is recognized by milk allergic patients (8,9). However, for α-lactalbumin, a widely varying sensitivity has been reported in the literature (10,5).

The α-lactalbumin is a 14.2 kDa calcium binding protein, which plays an important role in the biosynthesis of lactose through the interaction with lactose synthase (11). It is expressed exclusively during lactation in the mammary gland and accounts for 20% of bovine whey proteins (12). Different studies have been reported concerning the effect of treatments on the allergenicity of cow’s milk proteins, indicating either a decrease or an increase in the sensitivity of patients (13,14,15,16,4). However, studies about the modification of allergenicity of α-lactalbumin were limited. From the above, the purpose of this research is to determine the effect of thermal treatment and enzymatic hydrolysis on the antigenicity as well as the allergenicity of α-lactalbumin in a population from Fez-Meknes region of Morocco, using ELISA and Dot-blot assay.

Materials and methods

Collect of patient’s sera

A transversal study was conducted in public and private laboratories of Fez-Meknes Hospitals, in order to collect information...
about milk sensitivity as well as blood serum samples. Before any serum sample taking, a questionnaire was carefully completed with each patient, and a formal consent of each patient or of the children’s parents was signed. The questionnaire contains data relating to age, sex and if there were any possible reaction to milk. Then, the collected sera were centrifuged at 3000 rpm/5 min and stored at -20 °C until use. The patients had not been sensitized beforehand with regards to milk proteins. They were patients who came for different medical tests, and they accepted to participate in the study benevolently. This study was approved by the ethic committee of the University Hospital Center of Fez.

**Extraction of α-lactalbumin**

The extraction of α-lactalbumin was realized according to Wal et al. (1995) (5) with some adjustments. In fact, a volume of 100 ml of raw cow’s milk was skimmed, its pH was adjusted to 4.6 by HCl (3 mol/l) and centrifuged at 5000 rpm/20 min. The whey proteins fraction was extracted in the supernatant, and dialyzed against bi-distilled water. The dialyzed extract was separated using gel filtration (G-100 Sephadex) column and the absorbance of fractions (50 fractions; 2 ml per tube) was determined by an UV-Visible Spectrometer at 280 nm. The fraction presenting α-LA was then concentrated in a 10% polyethylene glycol solution (PEG). The quality of protein extracted was characterized by sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

**SDS-PAGE of α-lactalbumin**

SDS-PAGE was performed under denaturation conditions in 20% polyacrylamide gel. A volume of 100 µl of the purified α-LA was mixed with loading buffer (10% SDS, 10% glycerol, 10% β-mercaptoethanol, and 2.5% bromphenol blue) and heated at 100 °C for 5 min. Then, the gel was fixed and stained using Coomassie Brilliant Blue R-250 (0.1%).

**Dot-blot assay**

Dot-blot assay was realized as described before (3). Briefly, 5 µl of purified α-LA was spotted on nitrocellulose membranes and incubated at 37 °C for 2 hours. Then, the dried spotted membranes were saturated by borate buffered saline (BBS) containing 2.5% Tween-20 for 1 h at 37 °C, in order to block the non-specific binding sites. Afterward, the membranes were incubated with human sera overnight at 4 °C, and later with anti-IgE peroxidase conjugate for 1 h at 37 °C. Finally, the reaction was revealed by the incubation of membranes in a solution containing 0.05% of diaminobenzidine (DAB) in BBS tampon. As indication, after each incubation step, the membranes were washed 3 times by BBS containing 0.1% Tween-20.

**Heat-treatment and pepsin hydrolysis**

The treatment of α-LA was performed on three sets of experiments; the first one was heat-treatment conducted in a thermostatic water bath (70, 80, and 90 °C) for 30, 60, and 120 min, the second one was pepsin hydrolysis (hog stomach, 3354 U/mg) at a concentration of 50 µg/ml in an acidic medium (pH = 2) during 30, 60 and 120 min at 37 °C, and the third one was the combination of the two treatments, heat-treatment followed by enzymatic digestion.

**Production of polyclonal antibodies anti α-lactalbumin**

Anti α-LA antibodies were prepared by immunizing rabbits against the native protein (α-LA) using Freund adjuvant. After five weeks, animals were sacrificed according to National Ethical Laws and blood samples were collected in dry tubes. After centrifugation for 15 minutes at 3000 rpm at 4 °C, serum was separated and frozen at -20 °C until use.

**Specific IgE determination**

In order to determine levels of specific IgE to milk and α-LA, indirect ELISA was used as described beforehand (17,18,4). Firstly, 100 µl of skimmed raw milk or α-LA (0.5 mg/ml) in PBS (Phosphate Buffered Saline, pH 7.4) was deposited on the wells of micro-titration plate (100 µl/well). Next, wells were saturated by BBS (borate buffered saline, pH 8.4) containing 2.5% Tween 20, and 100 µl of the human serum added. The revelation was made by adding the anti-human IgE conjugated to peroxidase, followed by addition of the ortho-phenylenediamine (OPD 0.05%) substrate. After incubation at 37 °C during 20 min, the reaction was stopped by adding 50 µl of HCl (3 mol/l) and the absorbance was measured at 490 nm by an ELISA reader.

**Statistical analysis**

Descriptive statistics were presented as numbers with percentages or as average values. Statistical analysis was based on the student’s t-test taking p < 0.05 as the limit of significant value. All statistical analyses were performed using Excel software.

**Results**

The questionnaire was fulfilled in by 832 subjects, represented by 54.5% of men and 45.4% of women. The age of the studied population ranged between 2 and 60 years old, among whom 18.8% were children (2-20 years) and 80.2% were adults (20-60 years).

Adverse reaction to milk was reported by 3.6% of the studied population, where children (2-10 years) and adults (20-40
Reduction of the allergenicity of α-lactalbumin under heat-treatment and enzymatic hydrolysis

The results of extracted α-LA native and treated by different treatments were presented in figure 1. The band of α-LA corresponded to a molecular weight of 14 kDa. The treatment of this protein by heat at 90 °C for 1 hour showed a very slight reduction in its band, but when it underwent pepsin hydrolysis for 1 hour with or without previous heating, the band totally disappeared.

Effect of heat-treatment and pepsin hydrolysis on the detection of α-LA by rabbit IgG by means of ELISA and Dot-blot assay

In order to determine the parameters of reduction of the immunoreactivity of α-LA to specific antibodies, we firstly studied its recognition by rabbit IgG anti-α-LA under heat-treatment, pepsin hydrolysis and under their combination. Under treatment by temperature (figure 2), the detection of α-LA was reduced after heating within 30 min at different temperatures, and was slightly modified for more heating time. Temperatures of 80 °C and 90 °C highly changed the liaison to IgG, more than 70 °C. Maximal reduction of IgG binding to α-LA were: 74% at 70 °C, 94% at 80 °C, and 97% at 90 °C. Concerning the hydrolysis by pepsin, we noticed that the detection of α-LA decreased progressively, until it reached a rate of 62% of decrease after 120 min of hydrolysis. While, when the two treatments were used, the detection of this protein by IgG was highly

Figure 1 - Electrophoresis profile of α-lactalbumin. 1, native α-LA; 2, α-LA processed by heat-treatment; 3, α-LA hydrolyzed by pepsin; 4, α-LA treated by heat followed by pepsin hydrolysis. M, Molecular weight marker.

Figure 2 - Effect of heat-treatment on α-LA binding to rabbit IgG.
attenuated within 60 min of treatment, reaching a maximum of 91% of reduction (figure 3).
Similar results were observed using the Dot-blot assay. The presence of the blotting spot indicated that native α-LA reacted to IgG. However, when α-LA underwent different treatments for 60 min each, its recognition by IgG was modified. This modification was slight under heat-treatment for 60 min, while it was more important under pepsin hydrolysis as well as under the combination of treatments (figure 4).

Figure 3 - Effect of heat-treatment and pepsin hydrolysis on the recognition of α-LA by rabbit IgG.

Figure 4 - Dot-blot assay of α-LA with rabbit IgG anti native α-lactalbumin. 1, Dot-blot of native α-LA; 2, Dot-blot of α-LA processed by heat-treatment; 3, Dot-blot of α-LA treated by pepsin; 4, Dot-blot of α-LA treated by heat followed by pepsin hydrolysis.

Figure 5 - Effect of heat-treatment and pepsin hydrolysis on the recognition of α-LA by human IgE.
Reduction of the allergenicity of α-lactalbumin under heat-treatment and enzymatic hydrolysis

Effect of heat-treatment and pepsin hydrolysis on the detection of α-LA by human IgE by means of ELISA and Dot-blot assay

Sera of 17 patients presenting high levels of IgE to α-LA (≥ 90 IU/ml) were used to study the effect of treatments on the recognition of α-LA by human IgE basing on ELISA. The results showed that under heat-treatment at 90 °C during 60 min, all patients showed an important decrease in the recognition of heated α-LA. This reduction was more than 50% for 11 patients, with a maximum of 88% and an average of reduction of 59%.

Under pepsin hydrolysis, the results showed that all patients presented high reduction in the recognition of treated α-LA. This reduction was varying from 27% to 96%, and it was more than 40% for 82% of studied patients (n = 14 from 17). The average of this reduction was 74%.

In the same manner, high decrease reactivity to treated α-LA by a combination of heat-treatment and pepsin hydrolysis was observed for all studied patients, with an average of 89% and a maximum of 97%. Also, 82% of patients presented a significant decrease in the recognition of α-LA when the treatments were combined more than each one solely (figure 4).

Basing on Dot-blot assay, sera of 14 patients with high specific IgE levels to α-LA (≥ 65 IU/ml) were tested. The presence of spots indicated that all these patients reacted to native α-LA. Thus, their sera were used to study their reactivity to treated α-LA. The results showed that the intensity of the spots highly decreased when the protein was processed by heat-treatment for almost all patients, except two of them (14 and 17) still reacting to heated α-LA. As regards the pepsin hydrolysis and the combination of the two treatments, we remarked an absence of spots under the two treatments, indicating that the allergenicity of α-LA was strongly attenuated for all studied subjects (figure 6).

Discussion

This research aimed to determine the effect of thermal treatment and enzymatic hydrolysis on the allergenicity of α-lactalbumin in a population from Fez-Meknes region of Morocco using ELISA and Dot-blot assay.

Our results showed that 3.6% of our population reported sensitivity to milk. We remarked that milk allergy was mostly reported in our studied population by children of less than 10 years and by adults of 20-40 years old. This result is in accordance with previous works concerning food allergy in Morocco, where our team research was involved (2,3,4,19).

Regarding specific IgE levels to cow milk, the results showed a strong sensitivity of the studied population to cow milk, especially in adult population aged 20-40 years. Therefore, we screened 31 sera sample presenting high levels of specific IgE to cow milk α-LA, in order to study the effect of thermal and enzymatic treatments on the allergenicity of α-lactalbumin.

Results showed that treatment by temperature reduced slightly α-LA band profile, while we observed by ELISA a high reduction in the IgE binding to heated α-LA for 70% of tested patients. This reduction presented an average of 59%, and varied from 23% to 88%. This result was confirmed by Dot-blot assay. This finding suggests that our studied population presented mostly conformational epitopes which has been reported to be denaturized under heat-treatment (20,21,22,4). Our results showed that the allergenicity of α-LA was decreased in all patients, and this decrease varied between 11% and 80%, indicating that there is a residual reactivity to heated α-LA persisted in some patients. This result of decreased allergenicity was in line with works of Bloom et al. (2014) (23) and Xu et al. (2015) (24), while the important residual allergenicity found is in ac-

![Figure 6 - Results of Dot-blot assay with sera of 14 patients. A, Dot-blot of native α-LA; B, Dot-blot of α-LA processed by heat-treatment; C, Dot-blot of α-LA treated by pepsin; D, Dot-blot of α-LA treated by heat followed by pepsin hydrolysis.](image-url)
cording with a study of Lee et al. (2014) (25). This difference in results might be explained by the differences in studied subjects, as well as by heterogeneity in the method of study as these previous works based on pooled sera, while our study was done using individual sera and purified α-LA.

The results of pepsin hydrolysis exhibited the disappearance of α-LA band in SDS-PAGE profile, accompanied with an important decrease in rabbit IgE binding as well as in human IgE binding antibodies. This finding showed that α-LA lost its allergenic effect under pepsin hydrolysis, as it was reported in previous works (26,22,4). Regarding the antigenicity of hydrolyzed α-LA, our result was in line with the study of Kim et al. (2007) (27) who found that the antigenicity of α-LA decreased significantly when the concentrate of whey proteins was hydrolyzed by pepsin. Concerning the treatment by heat-treatment followed by pepsin hydrolysis, the binding to IgE antibodies was totally attenuated. This attenuation reached a maximum of 97% of the protein allergenicity. All studied subjects showed a significant decrease in IgE binding to treated α-LA under the combination of treatments which was more predominant than under each treatment solely (heat or hydrolysis). This indicated that the pre-heating may ameliorate the enzymatic action, as was reported previously (28).

These findings indicate that the majority of studied subjects recognize conformational epitopes, as there was an important decrease in the IgE binding after heat-treatment, while some patients showed slight decrease in IgE binding indicating that they recognize mostly linear epitopes. Furthermore, the pepsin hydrolysis alone or preceded by heat-treatment, caused an important decrease in the recognition of α-LA for all studied subjects. However, in previous works of our laboratory, the pepsin hydrolysis preceded by heat showed an apparition of new epitopes (18,2,3,22,4).

Conclusion

In conclusion, our study focused on the effect of heat-treatment and pepsin hydrolysis on the allergenicity of cow milk α-LA as one of allergens incriminated in milk allergy. The results showed that milk allergy could be related to α-LA sensitivity. We observed that there was a significant decrease in the α-LA allergenicity after heating and with hydrolyzed α-LA in all studied subjects. This indicated the implication of conformational epitopes in this allergenicity. Furthermore, the residual reactivity of IgE to heated α-LA, indicated that sequential epitopes were also implicated in the sensitivity of this population, but at less level.

Acknowledgements

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Conflict of interest

The authors declare that they have no conflict of interest.

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References

13. Rytkönen J, Karttunen TJ, Karttunen R, Valkonen KH, Jnenalm MC, Alatossava T, ... and Kokkonen J. Effect of heat denaturation on beta-lactoglobulin-induced gastrointestinal sensitization in rats:
Denatured βLG induces a more intensive local immunologic response than native βLG. Pediatr Allergy Immunol 2002; 13(4):269-77.


1. DENOMINAZIONE DEL MEDICINALE. AYRINAL 20 mg compresse.

2. COMPOSIZIONE QUALITATIVA E QUANTITATIVA. Ogni compresse contiene 20 mg di bilastina. Per l’elenco completo degli eccipienti, vedere paragrafo 6.1.

3. FORMA FARMACEUTICA. Compressa. Compresse bianche, ovali, biconvessle con linea di incisione (lunghezza 10 mm, larghezza 5 mm). La linea di incisione sulle compresse serve solo per agevolare la rottura al fine di ingerire la compresse più facilmente e non per dividerla in dosi uguali.

4. INFORMAZIONI CLINICHE. 4.1 Indicazioni terapeutiche. Trattamento sintomatico della rinocoangiuntivite allergica (stagonale e perenne) e dell’urticaria. AYRINAL è indicato negli adulti e negli adolescenti (12 anni di età ed oltre). 4.2 Posologia e modo di somministrazione. Posologia: Adulti e adolescenti (12 anni di età ed oltre): 20 mg di bilastina (1 compresse) una volta al giorno per alleviare i sintomi della rinocoangiuntivite allergica (SAR e PAR) e dell’urticaria. La compresse deve essere assunta un’ora prima o due ore dopo l’assunzione di cibo o succhi di frutta (vedere paragrafo 4.5).

4.3 Interazioni medicamentos e con alimenti. La bilastina è insoluble in acqua. Si raccomanda di assumere la dose giornaliera in un’unica somministrazione. 4.4 Avvertenze speciali e precauzioni d’impiego. Popolazione pediatrica: La bilastina non è stata studiata in pazienti di età inferiore ai 12 anni. Durata del trattamento: Per la rinite allergica il trattamento deve essere limitato al periodo di esposizione agli allergeni. Per la rinocoangiuntivite stagionale, l’urticaria e l’allergia da stoccaggio, l’intervallo di durata varia da 1 a 2 mesi. 4.5 Interazioni con altri medicinali ed altre forme di interazione. Intossicazione con cibo: la bilastina ha una bassa affinità per gli enzimi degradanti della bilastina nella bocca. L’incidenza di eventi avversi in pazienti affetti da rinocoangiuntivite allergica o da orticaria (stagionale e perenne) e dell’urticaria.

5. EFFETTI AVVERSI. 5.1 Incidenza. Le reazioni rare, molto rare e con frequenza non nota non sono state incluse nella tabella.

5.2 Incidenza in adulti. Le reazioni avverse più comuni sono state segnalate nei pazienti trattati con AYRINAL 20 mg. Le reazioni avverse più comuni segnalate nei pazienti trattati con 20 mg di bilastina nel corso dello studio clinico (N = 1697). Le frequenze sono assegnate come segue: Molto comune (≥1/10); Comune (da ≥1/100 a <1/10); Non comune (da ≥1/1000 a <1/10000); Raro (da >1/10000 a ≤1/10000). Tutte le dosi N=2525

<table>
<thead>
<tr>
<th>Classificazione per Sistemi ed Organi</th>
<th>Bilastina 20 mg</th>
<th>Bilastina Tutte le dosi N=2525</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infezioni e infestazioni</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non comune</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Herpes orale</td>
<td>2 (0,12%)</td>
<td>2 (0,08%)</td>
</tr>
<tr>
<td>Disturbi del metabolismo e della nutrizione</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non comune</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aumento dell’appetito</td>
<td>10 (0,59%)</td>
<td>11 (0,44%)</td>
</tr>
<tr>
<td>Disturbi psichiatrici</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non comune</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ansia</td>
<td>6 (0,35%)</td>
<td>8 (0,32%)</td>
</tr>
<tr>
<td>Insomnia</td>
<td>2 (0,12%)</td>
<td>4 (0,16%)</td>
</tr>
<tr>
<td>Disturbi del sistema nervoso</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Comune</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sonnolessness</td>
<td>52 (3,06%)</td>
<td>82 (3,25%)</td>
</tr>
<tr>
<td>Cefalea</td>
<td>68 (4,01%)</td>
<td>90 (3,56%)</td>
</tr>
<tr>
<td>Non comune</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Capogiri</td>
<td>14 (0,83%)</td>
<td>23 (0,91%)</td>
</tr>
<tr>
<td>Disturbi dell’orecchio e del labirinto</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

6. CONSERVAZIONE. Il farmaco deve essere conservato a temperatura ambiente, il pacchetto protetto dalla luce. Il farmaco è contraddistinto con un’etichetta di avvertimento a rilievo, con le informazioni essenziali per l’uso del farmaco.

7. LEGGE SUZUKI. Il farmaco AYRINAL deve essere utilizzato esclusivamente ai fini di terapia e non deve essere usato da persone non in possesso di un ordine di prescrizione o di un attestato di salute.

8. DEPOSITO. Il farmaco AYRINAL deve essere depositato in un ambiente a temperatura ambiente e protezione dalla luce. Il farmaco AYRINAL deve essere depositato in un ambiente a temperatura ambiente e protezione dalla luce.
Classificazione per Sistemi ed Organi

<table>
<thead>
<tr>
<th>Frequenza</th>
<th>Classificazione</th>
<th>Reazione avversa</th>
<th>Bilastina 20 mg N=1697</th>
<th>Bilastina Tutte le dosi N=2525</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non comune</td>
<td>Tinito</td>
<td>2 (0,12%)</td>
<td>2 (0,08%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Vertigini</td>
<td>3 (0,18%)</td>
<td>3 (0,12%)</td>
<td></td>
</tr>
</tbody>
</table>

Patologie cardiche

| Non comune | Blocco di branca destra | 4 (0,24%) | 5 (0,20%) |
| Holter | Artrtitia sinussale | 5 (0,30%) | 5 (0,20%) |
|          | Prolungamento del tratto QT all'elettrocardiogramma | 9 (0,53%) | 10 (0,40%) |
|          | Altre anomalie all'EKG | 7 (0,41%) | 11 (0,44%) |

Patologie respiratorie, toraciche e mediastiche

| Non Comune | Disprea | 2 (0,12%) | 2 (0,08%) |
|            | Fascito nasale | 2 (0,12%) | 2 (0,08%) |
|            | Secchezza del naso | 3 (0,18%) | 6 (0,24%) |

Patologie gastrointestinali

| Non comune | Dolore all'addome superiore | 11 (0,65%) | 14 (0,55%) |
|            | Dolore addominale | 5 (0,30%) | 5 (0,20%) |
|            | Nausea | 7 (0,41%) | 10 (0,40%) |
|            | Fasticto gastrico | 3 (0,18%) | 4 (0,16%) |
|            | Diarrea | 4 (0,24%) | 6 (0,24%) |
|            | Bocca secca | 2 (0,12%) | 6 (0,24%) |
|            | Dispepsia | 2 (0,12%) | 4 (0,16%) |
|            | Gasbrite | 4 (0,24%) | 4 (0,16%) |

Patologie della cute e del tessuto sottocutaneo

| Non comune | Prunito | 2 (0,12%) | 4 (0,16%) |

Disturbi generali e condizioni relative alla sede di somministrazione

| Non comune | Afficalcamento | 14 (0,83%) | 19 (0,75%) |
|            | Selle | 3 (0,18%) | 4 (0,16%) |
|            | Miglioramento della condizione pre-esistente | 2 (0,12%) | 2 (0,08%) |
|            | Pissresa | 2 (0,12%) | 3 (0,12%) |
|            | Astenia | 3 (0,18%) | 4 (0,16%) |

Esami diagnostici

| Non comune | Aumento della gamma-glutamiltransferasi | 7 (0,41%) | 8 (0,32%) |
|            | Aumento dell'alaina amino trasferasi | 5 (0,30%) | 5 (0,20%) |
|            | Aumento dell'aspartato amino trasferasi | 3 (0,18%) | 3 (0,12%) |
|            | Aumento della creatinina nel sangue | 2 (0,12%) | 2 (0,08%) |
|            | Aumento dei trigliceridi nel sangue | 2 (0,12%) | 2 (0,08%) |
|            | Aumento del peso corporeo | 8 (0,47%) | 12 (0,48%) |

Frequenza non nota (non può essere definita sulla base dei dati disponibili): palpitazioni, tachicardia e reazioni di ipersensibilità (quali anafilassi, angioedema, dispnea, eruzione cutanea, edema localizzato/gonfiore locale ed eritema) sono state osservate nel periodo post-marketing. Descrizione di alcune reazioni avverse: Le reazioni avverse segnalate con maggior frequenza sono state due comuni (sonnolenza e cefalea) e due non comuni (capogiri e affaticamento). Le loro frequenze in pazienti trattati con bilastina rispetto ai pazienti trattati con placebo sono state 3.06% vs. 2.86% per la sonnolenza; 4.01% vs. 3.38% per la cefalea; 0.83% vs. 0.59% per i capogiri; 0.83% vs. 1.32% per l'affaticamento. Quasi tutte le reazioni avverse, incluse nella tabella sopra riportata, sono state osservate con un’incidenza simile in pazienti trattati con 20 mg di bilastina che in quelli trattati con placebo. Le informazioni raccolte nel corso della vigilanza post-marketing hanno confermato il profilo di sicurezza osservato durante lo studio clinico. Popolazione pediatrica: La frequenza, la tipologia e la severità delle reazioni avverse negli adolescenti (di età compresa tra 12 e 17 anni) durante lo studio clinico, sono state le stesse osservate negli adulti. Le informazioni raccolte in questa popolazione (adolescenti) durante la vigilanza post-marketing hanno confermato i risultati degli studi clinici. Segnalazione delle reazioni avverse sopresse: La segnalazione delle reazioni avverse sopresse che si verificano dopo l’autorizzazione del farmaco è importante, in quanto permette un monitoraggio continuo del rapporto beneficio/rischio del medicinale. Agli operatori sanitari è richiesto di segnalare qualsiasi reazione avversa soppressa tramite il sistema nazionale di segnalazione all’indirizzo: http://www.agenziafarmaco.gov.it/contenuti/come-segnala-una-sospetta-reazione-avversa.

4.9 Sovradosaggio. Le informazioni riportate nel sovradosaggio acuto di bilastina derivano dalle esperienze raccolte in trial clinici con adolescenti con rinocongiuntivite allergica (stagionale e perenne). L’efficacia e la sicurezza di questo dosaggio hanno confermato i risultati degli studi clinici. L’agenzia Europea dei medicinali ha rinviamo l’obbligo di presente i risultati degli studi con AYRINAL in uno o più sottogruppi della popolazione pediatrica per il trattamento della rinocongiuntivite allergica e per il trattamento dell’orticaria (vedere paragrafo 4.2 per informazioni sull’uso pediatrico). 5.2 Proprietà farmacocinetiche. Assorbimento: La bilastina viene rapidamente assorbita dopo la somministrazione orale raggiungendo la massima concentrazione nel plasma in circa 1,3 ore. Non si è osservato fenomeno di accumulo. La biodisponibilità media della bilastina dopo somministrazione orale è del 61%. Distribuzione: Studi in vitro e in vivo hanno mostrato che la bilastina è un substrato del Pgp (vedere paragrafo 4.5 "Interazione con chetconazolo", cefalosporine e diiltiazem, ciprofloxacin e paragagrafo 4.5 "Interazione con succo di pompelmo"). La bilastina non risulta essere un substrato del trasportatore BCRP o dei trasportatori renali OCT2, OAT1.
In base agli studi in vitro, non si prevede che la bilastina inibisca i seguenti trasportatori nella circolazione sistemica: P-gp, MRP2, BCRP, BSEP, OATP1B1, OATP1B3, OATP2B1, OAT1, OAT3, OCT1, OCT2 e NTCP, poiché solo una modesta inibizione è stata rilevata per P-gp, OATP2B1 e OCT1, con una IC₅₀ stimata ≥ 300 μM, molto più elevata rispetto alla Cₚₚₚₚ plasmatica clinica calcolata e per ciò queste interazioni non saranno clinicamente rilevanti. Tuttavia, sulla base di questi risultati, l’azione inibitoria della bilastina sui trasportatori presenti nella mucosa intestinale, per esempio P-gp, non può essere esclusa. Alle dosi terapeutiche la bilastina è legata per l’84-90% alle proteine del plasma. Biotrasformazione: La bilastina non ha indotto o inibito l’attività degli isoenzimi CYP450 negli studi in vitro. In uno studio di bilanciamento di massa condotto su volontari sani, dopo la somministrazione di una singola dose di 20 mg di ¹⁴C-bilastina, quasi il 95% della dose somministrata è stata recuperata nelle urine (28,3%) e nelle fes (66,5%) come bilastina immodificata, confermando quindi che la bilastina non è significativamente metabolizzata nell’uomo. L’emivita di media di eliminazione calcolata in volontari sani è stata di 14,5 h. Linearietà: La bilastina presenta una farmacocinetica lineare nell’intervallo di dosi studiato (da 5 a 220 mg), con bassa variabilità interindividuale. Compromissione renale: In uno studio in soggetti con compromissione renale, la media (DS) dell’AUC₀₋∞ è aumentata da 737,4 (±260,8) ng·h/ml nei soggetti senza compromissione renale a 967,4 (±140,2) ng·h/ml nei soggetti con compromissione lieve (GFR: 50-80 ml/min/1,73 m²), 1384,2 (±263,23) ng·h/ml nei soggetti con compromissione moderata (GFR: 30-50 ml/min/1,73 m²), e 1708,5 (±699,0) ng·h/ml nei soggetti con compromissione grave (GFR: < 30 ml/min/1,73 m²). L’emivita media (DS) della bilastina era 9,3 h (± 2,8) nei soggetti senza compromissione, 15,1 h (± 7,7) nei soggetti con compromissione lieve, 10,5 h (± 2,3) nei soggetti con compromissione moderata e 18,4 h (± 11,4) nei soggetti con compromissione grave. L’esecuzione unaria della bilastina era essenzialmente completa dopo 48-72 h in tutti i soggetti. Questi cambiamenti farmacocinetici non si prevede presentino un’influenza clinicamente rilevante sulla sicurezza della bilastina, dato che i livelli di bilastina nel plasma nei pazienti con compromissione renale rientrano ancora nell’intervallo di sicurezza della bilastina. Compromissione epatica: Non esistono dati sulla farmacocinetica per i soggetti con compromissione epatica. La bilastina non viene metabolizzata negli umani. Dato che i risultati dello studio sulla compromissione renale indicano che l’eliminazione renale è il maggior contributore dell’eliminazione, si prevede che l’esecuzione biliare sia coinvolta solo marginalmente nell’eliminazione di bilastina. Non si prevede che le alterazioni nella funzione epatica abbiano un’influenza clinicamente rilevante sulla farmacocinetica di bilastina. Anziani. Sono disponibili solo un quantitativo limitato di dati di studi farmacocinetici in soggetti oltre i 65 anni di età. Non sono state osservate differenze statisticamente significative nella farmacocinetica della bilastina negli anziani oltre i 65 anni di età rispetto alla popolazione di adulti di età compresa tra 18 e 35 anni. Popolazione pediatrica: Non sono disponibili dati di farmacocinetica negli adolescenti (di età compresa tra 12 e 17 anni) in quanto, per questo prodotto, l’estrapolazione dei dati nell’adulto sono ritenuti appropriati. 5.3 Dati preclinici di sicurezza. I dati non-clinici sulla bilastina non evidenziano rischi particolari per l’uomo sulla base di studi convenzionali di sicurezza farmacologica, tossicità a dosi ripetute, genotossicità e potenziale carcinogeno. Negli studi di tossicità ripetuta, gli effetti della bilastina sul feto (perdita pre e post-impianto nei ratti ed ossificazione incompleta delle ossa craniali, dello sterno e degli arti nei conigli) sono stati osservati solo a dosi tossiche per la bilastina. L’effetto teratogenico della bilastina non è stato analizzato in studi di tossicità riproduttiva. Non si prevede che l’uso di bilastina provochi ai danni fetali eventuali interazioni farmacologiche, tossicità a dosi ripetute, genotossicità o potenziale cancerogeno. Alcune interazioni farmacologiche, tossicità a dosi ripetute, genotossicità, potenziale cancerogeno e potenziale genotossicità sono state effettuate in studi in vivo e in vitro. Nei ratti, la bilastina non ha indotto o inibito l’attività degli isoenzimi CYP450 negli studi. Tuttavia, sulla base di questi risultati, l’azione inibitoria della bilastina sui trasportatori presenti nella mucosa intestinale, per esempio P-gp, non può essere esclusa. 6.1 Elenco degli eccipienti. Cellulosa microcristallina, Sudic Amido glicolato (tipo A) (derivato dalle patate), Sillie colloidale anidra, Magnesio stearato. 6.2 Incompatibilità. Non pertinenti. 6.3 Periodo di validità. 5 anni. 6.4 Precauzioni particolari per la conservazione. Questo medicinale non richiede alcuna condizione particolare di conservazione. 6.5 Natura e contenuto del contenitore. Il medicinale è confezionato in un blister, che consiste di due parti: laminato, composto da poliamide orientata (lato esterno del laminato), alluminio e PVC (lato interno del laminato); Foglio in alluminio: Il foglio in alluminio è termosaldato con una lacca termosaldante (copolimero PVC-PVAC e resine di butilmetacrilato) al laminato dopo la formatura e il riempimento. La vendita: Confezioni da 10, 20, 30, 40 o 50 compresse. - A.I.C 040854034, 40 compresse - A.I.C 040854046, 50 compresse - A.I.C 040854059
An unusual case of wheat dependent exercise induced anaphylaxis (WDEIA) triggered by Tri a 14 in a pediatric patient: a case report

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Summary
Wheat dependent-exercise-induced anaphylaxis (WDEIA) is a food allergy characterized by anaphylaxis elicited when wheat ingestion is time-related with physical exercise. We report a case of WDEIA in an asthmatic boy admitted to our Unit with suspected mushroom acute toxicity. The symptoms occurred during a gym session, approximately 2 hours after the ingestion of a meal based on pasta and cooked mushroom found in the family’s garden. Acute toxicity due to mushroom ingestion was then excluded. Triptase serum levels resulted elevated in acute phase and normal after 24 hours. Food specific IgE showed a sensitization to lipid transfer protein Pru p 3 and to Tri a 14. This case highlights that WDEIA is underdiagnosed, especially when patients are firstly visited in Emergency Unit. Moreover, Tri a 14 is seldom described as responsible for WDEIA, compared to omega 5 gliadin.

Introduction
In 1979 Maulitz first reported food-dependent exercise-induced anaphylaxis (FDEIA) in an adult long-distance runner after a shellfish-based meal (1). Bread wheat (Triticum aestivum) is the most widely grown crop worldwide. Moreover, wheat is highly consumed and often used in food preparations (2). In genetically predisposed individuals, wheat can cause specific immune responses, both immediate (IgE-mediated, such as anaphylaxis, FDEIA, rhinitis and asthma, urticaria) and delayed (non-IgE-mediated, such as celiac disease and dermatitis herpetiformis) (2,3).

FDEIA is a special kind of food allergy. In patients with FDEIA, allergic symptoms are elicited when the ingestion of a specific food is time-related with a triggering factor, such as physical exercise (3).

We report a case of WDEIA in a young boy, which was uncommon for age presentation, for the confounding anamnestic data and hospital admission clinical suspicion.

Case report
M.B. is a 13-year-old boy affected by allergic asthma from the age of 7-years-old (sensitization to dust mite and grass pollen) on steroid inhalatory preventive treatment. Skin prick tests and specific serum IgE were positive to grass and birch pollens, alternaria, dust mite. He had no previous history of food allergy of anaphylaxis. M.B. was referred to our Hospital Emergency Department by the Territorial Emergency Unit for vomiting, flushing and breathing impairment. All the symptoms occurred approximately 2 hours after the ingestion of a meal based on pasta with tomato sauce, chard and cooked mushroom found in
the family's garden. No assumption of any other food/juice, alcohol, drugs nor medications (NSAID) was reported, no insect bite was reported.

M.B. initially showed symptoms while he was exercising in gym, running on a tapis-roulant. Emergency Territorial Unit was alerted, suspecting a toxic reaction to mushroom. Inhalatory salbutamol and endovenous administration of saline solution were given on Ambulance.

M.B. was admitted to Hospital in fair general condition, showing a state of anxiety; oxygen peripheral saturation, blood pressure and heart were all within normal range. He had generalized urticaria and initial lips swelling, with no respiratory distress. Endovenous methylprednisolone and oral cetirizine were administered, with progressive resolution of cutaneous manifestations.

Myelogram analysis showed that the mushroom eaten was *Pleurotus ostreatus*, a well-known edible species. Acute toxicity due to mushroom ingestion was then excluded.

Triptase serum levels and specific food IgE test were performed for egg (0.09 kUA/l), peanut (6.99 kUA/l), nut (7.92 kUA/l), milk (0.10 kUA/l), shrimp (1.54 kUA/l), cod (0.12 kUA/l), wheat (4.32 kUA/l), gluten (0.20 kUA/l), omega-5-gliadin (0.07 kUA/l), LTP Tri a 14 (6.68 kUA/l), LTP Pru p 3 (25.1 kUA/l), Bet v2 profilin (0.10 kUA/l), Bet v1 PR10 (0.07 kUA/l), tomato (0.1 kUA/l), and were determined by ImmunoCAP (Thermo Fisher Scientific, Sweden). The involvement of three different apparatus made the diagnosis of anaphylactic reaction more likely. Triptase level was elevated in acute-phase (19 ug/L, normal values < 11 ug/L), whereas the levels were normal 24 hours after the allergic event. These data confirm the diagnosis of anaphylaxis. Food specific IgE, performed during the clinical observation, showed a sensitization to lipid transfer protein Pru p 3 (25 Ku/l) and to Tri a 14 (6.68 Ku/L), which is a wheat lipid transfer protein. The dosage of Tri a 19 Omega 5 gliadin was negative. Prick-by-prick tests with cooked chard, commeal mush, nut and hazelnut were performed after 3 weeks, all with positive results, whereas prick-by-prick with tomato were negative. All these foods contain LTP. Prick-by-prick for peach–tea, wheat, peach and apricot jam were negative: they were tested to evaluate the sensitization toward widely consumed food in infancy and to reduce the risk of a relapse. Oral challenge was proposed, but parents did not give their consent. During allergologic follow up, M.B. reported eating wheat daily with no symptoms, but never in relation with physical exercise, as he was instructed not to. Self-injectable adrenaline was prescribed. He was not instructed to avoid chard, that was assumed even before physical exercise, without allergic reactions. Instead, recently he developed generalized urticaria after the assumption of a piece of flat bread 2 hours before a gym session.

Based on patient's clinical history and allergy test results, we made a diagnosis of WDEIA.

FDEIA is a rare yet severe form of IgE-mediated allergy where ingestion of a putative food associated with physical exercise within 4 hours triggers anaphylaxis (2,4). In FDEIA, both food allergen ingestion and physical exercise are independently tolerated, but their association can elicit anaphylactic reaction (5). Various types of food can be responsible of FDEIA. The list of possible triggering food is constantly under revision and influenced by geographical location and local diet (6).

WDEIA is elicited by wheat proteins (7). Palosuo et al. identified a gamma-like gliadin, later classified as omega-5-gliadin (Tri a 19), as the main allergen involved in WDEIA (8). Patients with WDEIA negative for Tri a 19 are described in literature to have sensitization to high-molecular-weight glutenin, alpha-gliadin, beta gliadin or gamma gliadin (8).

**Discussion**

In our case report, no sensitization against these common wheat antigens was found. Surprisingly, analysis performed in our patient showed a sensitization to a lipid transfer protein (Tri a 14). Tri a 14 is an allergen often involved in Baker’s asthma (9), but few cases of Tri a 14 involvement in WDEIA are described. To our knowledge, only one pediatric case was identified in Europe so far (10). In this study, LTP resulted to be a major allergen only in Italian patients. The role of important cereal allergens, such as lipid transfer proteins, is still unknown in wheat (10).

The pathogenesis of WDEIA is still partly unknown. Physical exercise can cause a re-distribution of blood flow or can interact with mastocyte function, leading to a higher bowel permeability to a specific allergen (2,4). According to a recently published review on WDEIA, wheat/exercise challenge is not a necessary diagnostic test if clinical and laboratory data are suggestive of WDEIA (11). To our knowledge, up to now a universally approved challenge-performance protocol has not been validated yet.

Our case report shows how clinical presentation of anaphylaxis can be misleading, especially when multiple confounding factors are present. In case of anaphylactic reaction in allergic patients with a positive personal history for inhalatory sensitization (asthma), food sensitization might be suspected. In acute phase, serum triptase levels must be assessed, as this test and its variation after 12 to 24 hours are of crucial importance in the diagnostic pathway.

WDEIA is underdiagnosed, especially when patients are firstly visited in Emergency Unit, considering that a patient might have tolerated wheat until its association with exercise.

In our patient, both Tri a 14 and Pru p 3 could be responsible for the anaphylactic event described, as they have a 45% homology, but Tri a 14 seems to be most likely involved, considering that the only food containing LTP (except wheat) assumed by our patient was tomato, and specific IgE for tomato resulted negative. What is more, Pru p 3 reactions are almost always im-
mediate, whereas our patient had a delayed reaction. Moreover, wheat is more often involved in exercised induced anaphylaxis. Tri a 14 is seldom described as responsible for WDEIA, compared to omega 5 gliadin. The assumption of chard before physical exercise did not induce symptoms, whereas the assumption of flat-bread in relation with a gym session caused a generalized urticaria.

Further investigation should be advisable, in order to identify the real importance and incidence of Tri a 14 in WDEIA in pediatric patients, especially in Southern Europe.

Patient consent

Obtained.

Conflict of interest

The authors declare that they have no conflict of interest.

References

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Galactose-α-1,3-galactose allergy: a rare syndrome and an atypical presentation

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Summary
Allergies to red meat associated with galactose-α-1,3-galactose, commonly known as α-gal, are rare and have only recently been described. At this time, the literature reports only one case documented in Portugal. In this study, we report the case of a 76-year-old male with an immediate reaction following the ingestion of red meat. Rigorous diagnostic exams, including prick test, prick-to-prick tests, serum specific IgE and SDS-PAGE IgE-immunoblotting, were performed. The α-gal epitope IgE returned a value of 35.3 kUA/L, leading the authors to believe that this is an atypical case of α-gal allergy.

Key words
allergy; anaphylaxis; galactose-α-1,3-galactose; red meat; SDS-PAGE IgE-immunoblotting

Introduction
Recent studies have shown that mammalian meat allergy is an emergent allergy worldwide. Many cases of delayed anaphylaxis to red meat have been described, especially in the United States (1). The basis for these reactions appears to be the presence of specific immunoglobulin E (IgE) antibodies against the oligosaccharide α-gal, a carbohydrate in non-primate mammals that does not exist in humans. Exposure to this oligosaccharide occurs with the ingestion of meat, offal and gelatin with an origin in beef, pork, lamb, horse and deer. This epitope may also be present in drugs, vaccines, and dairy products. The typical presentation of α-gal allergy involves a delayed onset reaction, i.e., occurring 4 to 8 hours after the consumption of mammalian meat products (2). The symptoms usually include urticaria and angioedema, and can progress to potentially fatal anaphylaxis. The α-gal hypersensitivity has been reported in association between an episode of tick bite and the subsequent development of symptoms in response to the ingestion of red meat. Reactions to cetuximab, a monoclonal antibody against EGFR (epidermal growth factor receptor), occur immediately after the first administration due to the presence of the epitope α-gal (3,4).

Case Report
Herein, we report the case of a 76-year-old Caucasian male who experienced an episode of anaphylaxis (sickness, diarrhea, vomiting and cutaneous lesions scattered over the body) 1 h after a meal consisting only of rice and beef. After self-medication with
oral corticosteroid and antihistamines, the reaction resolved completely within a few hours, and without any residual damage. Since then, the patient has self-excluded this specific type of food from his diet.

Two months later and following a continuous period of abstinence from eating red meat, the patient unintentionally ingested a blend of cooked meats (beef, pork, and sausages) and immediately experienced anaphylactic shock (diarrhea, vomiting, cutaneous lesions, hypotension and syncope). Upon admission to the emergency department, the patient was treated with antihistamines, epinephrine, corticosteroids and intravenous fluid therapy, with a full resolution of his symptoms. The patient was referred to the immunoallergy unit.

The patient presented no family history of atopy or allergic disorders and no personal comorbidities such as asthma or other allergies. Neither of the anaphylactic episodes was preceded by physical exercise or ingestion of any kind of drug, and the patient denied having been stung or bitten by an insect.

In the allergy unit, the patient underwent a diagnostic workup including skin-prick testing with a panel of aeroallergens and food allergens (Dermatophagoides pteronyssinus, cat and dog epithelia extracts, grass, cow’s milk, egg, wheat and fish (Bioportugal®, ALK-Abelló, Madrid, Spain). The tests returned positive results for cow’s milk and for cat and dog epithelia extracts. Prick-to-prick tests were also performed for raw and cooked pork and beef, returning positive results for raw pork and cooked beef.

Laboratory tests revealed an elevated serum total IgE value by returning positive results for raw pork and cooked beef. Serum-specific IgE values were also determined for cat epithelia (4.82 kUA/L), dog epithelia (1.04 kUA/L), cow’s milk (2.72 kUA/L), beef (8.6 kUA/L) and pork (6.67 kUA/L). Due to the IgE value for cow’s milk, together with several non-specific gastrointestinal complaints associated with milk ingestion, we conducted an oral provocation test with milk that was negative (cumulative dose of 200 ml).

With the collaboration of the research department of BIAL Arístegui, SDS-PAGE IgE-immunoblotting with pork, beef, bovine serum albumin (BSA), cat and dog epithelia was performed.

As presented in figure 1, beef and pork IgE binding were detected in the bands of the same molecular mass: batch of molecular mass > 45 kDa. Regarding extracts from cat and dog epithelia, IgE binding is also observed in bands of molecular mass > 45 kDa; in the dog epithelia extract, IgE binding is observed in bands of approximately 67 kDa and 45 kDa. Bovine serum albumin (BSA) also had the same molecular mass for the bands of approximately 67 and 45 kDa (figure 1). IgE binding bands of the same molecular mass as mammalian serum albumin (BSA and cat albumin) have been detected (approximately 67 e 45 kDa). An important differential diagnosis with α-gal allergy is the pork syndrome, since they have several characteristics in common. Both involve IgE-mediated reactions triggered by the ingestion of mammalian meat. Both syndromes may show similar results in skin-prick tests and immunoassay by cross-reactivity. Patients with allergy to red meat from hypersensitivity to α-gal have high specific IgE levels for beef, pork, lamb, cat epithelia, dog epithelia and cow’s milk. These patients have high specific IgE to cat epithelia, due to the α-gal residues present in cats’ IgE and not due to a positivity of Fel d 1, the cat’s main allergen (5).

Specific IgE against cat serum albumin was determined with values of < 0.35 kUA/L. Finally, an α-gal epitope IgE was performed, returning a value of 35.3 kUA/L. The levels of α-gal were calculated by the ImmunoCAP method (Thermo Fisher, Vitoria, Basque Country).

The study was conducted in accordance with the ethical standards established in the Declaration of Helsinki of 1946, and that informed consent was obtained from the patient before enrolment in the study.

Conclusions

The major allergens involved in allergic reactions to mammalian red meat are serum albumin and immunoglobulins. In these patients, it may be difficult to identify a cause for the reactions, especially if there is no history of tick bite or exposure to cetuximab. Therefore, skin tests have limited value for diagnosis, making IgE for specific α-gal essential for diagnosis. We report an unusual case of immediate reaction to meat, with the detection of high serum specific IgE values to α-gal, in a patient with no history of tick bite or exposure to intravenous cetuximab. The patient has now been avoiding red meat for two years and has had no further reactions.

Figure 1 - SDS-PAGE IgE-immunoblotting. A, milk extract; B, beef (raw); C, beef extract (raw); D, pork (raw); E, pork extract (raw); F, BSA (bovine serum albumin); G, cat epithelium extract; H, dog epithelium extract; I, dog dander extract; J, cat serum albumin; P, patient serum; G, serum from negative control group; M, standard molecular masses.

![Figure 1 - SDS-PAGE IgE-immunoblotting](image-url)
Conflict of interest

The authors declare that they have no conflict of interest.

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