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Biomarkers of treatment efficacy in patients with chronic spontaneous urticaria

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Summary

Background. *Currently there are no biomarkers useful to predict the future evolution and the therapeutic response in patients with chronic spontaneous urticaria (CSU).* ***Objective.*** *To review the available information on biomarkers that might be applied for the follow up of the response to guideline recommended therapies for CSU.* ***Methods.*** *A review of the medical literature on CSU potential clinical and laboratory biomarkers in PubMed and MEDLINE including the terms urticaria, chronic urticaria, chronic idiopathic urticaria, chronic spontaneous urticaria, antihistamines (AHs), omalizumab (OMA), cyclosporine (CyA), and treatment.* ***Results.*** *Clinical manifestations that were associated to poor responses to AHs were atopy, asthma, rhinitis / rhinosinusitis, thyroid disease, hypertension, higher disease activity and duration. Laboratory markers of AH resistance that have been reported include Complement C5a fraction, Autologous Serum Skin Test (ASST), Basophil Activation Test (BAT), D-dimer and LCN2 adipokine. Basophil Histamine Release Assay (BHRA), ASST, and basophil CD203c-upregulating activity in the serum correlated with favorable response to OMA, whereas disease duration and severity, BAT, BHRA, and D-dimer levels were associated with better responses to CyA.* ***Conclusion.*** *Some promising biomarkers useful for patient management in CSU, have been identified in the literature. There is, however, an urgent need of new, easy-to-perform markers that can be made widely available for the optimal care of patients suffering CSU.*

Introduction

Chronic spontaneous urticaria (CSU) is a highly prevalent condition affecting 0.3 to 0.6% of the population (1,2). Although currently CSU is regarded as a systemic inflammatory disease, its pathogenesis is not completely understood (3). In consequence, treatment recommendations are in general oriented to the control and prevention of symptoms and exacerbations (4). Current international guidelines recommend a 3-step system, beginning with second generation, non-sedating antihistamines at conventional doses, followed by increased doses up to 4 times if there is no response after 2 weeks. For patients who do not respond to antihistamines, a third line therapy consists of adding omalizumab, cyclosporine or montelukast (4).

When a new patient suffering CSU is diagnosed, it would be important for the treating physician to have available clinical biomarkers, useful to predict the future evolution of the disease and the most likely response to the treatment. A previous article from our institutions presented a summary of the clinical and laboratory markers that have been proposed for the assessment of the severity of CSU (5). The present article reviews the information currently available on biomarkers that could be utilized to identify who are the patients more suitable for a particular form of treatment. We have to mention that in this literature search, we did not find adequate investigations dealing with clinical or complementary markers associated to a favorable or negative response to corticosteroids.

Methods

A review of the medical literature was performed on PubMed and MEDLINE, including the terms urticaria, chronic urticaria, chronic idiopathic urticaria, chronic spontaneous urticaria, antihistamines, omalizumab, cyclosporine, and treatment. All reference types were included in the search.

Results

Therapeutic guidelines for chronic spontaneous urticaria

Current international guidelines for the management of urticaria and angioedema recommend therapy with conventional or increased doses of second generation antihistamines (AHs) for patients with chronic urticaria (CU) (4).

Although the definition of antihistamine-resistant urticaria, as given in the guidelines, is "urticaria which is not controlled after updosing of non-sedating AHs up to 4 times the usual dose" (4), other investigators have used their own definitions. For example, the following have been proposed: "steroid dependent rash poorly responsive to multiple AHs and immunosuppressive agents" (6), "patients not responding to 10 mg cetirizine per day for 1 week" (7), or "those on 5 mg of levocetirizine or 10 mg of cetirizine twice a day for 15 days and a combination of fexofenadine 180 mg and hydroxyzine 25 mg for another 15 days without > 50% reduction of baseline urticaria activity scores" (8).

Between 40 and 65% of patients with CSU do not respond to the treatment with AHs even at doses 4 times higher than the approved ones (9). Patients refractory to increased doses of H1 receptor antagonists require the addition or substitution of alternative agents, being corticosteroids, cyclosporine and omalizumab more efficacious than others, such as H2-receptor antagonists, leukotriene antagonists, hydroxychloroquine, dapsone, colchicine, and sulfasalazine. Long term use of corticosteroids is discouraged due to safety concerns, while they are recommended exclusively for short periods of time during disease exacerbations.

Biomarkers of response to antihistamines

Nonsedating AHs are the first line of therapy for the control of symptoms in patients with CSU (4). As mentioned, for patients who do not respond to approved doses, the recommendation is to administer increased doses of second generation AHs. This is based on controlled studies showing better efficacy (and acceptable tolerance) of higher doses over the usual doses, and also its superiority when compared to the combination of two different AHs (4,10,11). However, updosing antihistamine to obtain a better control of CSU is not always successful (12).

Insufficient response to treatment is a defining feature of severe CSU. In general, there is scarce information on the factors that may predict a favorable therapeutic response. In a recent study, it was reported that antihistamine-resistant CU was associated to other clinical indicators of severity, including atopic asthma, rhinitis and rhinosinusitis, thyroid disease and hypertension (13).

Another investigation showed that antihistamine-resistant CU shows increased complement C5a fraction in the serum, higher disease activity, longer duration of wheals and higher positivity of the autologous serum skin test (ASST) (14). According to Staubach and coworkers, and to Ye and coworkers ASST and Basophil Activation Test (BAT) positivity is associated with a poor response to antihistamine treatment in patients with CU (15,16). However, the usefulness of ASST to predict the response to treatment with AHs and corticosteroids has been challenged by Belot and coworkers, who proposed that ASST results do not have bearing on the treatment and are not associated with greater resistance to antihistamine treatment (17).

In a different approach, Asero has proposed that elevated D-dimer plasma levels should be considered a marker of antihistamine-resistant CU (18). Plasma D-dimer levels were elevated in 0 of 41 patients (0%) showing an "excellent" response to cetirizine, 3 of 14 (21%) patients showing a "good" response, 3 of 5 (60%) patients showing a "partial" response, 18 of 23 (78%) patients showing a "poor" response, and 7 of 8 (88%) non-responders. On comparing patients with a disease of similar severity showing either normal or elevated D-dimer levels, it turned out that the latter were much more frequently cetirizine-resistant.

We found no published evidence that failure to respond to treatment in adult CSU patients is linked to long disease duration. In children with CSU, remission rates of patients who respond to standard dosed antihistamine treatment are reportedly higher than those of patients who require high-dose antihistamine or combination medications, indicating that urticaria controlled by a standard dose of antihistamine may predict a shorter time to remission in the pediatric CSU population. The mean duration of disease at the first visit in the non-remission group was higher than in the remission group, at the end of the study (19).

An imbalance in pro- and anti-inflammatory adipokines in CU patients has been recently observed by Trinh and coworkers. Mean levels of serum Lipocalin-2 (LCN2), TNF-α, IL-6, and IL-10 were significantly higher in CU patients than in controls, whereas adiponectin levels were significantly lower in patients with CU than in controls. While serum IL-6 levels were significantly higher in refractory CU patients, compared to responsive CU individuals, LCN2 showed a direct relationship with the Urticaria Activity Score (UAS). Authors suggested that LCN2 could be a differential marker for disease activity and the clinical responses to antihistamine treatment in CU patients (20).

Table I *- Biomarkers of antihistamine resistance in chronic spontaneous urticaria.*

Clinical	References	Laboratory	References
asthma	13	complement C5a fraction	14
rhinitis / rhinosinusitis	13,21	positivity of the autologous serum skin tests (ASST)	14-16
thyroid disease	13	basophil activation test	15,16
hypertension	13	D-dimer	18
higher disease activity	14	LCN2 adipokine	19
longer duration of wheals	14,15		
atopy	21		

According to one study from Taiwan, the presence of atopy and allergic rhinitis is associated with a poor therapeutic response to second generation antihistamines, since non-responders tended to have atopy, especially allergic rhinitis (21). Potential biomarkers of response to AHs are presented in **Table I**.

Biomarkers of response to omalizumab

The efficacy and safety of recombinant monoclonal anti-human IgE (omalizumab) in the treatment of chronic urticaria was clearly established in various seminal studies (22-24). Recent investigations have tried to identify possible markers of response to omalizumab (**Table II**).
According to Gericke et al., there are significant correlations between a positive Basophil Histamine Release Assay (BHRA) and ASST and the time to symptom relief with omalizumab. The fact that a positive BHRA is predictive of a slow response to omalizumab, suggests that omalizumab works via reducing FcεRI expression in these patients (25).
In a second study that investigated markers of the response to omalizumab, Palacios et al. reported that the lack of basophil CD203c-upregulating activity in the serum of patients with CU correlates with the clinical response to this monoclonal anti-IgE antibody (26).

Table II *- Biomarkers of omalizumab effectiveness in chronic spontaneous urticaria.*

Laboratory	References
basophil histamine release assay (BHRA)	25
ASST	25
lack of basophil CD203c-upregulating activity in the serum	26
D-dimer plasma levels	29

It has been reported that serum IL-31 levels of patients with CSU are significantly higher than in healthy controls (27). A recent investigation reported that omalizumab, but not placebo, significantly reduced IL-31 levels of patients with CSU. Nevertheless, no correlation between IL-31 and urticaria activity score, wheal, or itch scores were present (28). From those results authors concluded that the functional relationship of IL-31 to the pathogenesis and symptom severity of CSU remains to be clarified.
New data from Asero and coworkers has demonstrated that D-dimer plasma levels parallel the clinical response to omalizumab (29).

Biomarkers of response to cyclosporine

The second alternative drug that has been shown to be effective for patients with treatment-resistant CSU is cyclosporine A (30-32).
Although effective, due to its nephrotoxicity it is advised that this medication is managed under the supervision of specialists experienced in its use.
Patients with CSU with positive BAT results respond better to cyclosporine treatment than those with negative BAT results (30). This finding was further supported by Iqbal's paper, who reported that in patients treated with cyclosporine, a positive BHRA indicated a higher probability of response (33). Furthermore,

Table III *- Biomarkers of cyclosporine effectiveness in chronic spontaneous urticaria.*

Clinical	References	Laboratory	References
disease duration	33	BAT	27
initial severity	33	BHRA	31
		D-dimer	33,34

shorter duration of the disease and higher initial severity predict a successful response to treatment with cyclosporine (34).
Baseline D-dimer levels show a highly significant negative correlation with the response to cyclosporine. (35). Other authors have postulated that baseline D-dimer is a good marker of disease activity in most patients with CSU, and may be useful to monitor the clinical response to cyclosporine treatment (36) (**Table III**).

Conclusions

Due to the scarcity of published investigations, few markers are currently available for predicting the response to treatment in patients with CSU. However, some clinical features, such as disease activity and duration as well as laboratory markers that include BAT, BHRA, ASST, and D-dimer, are potentially useful for such purposes. Additional studies that provide easy to perform and widely available markers are needed in order to deliver a better care for CSU patients.

Conflict of interest

M. Sánchez-Borges has received honorary for lecturing from Novartis Pharma AG. F. Caballero-Fonseca has received speaker honorary from Sanofi Aventis. A. Capriles-Hulett and L. González-Aveledo declare that they do not have conflicts of interest.

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Skin prick test analysis reveals cross-sensitization to tomato profilin and grass pollen in nasobronchial-allergic patients with history of tomato food allergy

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Summary
The association between grass pollen sensitization and food allergy to tomato is of great interest. We report here, the first such study in Indian population.
We investigated 246 allergic rhinitis / asthma patients by diagnostic case history and skin prick test (SPT); grass pollen mix, tomato extract and purified tomato profilin were used for SPT. Tomato profilin was purified by affinity chromatography, and analyzed by HPLC (95% purity) and SDS-PAGE (14 kDa).
We observed that 38% of the patients had sensitization to both grass pollen and tomato fruit, of which 92% were sensitized to tomato profilin. Among patients with a history of food allergy to tomato fruit, the association was more pronounced (66%).
Tomato profilin appears to be an important cross-sensitizing panallergen in respiratory allergic patients in the Indian subcontinent.

Abbreviations
OAS, oral allergy syndrome; PBS, phosphate-buffered saline; PLP, poly-L-proline; SPT, skin prick test.

Introduction

Allergic rhinitis is an inflammation of the mucous membranes that occurs when allergens to which the patient is previously sensitized, come in contact with the lining of the nose. It is characterized by sneezing, congestion, itching and runny nose, and itchy, watery eyes. The common allergens causing allergic rhinitis are house dust, pollens from weeds, grasses and trees, fungi and mold spores, animal dander and some foods. Seasonal allergic rhinitis (SAR; hay fever) affects 70 million people around the globe (1,2). It is the most common allergic disease encountered in the clinics and constitutes ~55% of all allergies seen in India (3). People who suffer from perennial rhinitis have symptoms year round, usually triggered by dust mites and cockroach droppings, indoor molds, and/or animal dander (1-3). Asthma is a chronic inflammatory disease of the airways associated with recurrent episodes of bronchoconstriction and increased bronchial hyper-responsiveness (3,4). In susceptible individuals, this inflammation causes recurrent bouts of wheezing, chest tightness, and cough; those with asthma often have atopy as a significant predisposing factor. Nearly 80% of all asthmatics also have allergies and their symptoms are triggered by specific allergens. Asthma is known to commonly co-occur with allergic rhinitis (1-4).

The association between respiratory allergies and food allergy has been established (5-8). Complex food allergy syndromes

involving allergy to fresh fruits / vegetables and sensitization to inhalant pollen allergens are commonly encountered in allergy clinics, sometimes extending over a range of botanically unrelated plant species (9-11). Typically, these patients experience itchy mouth, tongue (oropharyngeal pruritus), and glottic edema, commonly recognized as pollen-food allergy syndrome or oral allergy syndrome (OAS) (12); it has been shown that ~2-4% of European adults experience this syndrome. OAS is caused by sensitization to cross-reactive determinants shared by various pollens and different fruits / vegetables. Well-known examples of such cross-allergenicity are: (i) birch pollen (apple, hazelnut, carrot, potato, celery, kiwi fruit, etc), (ii) mugwort pollen (celery, carrot, spices), (iii) grass pollen (melon, tomato, peanut, watermelon, orange), and (iv) ragweed pollen (melon, watermelon, banana, zucchini, cucumber). Approximately 35% of patients with such types of pollinosis show hypersensitivity to antigenically-related fruits and vegetables (9-12).

Profilins (12-15 kDa) are a family of highly conserved plant pan-allergens (13-15). The ubiquitous occurrence of profilins in distantly-related plants provides a molecular basis for the frequently observed phenomenon of cross-sensitization towards unrelated plant species in allergic patients. Profilin was first identified as an allergen in birch pollen, and has since been shown to be present as a multigene panallergen family in many plants (13-15); it is described as a pan-allergen, because it was shown to be recognized by the serum IgE of > 20% of pollen-allergic patients (13-15). Profilins from tree, grass and weed pollens, many fruits and vegetables, and latex have been identified as cross-reactive allergens, and studied extensively (16-18).

Profilin binds to poly-(L-proline) (PLP) with high affinity (19-21), a property that is exploited in the purification of many profilins (16-18). The amino acid sequence of profilins is not very highly conserved across many phyla (protozoa, mammalian, yeast, insect, echinoderm and plant); despite this, the structure is conserved (22,23). Possibly connected with the sequence diversity, ubiquity, and abundance of profilins in eukaryotes is the fact that plant profilins are important cross-reactive allergens, and are identified as pan-allergens (23).

The prevalence of tomato (*Solanum lycopersicum* L.) allergy was estimated to range from 1.5% to 16% among food-allergic populations (24-27), and up to 39.2% among grass pollen-allergic patients (28). Profilin from tomato fruit (14 kDa; Sola l 1) has been identified as a food allergen, and has been cloned and characterized (29,30). It was observed at the Allergy, Asthma and Chest Centre in Mysuru (India) that most of the allergic rhinitis and/or asthma patients (patients sensitized to pollen) had allergy to one or more raw vegetables and fruits. The major symptoms were triggering or worsening of allergic rhinitis / asthma, and OAS. Tomato, banana, cucumber, and citrus fruits were the most common offending foods. The association between grass pollen sensitization and food allergy to tomato fruit is known, and the heat-labile cross-reactive allergen profilin has been implicated in such cases. Until now, studies have not been reported from India with respect to tomato allergy in pollen-allergic patients, and the importance of profilin. The present study was undertaken to investigate the spectrum of cross-allergenicity between tomato and grass pollen among allergic rhinitis / asthma patients, and to assess whether tomato profilin is a cross-reactive allergen in a representative population from Mysuru city in South India.

Materials and methods

Reagents and allergenic extracts

Poly-(L-proline) (average mol. wt. 5000 Da; PLP), urea, DMSO (dimethyl sulfoxide), phenylmethanesulfonyl fluoride (PMSF) and trifluoroacetic acid (TFA) were products of Sigma-Aldrich Chemical Co., St Louis, MO, USA. Sepharose-4B was obtained from Pharmacia, Uppsala, Sweden. Cyanogen bromide (CNBr) was a product of Sisco Research Laboratories, Mumbai, India. Grass pollen mix extracts were obtained from Bayer Corp., Spokane, WA, USA and Greer Laboratories, Lenoir, NC, USA. Southern grass pollen mix (#1651, Bayer Corp.) contained pollens from Bermuda, Johnson, Kentucky blue, Orchard, Redtop, sweet Vernal, and timothy grasses. Grass pollen mix (#P28, Greer Laboratories) contained pollens from Bermuda, Johnson, Kentucky blue, Orchard, Redtop, Timothy, sweet Vernal meadow, fescue, and perennial rye grasses. All other chemicals and reagents were of analytical grade.

Preparation of tomato extract

A 50% w/v tomato extract was prepared by blending ripe tomato fruits in a blender using cold phosphate-buffered saline (PBS), and filtering the extract to obtain a clear filtrate. The filtrate thus obtained was dialyzed against PBS using 3500 MWCO dialysis membrane, sterile filtered, and used for SPT. For the purification of profilin, tomato extract was prepared in 10 mM Tris-HCl buffer, pH 7.8 containing 0.1 M KCl, 0.1 M glycine, 0.2 mM PMSF, 0.5 mM dithiothreitol, and 0.2% sodium azide (*buffer A*). Protein content was determined by the method of Bradford (31).

Allergic subjects and case history

Subjects diagnosed as having allergic rhinitis or bronchial asthma or both, based on diagnostic tests amongst the incoming subjects at the Allergy, Asthma and Chest Centre, Mysuru and at the 'Asthma and Allergy' medical camps conducted by the center in and around Mysore city (over a period of 2 calendar years) were enrolled in this study.

A detailed case history was obtained for each subject based on a questionnaire which mainly included age, family history of allergic diseases, history and symptoms of respiratory allergy, history of food allergy, symptoms of allergy to tomato and/or any other foods, any other allergic symptoms, onset, frequency and status of symptoms, diagnostic tests undergone earlier and medications taken, if any.

All procedures performed and studies involving human participants were approved by the institutional research ethics committee, and were conducted in accordance with the ethical standards established in the Declaration of Helsinki of 1946 and its later amendments or comparable ethical standards. Informed consent was obtained from all participants before enrollment in the study.

Preparation of poly-(L-proline)-Sepharose-4B affinity matrix

The preparation of activated Sepharose 4B was performed by the cyanogen bromide activation method described in the literature (32). Briefly, 30 mL of slurry of washed agarose beads (Sepharose 4B), consisting of equal volumes of gel and water, was added to 30 mL of 2 M sodium carbonate and mixed by stirring slowly. The rate of stirring was increased and 1.5 mL of an acetonitrile solution of cyanogen bromide (2 g cyanogen bromide per mL of acetonitrile) was added all at once. The slurry was stirred vigorously for 1-2 min, after which the slurry was poured on to a sintered glass funnel, washed sequentially with 300 mL each of 0.1 M sodium bicarbonate (pH 9.5), water, and coupling buffer (0.1 M $KHCO_3$, pH 8.3, containing 0.5 M KCl). The coupling of PLP was done according to the method of Lindberg et al (19). After the last wash, the slurry was filtered under vacuum to a moist, compact cake and transferred to a plastic bottle containing the ligand solution; the ligand solution contained 100 mg of PLP (average mol. wt. 5000) dissolved in 30 mL coupling buffer. The coupling mixture was incubated at room temperature for 2 h and continued at 4 °C overnight with gentle stirring. The coupling was followed by recording the absorbance at 230 nm; after completion of the reaction, about 50-60% of PLP had bound to the matrix. Uncoupled ligand was removed by washing the gel in coupling buffer, after which the remaining active groups on Sepharose were deactivated by incubating with 0.1 M Tris-HCl buffer, pH 8.0.

Purification of tomato profilin by affinity chromatography

The purification of tomato profilin by affinity chromatography was carried out as described in the literature (20,21). The poly-(L-proline)-Sepharose 4B affinity matrix was packed in to a glass column (1.6 cm i.d. × 7.5 cm) and was equilibrated with *buffer A* (described under "*Preparation of tomato extract*"). Tomato extract was passed through the column at 4 °C, at a flow rate of 25 mL/h. The column was then washed with *buffer A* until the absorbance at 280 nm of the effluent decreased to that of the buffer. The bound profilin was eluted using *buffer A* containing either 30% DMSO, or 7 M urea (after washing initially with 3 M urea). Profilin-containing fractions were pooled, dialyzed, concentrated by lyophilization, and stored in aliquots at -20 °C. The purity of the purified tomato profilin was determined by SDS-PAGE and reverse-phase HPLC.

Skin prick tests

Glycerinated (50%) allergen extracts were used for SPT by following the standard procedure (33) using sterile prick lancetter (Bayer Pharmaceutical Division, Spokane, WA, USA). Affinity-purified tomato profilin was used at 100 µg/mL concentration in glycerinated PBS. Glycerinated PBS (pH 7.4) and histamine phosphate (1.4 mg/mL, equivalent to 1 mg/mL histamine base) served as negative and positive controls, respectively. The diameter of the wheal was read after 20 min. SPT was graded based on the diameter of wheal produced by a sample in comparison to that of positive control (H): 1+ (less than ½H); 2+ (equal to or greater than ½H); 3+ (equal to or greater than H); 4+ (equal to or greater than 2H). The test was considered negative if the wheal diameter was equal to or less than that of the negative control (3 mm).

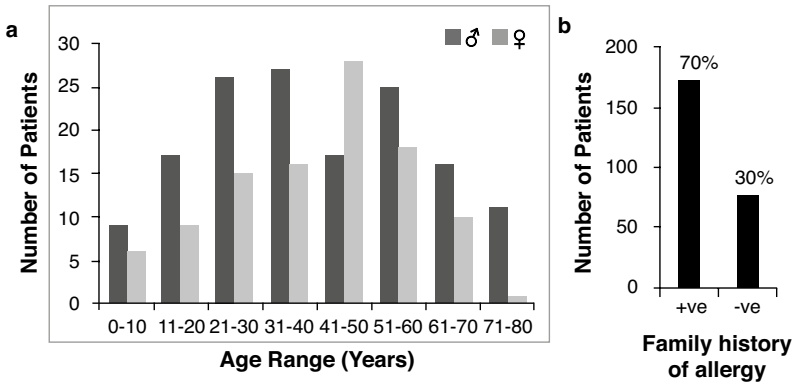
Results

Demographic analysis

A panel of 246 subjects (age range: 4-80 years) who had allergic rhinitis or asthma or both, were investigated by careful case history and SPT in order to understand the prevalence of grass pollen sensitization and tomato allergy, and the relevance of tomato profilin as a cross-reactive allergen among such a population. The number of subjects in different age groups based on gender is shown in **Figure 1** (panel **a**); nearly 70% had family history of allergy (**Figure 1**, panel **b**). The prevalence of clinical symptoms among the 246 subjects is shown in **Figure 2** (panel **a**). Wheeze was most prevalent; nasal symptoms (itchy nose and rhinorrhea) and pharyngeal symptoms (cough, tightness and itching in the oropharyngeal region) were also common. Among the cutaneous symptoms, urticaria was the most common. Considering the actual respiratory disease, 66% of subjects in the study group had allergic rhinitis and 60% of them had asthma, while 43% of subjects suffered from both allergic rhinitis and asthma (**Figure 2**, panel **b**).

The demographic and clinical symptoms of allergic rhinitis / asthma patients having a history of tomato allergy (n = 74) are presented in **Table I**. Majority of the subjects, who had histo-

Figure 1- *Panel **a**, distribution of subjects of the study group into different age groups and sex (n = 246); panel **b**, family history of allergy in the study group based on case history (n = 246; +ve, positive; -ve, negative).*



ry of adverse reactions to tomato, had experienced triggering or worsening of respiratory symptoms after eating raw tomato fruit (56%). The majority of the patients (-66%) had nasal / pharyngeal symptoms; patients with wheezing were even higher (74%). Other symptoms of allergy to tomato were OAS (28%), and rarely, skin rashes or urticaria. Other major offending foods in this study population are banana (57%), citrus fruits (40%), cucumber (32%) and eggplant (8%).

Figure 2 - *Panel **a**, symptoms of respiratory and cutaneous allergy in the study group (n = 246) based on case history; panel **b**, prevalence of allergic rhinitis and asthma in the study group (n = 246) based on case history.*

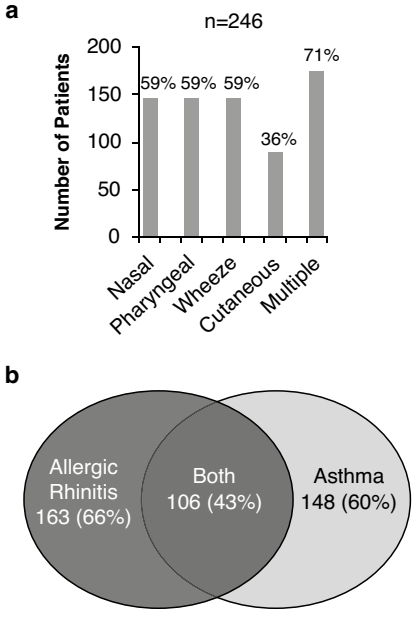


Table I - *Demographic characteristics of subjects with allergic rhinitis / asthma reporting history of allergy to tomato fruit.*

Feature	Number or percentage
patients (n)	74
age (y)	5 - 80
median age (y)	40.5
male	27 (36%)
female	46 (64%)
family history of allergy	26 (35%)
Symptoms	
nasal	49 (66%)
pharyngeal	48 (65%)
wheeze	55 (74%)
cutaneous (urticaria)	22 (30%)
multiple	10 (13%)
Other offending foods	
Major	
banana	42 (57%)
citrus fruits	30 (40%)
cucumber	24 (32%)
eggplant	6 (8%)
Minor	
apple, coconut, guava, grapes, mango, milk, onion, pulses, wheat, yam	< 1%

Affinity purification of tomato profilin from tomato extract for SPT studies

The elution profiles of affinity chromatography of tomato fruit extract on poly-(L-proline)-Sepharose-4B carried out to purify tomato profilin are presented in **Figure 3**. Two different eluants, viz., 30% DMSO (**Figure 3**, panel **a**) and 7 M urea (**Figure 3**, panel **b**) were used in separate experiments to elute the bound tomato profilin. The fractions were analyzed by SDS-PAGE, and the fractions containing the 14 kDa protein were pooled, dialyzed using 3500 MWCO dialysis membrane, and concentrated. The SDS-PAGE patterns of the affinity chromatographic eluates are shown in **Figure 4**. Elution using 7 M urea yielded highly pure 14 kDa protein (**Figure 3**, panel **b**), whereas the DMSO eluate showed the presence of other contaminating proteins around 10, 20, and 66 kDa regions in SDS-PAGE, besides the protein band at ~14 kDa (**Figure 3**, panel **a**). It is known that DMSO elutes both profilin and profilactin complexes, whereas 7 M urea (after the initial washing with 3 M urea) elutes profilin selectively (20). The

***Figure 3** - Affinity chromatography of 50% w/v tomato extract (500 mL) on poly-(L-proline)-Sepharose-4B column (1.6 cm i.d. × 7.5 cm) for the preparation of tomato profilin. Temperature, 4 °C; flow rate, 25 mL/h; fraction size, 2 mL/fraction. Panel **a**, elution using 30% DMSO; fractions 16-19 containing the 14 kDa protein were pooled. Panel **b**, elution using 7 M urea after washing with 3 M urea; fractions 4-8 containing the 14 kDa protein were pooled.*

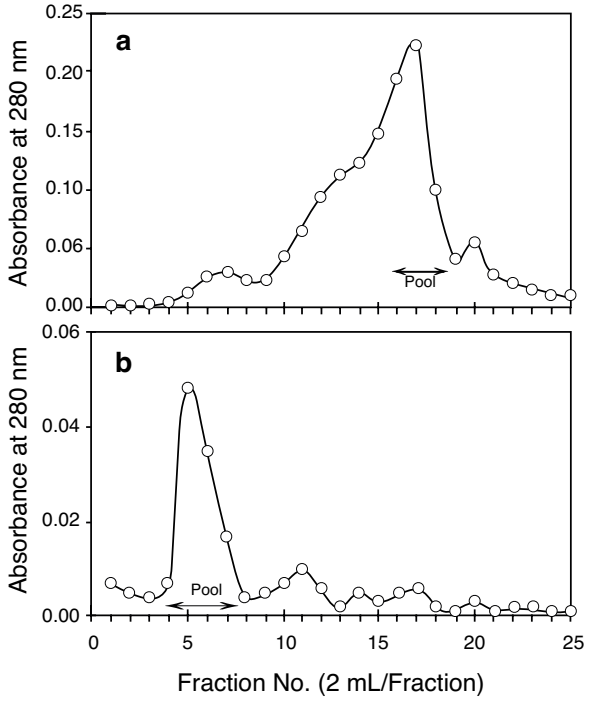
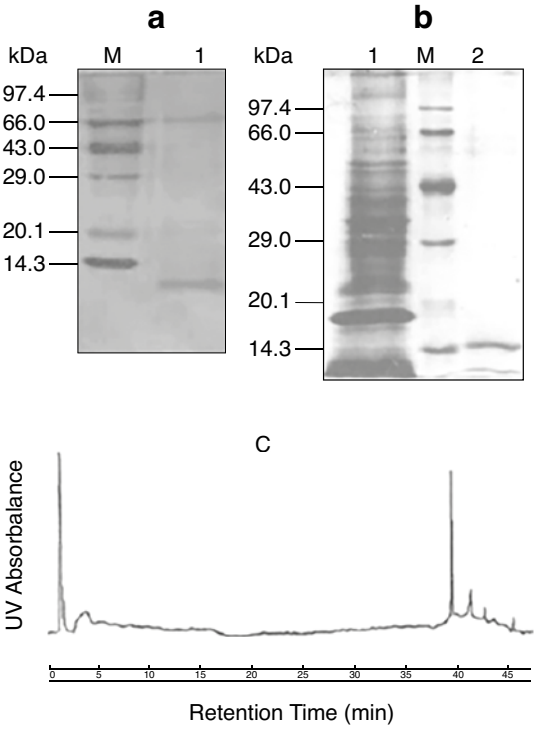


***Figure 4** - SDS-PAGE analyses of affinity-purified tomato profilin. Panel **a**, 15% gel (reducing). M, mol. wt. markers; lane 1, tomato profilin eluted using 30% DMSO. Panel **b**; 12% gel (reducing). Lane 1, tomato whole extract; M, mol. wt. markers; lane 2, tomato profilin eluted using 7 M urea. Panel **c**, reverse-phase HPLC profile of affinity-purified tomato profilin (eluted using 7 M urea). Column: Zorbax C8, DuPont, Bellefonte, PA, U.S.A.; elution: binary gradient of 0.1% TFA (solvent A), and 70% acetonitrile + 0.05% TFA (solvent B); flow rate, 1 mL/min; UV detection, 280 nm. The pure protein showed an RT (retention time) of 39 min.*



homogeneity of urea-eluted tomato profilin was determined by reverse-phase HPLC analysis where it showed ~95% purity (**Figure 4**, panel **c**).

Studies on the relevance of tomato profilin as a cross-reactive allergen in patients with respiratory allergy

In the study group of 246 allergic rhinitis / asthma subjects, sensitization to grass pollens, tomato fruit and affinity-purified tomato profilin was determined by SPT. As high as 182 (74%) subjects showed sensitization to grass pollen extract (**Figure 5**, panel **a**); sensitization to tomato fruit extract and tomato profilin was 43% and 51%, respectively. Out of 246 subjects, 95 (38%) showed positive SPT with both grass pollen mix and tomato extracts (**Figure 5**, panel **b**). If one considers only

the grass-pollen sensitized subjects, the percentage of subjects reactive to tomato extract comes to ~54%. Out of the 95 subjects who showed cross-sensitization to both grass pollen and tomato extracts, 82 subjects (92% of subgroup) showed positive SPT with affinity-purified tomato profilin (**Figure 5**, panel **b**). These results based on SPT indicate that cross-sensitization to grass pollen and tomato fruit is very prevalent at 38% among allergic rhinitis / asthma patients, and majority of them (92%) are sensitized to the cross-reactive panallergen, tomato profilin.

SPT studies in the subgroup of allergic rhinitis / asthma patients with a history of adverse reactions to tomato fruit

In this subgroup of 74 subjects, SPT with grass pollen mix extract, tomato fruit extract, and affinity-purified tomato profilin was positive in 84%, 67%, and 64% of subjects, respectively (**Figure 6**); the percentage of patients giving a 2+ reaction in SPT with all the 3 extracts was higher compared to those showing 1+ or 3+ reaction.

Out of 48 (65%) subjects who showed cross-sensitization to tomato fruit and grass pollen, 44 (91%) subjects showed sensitization to tomato profilin (**Figure 7**). These data indicate that tomato profilin is a very important cross-sensitizing allergen among the tomato allergens in allergic rhinitis / asthma patients sensitized to grass pollen and with a history of tomato allergy.

Figure 5 *- Pattern of sensitization to grass pollen, tomato fruit, and tomato profilin based on SPT. Panel **a**, the entire study group of allergic rhinitis / asthma patients (n = 246). Panel **b**, subgroup of patients cross-sensitized to grass pollen and tomato fruit as determined by positive SPT.*

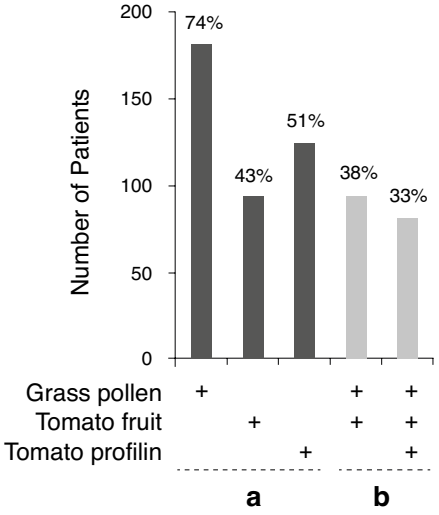


Figure 6 *- Gradation of SPT in the subgroup of allergic rhinitis / asthma patients with history of adverse reactions to tomato fruit ingestion (n = 74). SPT was graded based on the diameter of wheal produced by a sample in comparison with that of positive control (histamine, H): 1+, less than ½H; 2+, equal to or greater than ½H; 3+, equal to or greater than H; 4+, equal to or greater than 2H. SPT was considered negative if the wheal diameter was equal to or less than that of the negative control.*

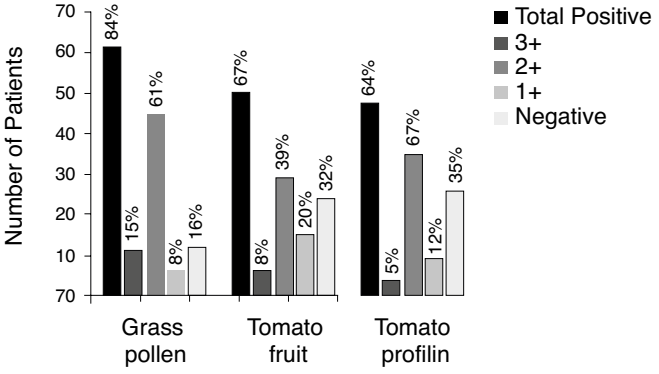
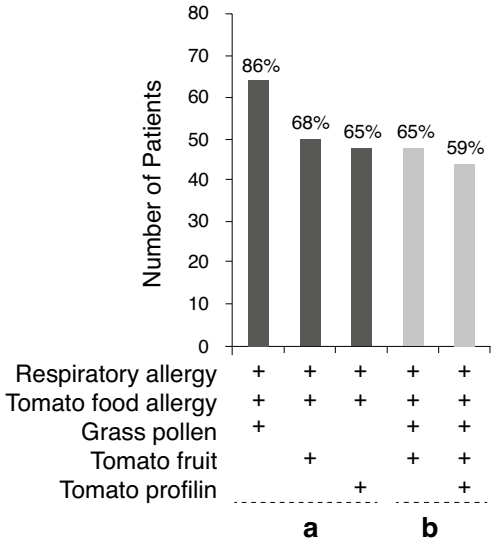


Figure 7 *- Pattern of sensitization to grass pollen, tomato fruit and tomato profilin based on SPT. Panel **a**, allergic rhinitis / asthma subjects with history of adverse reactions to tomato fruit ingestion (n = 74); and panel **b**, those with positive SPT to grass pollen and tomato fruit.*



Discussion

In this study, we have evaluated the role of tomato profilin in presenting cross-reactivity with grass pollen-sensitized allergic subjects having respiratory allergy. Petersen et al (25) identified the main tomato allergens using sera from 8 subjects with high

specific IgE to tomatoes that also had grass pollen allergies; 5 out of 8 sera reacted to β-fructofuranosidase (β-FF), and another 5 reacted to profilin. Foetish et al (28) studied the relevance of tomato allergy in pollen-allergic patients in a group of 32 birch pollen-allergic patients with history of adverse reactions to tomato fruit, and found that tomato allergy occurred with a prevalence of 9% in the study group, and the majority of pollen-associated allergies to tomato were due to ubiquitous allergenic structures such as profilin (44%) and cross-reactive IgE binding *N*-glycans (35.5%). However, in the present study the sensitization was estimated based only on SPT, in which a positive result shows the presence of mast cell-bound specific IgE to a particular extract or protein. Despite SPT producing false positive and false negative tests with about 5-10% margin of error, it is a simple and reasonably accurate test most widely used in allergy clinics as the first diagnostic test (33). A number of impactful studies have demonstrated the sensitivity of prick tests as better or comparable to *in vitro* tests or immunoassays for the detection of specific IgE using whole extracts or purified allergens, and have positively confirmed their reliability to assess allergen sensitization (34-38). Although not supported by data on serum allergen-specific IgE, the results presented here give an idea of the pattern of sensitization to tomato profilin in pollen-allergic patients in a South Indian city.

Tomato fruit profilin gene has been cloned and the recombinant protein was shown to be IgE-reactive with a prevalence of 22% in tomato-allergic patients (29). In subjects with tomato allergy and multiple sensitization to other foods and birch pollen, IgE directed against tomato profilin showed a strong cross-reactivity with profilins from plant food sources and birch pollen. Westphal et al (30) concluded that tomato profilin is a minor allergen in tomato fruit.

In studies of profilin sensitization by SPT, Asero et al (34,35) highlighted the clinical importance of diagnosing hypersensitivity to single food allergenic proteins immediately in the office; this is especially useful for the allergist if the relevant food allergen sources contain several allergenic proteins that show different physicochemical characteristics and, hence, completely different risk profiles. The large majority of profilin-allergic patients reported OAS as the only food-induced symptom, and was able to tolerate the offending foods if they were cooked or processed. Profilin should be considered a clinically relevant food allergen, and allergy to melon, watermelon, tomato, banana, pineapple and orange may be considered as a marker of profilin hypersensitivity (34,35).

In evaluating the clinical features and usefulness of current diagnostic methods for tomato allergy, Asero (39) found that tomato allergy was detected in 33% of subjects with plant food allergy, and was significantly associated with profilin hypersensitivity ($p < 0.001$), and concluded that tomato allergy occurs by sensitization towards different proteins; most cases are seen in profilin-hypersensitive subjects and are mild. In another study of 26 patients overexposed to grass who were referred for severe food reactions, oral provocation with purified profilin resulted in a significant number of patients showing severe positive food challenge test reactions at low doses of profilin (40). These investigators claim that profilins are complete food allergens in food-allergic patient populations that are exposed to high levels of grass pollen; this patient type constitutes an optimal model to understand the link between respiratory and food allergies. It is likely that intake through the oral mucosa might be a relevant route of exposure to food allergens.

Conclusions

The association between sensitization to pollen and allergy to raw vegetables and fruits is well established (5,9-11,13-15,18). Understanding the spectrum of cross-reactive pollen-food allergies in different populations is required for the proper diagnosis and treatment of such allergic disorders. The association between grass-pollen sensitization and allergy to tomato fruit is known from studies in Europe and Japan (24-26,28,30); however, there are no reports of such studies in the Indian population. The present study describes a preliminary investigation based on case history and SPT in a study group of 246 allergic rhinitis / asthma patients in Mysuru city, India, aimed at understanding the importance of tomato profilin as a cross-reactive allergen. Tomato profilin was purified by affinity chromatography, and was used in SPT along with tomato fruit extract and grass pollen mix extracts. The results showed that 38% of subjects had sensitization to both tomato fruit and grass pollen, out of which 92% were sensitized to tomato profilin. In the subgroup of allergic rhinitis / asthma patients with a history of adverse reaction to raw tomato fruit (32%), the result was even more pronounced, with 66% subjects showing cross-sensitization to tomato fruit and grass pollen, out of which majority of them (91%) showed positive SPT with tomato profilin. Our results clearly indicate that tomato profilin is a very important cross-sensitizing allergen in allergic rhinitis / asthma patients sensitized to grass pollen, and more so in patients with history of food allergy to tomato fruit. In conclusion, it may be advantageous to incorporate purified profilins (e.g., tomato profilin) in the routine SPT procedures for patients with respiratory allergy, and subsequently, in specific immunotherapy protocols in allergy clinics for desensitization of allergic subjects sensitized to profilin.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgments

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Allergy and high trait anxiety are related to increases in heart rate variability: results of naturalistic long-term design study

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Key words
heart rate variability; allergy; trait anxiety; stress; naturalistic design

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Summary
A number of studies report heart rate variability (HRV) changes in allergic as well as high trait anxious people, and associations between allergic inflammation and trait anxiety. This study investigated HRV of 20 low anxious allergic, 19 healthy high trait anxious and 18 healthy low anxious, in naturalistic setting. On arranged research days, subjects performed measurements using portable ECG device and subjective self-assessment of perceived stress. Five repeated measurements data from each subject have shown increases in overall HRV, as well as HRV on respiratory frequencies in both allergy and high trait anxiety. Subject's sex was an important factor, because HRV increases in allergy were only apparent in women. Data from self-assessment show no differences in experienced stress attributable to allergy, only to trait anxiety.

Abbreviations
ANS, autonomic nervous system; HR, heart rate; HRV, heart rate variability; IgE, immunoglobulin E; AR, allergic rhinitis; AD, atopic dermatitis; BMI, body mass index; STAI, state and trait anxiety inventory; ECG, electrocardiogram; PSS, perceived stress scale; DFA, detrended fluctuation analysis; HF, high frequencies; LF, low frequencies; VLF, very low frequencies; ApEn, approximate entropy; BIC, Schwarz's Bayesian Criterion; F, test statistic; df, degrees of freedom.

Introduction

An increasing number of studies show relevance of autonomic nervous system (ANS) dysregulations to both mental and somatic disorders, mainly those which are related to stress (1). Autonomic dysregulations, may emerge as consequences of allostatic load, cumulative wear and tear of bodily systems, that have been exposed to chronic or traumatic stress (2,3).

Heart rate (HR) is conditioned by both autonomic branches; normally it should exhibit a relatively large inter-beat variability. Heart rate variability (HRV) is used as a marker of healthy adaptive capacity of cardiovascular system, while cardiac pathological states show low or almost no variability and high predictability of heart rate (4,5). Psychobiological models of neurovisceral integration (6,7) underline associations of HRV with brain pathways connected to stress, therefore HRV may serve as a suitable psychobiological index of central inhibitory control and emotional regulation.

Anxiety is defined by Spielberger (8) as both an unpleasant emotional state grounded in fear (state anxiety), and as a relatively stable tendency (trait anxiety) to manifest anxiety states. High trait anxiety is related to failure in inhibition of threat responses on psychological as well as physiological level (9), which leads to perpetuation of the adaptive capacity of an individual.

High anxiety and anxiety disorders were previously associated with increased cardiovascular risk (10), reduced overall HRV, and vagal HRV indices in anxiety disorders were concluded in recent meta-analysis (11). While findings in pathological anxiety are quite consistent across various studies (for review, see 11), HRV changes in subclinical anxiety are not so univocal. The assumption that chronic anxiety may be associated with trait-like lowered vagal tone (9) was supported by some studies, which found lowered HRV in highly anxious people (12,13). However, there has been a problem with replication of these results on other data, with small effects attributable to anxiety, while acute stress related reductions of HRV were reliably observed (14).

Anxiety and allergy

Current neuroendocrinology research on allergy underlines the importance of psychological factors, such as stress, in the onset and pathogenesis of allergies (15). Cumulative allostatic load may play a part in allergic inflammatory process as well as changes in psychological states, e.g. feelings of anxiety (3). Increased trait anxiety was correlated with elevated levels of IgE and increase in Th2 immune response, which play a critical role in development of allergy (16). Other studies (17,18) found that personality traits such as anxiety, depression, a high emotional excitability, as well as higher comorbidity of anxiety disorders were linked to major allergic disorders (atopic dermatitis, allergic rhinitis, allergic asthma).

Based on associations between allergy and anxiety, as well as on expected discomfort due to the presence of allergic symptoms, reduced HRV in allergy would be expected. Recent HRV studies, however, show that allergic patients exhibited an increase in overall HRV, as well as in HRV on respiratory frequencies. Increased HRV in adults suffering from allergic rhinitis (AR) (19,20) as well as children (21), imply parasympathetic predominance and/or sympathetic withdrawal in AR. Similar findings were obtained for atopic dermatitis (AD) as well (22).

Based on described links between high trait anxiety and allergy, we aim to investigate HR, HRV and subjectively perceived stress differences, in allergic and high trait anxious people. Because of strong comorbidity of allergy and high trait anxiety, it is difficult to access effects of allergy and anxiety on psychological and physiological functioning separately (23). We studied differences between groups of low anxious allergic, and high anxious healthy subjects, and compared them to low anxious healthy controls, to examine factors of allergy and high trait anxiety independently.

Another important characteristic of this study is the naturalistic design of data acquisition. Following our previous studies with laboratory design (24), and based on naturalistic studies with salivary cortisol (25) and studies of long term HRV or HRV in natural conditions (19,26), we investigated HRV indices and subjective experience of research subjects in relation to stressors during their daily lives. A design with repeated measurements of HRV and subjective self-assessment of experienced stress during longer time period was applied here.

Method

Subjects

Subjects were recruited from students of Comenius University, Bratislava, while some allergy subjects were recruited from ambulant allergologists in Bratislava, Slovakia. Initially 64 people were recruited, however due to drop out of 7 of them the sample for analysis consisted of 57 subjects (26 males, 31 females) divided into three balanced groups based on allergy and anxiety. Assignment to allergy group was assessed based on individual history (occurrence of allergic symptoms for at least 2 years) and diagnosis of allergy based on a skin prick test performed by an allergologist. Both subjects with allergic rhinitis (AR) and atopic dermatitis (AD), as well as combined symptoms of both, were included. Subjects in non-allergy groups had no allergy symptoms and negative allergy anamnesis dating back to childhood.

Trait anxiety was measured via Slovak version of State and trait anxiety inventory (STAI-T) (11,27). Subjects were allocated to high and low anxiety groups based on cut off score: low anxiety < 39, high anxiety > 49. All who scored in between these values were not included in this study. This design was chosen based on prior stress research on Slovak sample (28) to include only very anxious, and on the other hand low anxious people.

Subjects suffering from cardiovascular, endocrine, psychiatric or any other chronic disease, which could affect HR and HRV variables, and subjects who regularly took medication (e.g. antihistamines, corticosteroids for allergy), or had any other possibly confounding conditions were excluded from the study. Some included allergic subjects took allergic medication seasonally, therefore only measurements from medication-free periods were included in data analysis.

Written informed consent was obtained from all subjects after explanation of all research procedures. The study was approved by ethics committee of the Trnava Self-Governing Region, Trnava, Slovakia.

Data Collection

This study used repeated measurements design, with HR and HRV data collected between November 2014 and November 2016 in separate measurements. Research subjects were given randomly selected dates, during their everyday lives, in which data collection was performed. Moreover, one stressful and one

relax day were individually agreed upon with each subject, relative to events in subjects' lives. This study includes data from 5 measurements from each subject, including both one stressful and one relax day.

ECG recording was performed via portable eMotion Faros 90° or Faros 180° ECG devices. Electrodes of ECG device were attached to subject's chest - one on the right side, under the clavicle, the other on the left under the ribcage.

At the beginning of data collection, all subjects were given instructions on how to apply the portable device and how to start and terminate the recording. All information was given to subjects in spoken as well as written form, and on research website (www.dlhodobymonitoring.sk), where they could also find dates of measurements, and all necessary information. Subjects could also contact research team via telephone, and were also regularly informed on research procedures.

Twenty-min ECG recordings and subjective emotional assessments were done in the morning, afternoon and evening, corresponding to sample collection for salivary cortisol, which will be analyzed and published separately. During all 20-min ECG measurements, subjects were instructed to be seated and relaxed. Evening ECG measurements were chosen as most appropriate for HRV analysis, due to the least missing data and movement artifacts. Morning and afternoon measurements were investigated via the same analyses and yielded congruent results to those described here.

Subjective assessment checklist was presented in paper form with separate pages for each measurement, containing questions about possible confound variables (e.g. alcohol, smoking, active movement or activity before measurement) and emotional states via list of adjectives compiled for this study. Used adjectives were related to feelings of stress, exhaustion and positive affect, which were proposed as latent factors via exploratory factor analysis.

For assessment of more long-term perception of stress, subjects filled Slovak version of perceived stress scale (PSS) (29) on each research day. PSS scale contains 10 statements that assess the extent in which a subject perceives their life as unpredictable, uncontrollable and overloading. Moreover, subjects were also instructed to write down stressful events which influenced them on the measurement day, and rate their stressfulness on a 5-point scale.

Heart rate variability analysis and measures

HR and HRV measures were obtained from ECG recording with sampling rate 250 Hz. For purposes of HRV, two 5-min long windows were extracted from each recording and averaged. HRV analysis was realized via Kubios HRV software (30), and all included R-R intervals were manually checked for artifacts and ectopic beats. Only continuous 5-min artifact free periods were included. Deleted ectopic beats represented less than 1% of used R-R intervals. Subsequently, data were analyzed using time domain parameters, spectral analysis via fast Fourier transform and non-linear analyses of Poincare plot characteristics, and fractal characteristics such as Entropy and Detrended fluctuation analysis (DFA).

As indicators of overall HR variability, we used SDNN (standard deviation of all R-R intervals) from time-domain analysis, Total power from spectral analysis, and SD2 (standard deviation of long term RR interval variability) from Poincaré plot. All these parameters are normally highly correlated.

Similarly, HRV on respiratory frequencies, which are predominantly associated with vagal activity, were used as follows: RMSSD (the square root of mean squared successive R-R differences) from time domain analysis, high frequency (HF) HRV fluctuations (frequency: 0.15-0.4 Hz) from spectral analysis, and SD1 (standard deviation of short term RR interval variability) from Poincaré plot. These indices capture fast beat-to beat changes on respiratory frequencies which are attributable to inhibitory control via vagal nerve (5,7,31). We also investigated HRV on lower frequencies (LF) (0.04-0.15 Hz) and very low frequencies (VLF) (< 0.04) that are thought to be modulated by a mix of both sympathetic and vagal influences as well as baroreflex influences, thermoregulatory processess etc. (31,32).

From non-linear HRV parameters, we analyzed approximate entropy (ApEn) as a measure of overall complexity and unpredictability of HR series (33) and Detrended fluctuation analysis (DFA). DFA is a scaling method, which estimates fractal (self-similarity) attributes on both short term $\alpha 1$ (4-11 heart beats) and long term $\alpha 2$ scale (33,34).

Statistical analysis

HRV measures naturally show large variances between measures. To enable statistical analyses, HRV variables from time domain, spectral and Poincaré plot analysis (SDNN, RMSSD, Total power, VLF, LF, HF, SD1 and SD2) were transformed by log10. Non-linear HRV variables (ApEn, DFAα1, DFAα2) were normally distributed, therefore remained untransformed.

To investigate both between and within subject effects, mixed regression modeling was used. This approach is appropriate for multiple repeated measures with both fixed and random parameters (35). Analysis was performed in IBM SPSS 20 software. Mixed regression modeling used HR and HRV, and subjectively perceived stress measures as outcome variables in separate subsequent analyses. Categories of sex and research groups were used as fixed factors, while subject identity during five repeated measurements was a random factor. Unstructured co-variance structure was used due to the number of repeated measurements and unknown co-variance structure of the data. Further reported model provided the best fit while comparing Schwarz's Bayesian Criterion (BIC) to models with different combination of factors or co-variation structures.

Table 1 *- General characteristics of research sample (body mass index and age - mean + std. error).*

	N	BMI		Age	
a, allergy + low trait anxiety	20 subjects (10 males, 10 females)	23.15	± 0.679	21.3	± 0.805
b, healthy + high trait anxiety	19 subjects (8 males, 11 females)	20.679	± 0.481	20.421	± 0.618
c, healthy + low trait anxiety	18 subjects (8 males, 10 females)	22.422	± 0.602	19.611	± 0.231

Furthermore, relationships between HRV measures and subjective stress measures were investigated via partial correlations controlled for base heart rate of the subject. This was done to control for large interindividual variability in basal HR.

Results

The sample analyzed in this study consisted of 57 subjects (26 males, 31 females). To compare allergy and non-allergy highly anxious subjects, three research groups were formed: a, Allergic subjects with low trait anxiety (n = 20); b, highly trait anxious subjects with no allergy symptoms (n = 19) and c, low anxious controls with no allergy symptoms (n = 18). All groups were balanced based on subjects' sex, age and body mass index (BMI) (for a summary of groups, see **Table I**).

Assessing effects of allergy and anxiety

Results of multiple regression model for individual outcome variables showed fixed effect of research group on overall HRV: SDNN: F (df = 2, 55.92) = 6.05, $p < 0.01$; Total spectral power: F (2, 55.18) = 4.21, $p < 0.02$; SD2: F (2, 58.29) = 6.49, $p < 0.01$. A similar effect was observed in HRV on respiratory frequencies: RMSSD: F (2, 58.83) = 3.82, $p < 0.03$; HF-HRV: F (2, 56.94) = 3.53, $p < 0.04$; SD1: F (2, 58.88) = 3.6, $p < 0.04$. Separate comparison of allergy and high trait anxiety group with controls is shown in following **Table II**, which summarizes mean values for individual variables and estimated difference of allergy and anxiety groups from controls.

HRV indices from spectral analysis, which represent slower changes in HR, have shown significant between group differences in VLF: F (2, 51.2) = 4.63, $p < 0.02$, but not LF F (2, 56.42) = 1.48, p = 0.24.

Differences in non-linear variables for allergy and trait anxiety were not significant: ApEn: F (2, 57.06) = 0.36, p = 0.7; DFA parameter $\alpha 1$: F (2, 58.81) = 0.83, p = 0.44; DFA parameter $\alpha 2$ F (2, 58.96) = 1.25, p = 0.3. Although no statistically significant differences were observed in non-linear parameters, $\alpha 1$ showed opposite trend in allergy and trait anxiety group to the rest of HRV variables in time domain and spectral analysis (see **Table II**).

Effects of research group on basal heart rate (in beats per minute, bpm) was not significant F (2, 56.28) = 2.31, p = 0.11, however difference between allergy and control group was significant (see Table II).

Assessing effects of sex

Differences between men and women in HR and HRV were assessed as a fixed factor. Women showed significantly higher heart rate (80.856 bpm) than men (74.014 bpm) F (1, 58.35) = 13.41, $p < 0.01$. Sex was also a statistically significant factor for overall HRV: SDNN: F (1, 58.07) = 4.64, $p < 0.04$; total spectral power: F (1, 57.34) = 7.18, $p < 0.01$; SD2: F (1, 60.34) = 6.68, $p < 0.01$. Mean values for men and women as well as estimated differences are summarized in **Table III**.

Table II *- Between group differences in HR and HRV.*

HR and HRV variables	Experimental group		
	allergy	high trait anxiety	control
HR (bpm)	75.355 (- 4.775)[1]	76.819 (- 3.311)	80.13
SDNN (log10)	1.838 (+ 0.119)[2]	1.830 (+ 0.111)[1]	1.719
RMSSD (log10)	1.651 (+ 0.152)[1]	1.648 (+ 0.149)[1]	1.499
TP (log10)	3.622 (+ 0.194)[1]	3.611 (+ 0.183)[1]	3.428
VLF (log10)	3.230 (+ 0.213)[2]	3.212 (+ 0.195)[1]	3.017
LF (log10)	3.154 (+ 0.123)	3.119 (+ 0.088)	3.031
HF (log10)	2.812 (+ 0.269)[1]	2.797 (+ 0.254)[1]	2.543
SD1 (log10)	1.501 (+ 0.148)[1]	1.499 (+ 0.146)[1]	1.353
SD2 (log10)	1.958 (+ 0.116)[2]	1.949 (+ 0.106)[2]	1.842
ApEn	1.113 (+ 0.012)	1.111 (+ 0.009)	1.101
DFAα1	1.194 (- 0.069)	1.212 (- 0.05)	1.263
DFAα2	0.852 (+ 0.001)	0.889 (+ 0.038)	0.852

Estimated marginal means values, parameter estimate values compared to control group in brackets. Statistical significance value (p): [1] < 0.05, [2] < 0.01.

Table III *- Sex differences in HR and HRV.*

HR and HRV variables	Sex		Parameter estimate
	female	male	
HR (bpm)	80.856	74.014	+ 6.841[2]
SDNN (log10)	1.763	1.829	- 0.067[1]
RMSSD (log10)	1.570	1.629	- 0.059
TP (log10)	3.472	3.635	- 0.164[2]
VLF (log10)	3.073	3.233	- 0.160[1]
LF (log10)	3.030	3.173	- 0.143[1]
HF (log10)	2.708	2.727	- 0.019
SD1 (log10)	1.423	1.479	- 0.056
SD2 (log10)	1.879	1.954	- 0.075[1]
ApEn	1.124	1.092	+ 0.032[1]
DFAα1	1.174	1.272	- 0.098[1]
DFAα2	0.854	0.875	- 0.021

Estimated marginal means values for men and women and parameter estimate values. Statistical significance value (p): [1] < 0.05, [2] < 0.01.

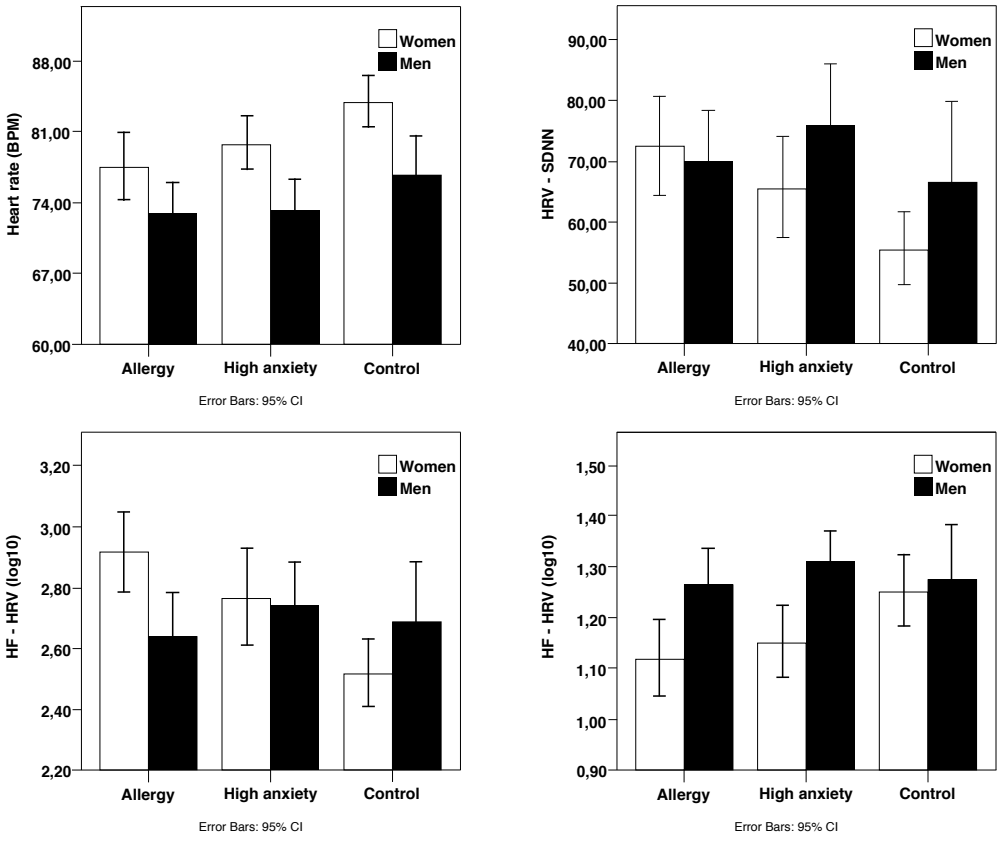
Respiratory frequencies HRV have shown no significant main effect of sex. However, the interaction of both research group and sex was observed for HF-HRV F (2, 50.76) = 4.41, p < 0.02 and similar not significant interaction for RMSSD: F (2, 50.97) = 2.75, p < 0.07. Different results in allergy and anxiety comparison between men and women were observed in other HRV variables as well; the interaction of factors was significant only in respiratory HRV parameters. Comparison of men and women in three research groups is shown in **Figure 1**.

Differences between sexes were also observed in non-linear HRV parameters of entropy (ApEn) F (1, 58.01) = 6.95, p < 0.01, which was higher in women; and detrended fluctuations analysis α1 F (1, 60.52) = 4.65, p < 0.04, higher in men (for summary see **Table III**).

Subjectively perceived stress differences

For assessing subjectively perceived stress, Slovak translation of perceived stress scale (PSS) (Cohen, Kamarck, and Mermelstein, 1983) (29), and assessment of adjectives describing emotional states related to stress, exhaustion and positive affect, were used.

Figure 1 *- Between group and between sex differences for HR, SDNN, HF-HRV and DFAα1.*



In multilevel regression model with the same factors as HR and HRV analysis, score from PSS differed significantly ($p < 0.01$) between high trait anxiety group (mean score 21.51) and two remaining groups (allergy 16.00; control 14.93). Differences between sexes were not significant ($p = 0.12$), women however showed slightly higher mean values (18.31) than men (16.66).
Group differences between scores from adjective scales were found for stress adjectives (non-parametric Kruskal-Wallis test, $p < 0.01$), with higher score in high trait anxiety group (10.29) compared to allergy (8.38) and control group (8.02), and positive emotion adjectives ($p < 0.01$) in high trait anxiety group (9.83), allergy (11.88), control (11.92). No statistically significant differences were found in exhaustion adjectives.
The relationship between HRV and subjective measures was investigated using partial correlations controlled for variable of heart rate, to eliminate individual differences in basal HR. Perceived stress (raw score from PSS test) correlated positively with VLF-HRV ($r = 0.23$, $p < 0.01$) and DFAα2 ($r = 0.22$, $p < 0.01$) and negatively with DFAα1 ($r = - 0.20$, $p < 0.02$). DFA α1 also correlated negatively with subjectively perceived exhaustion from adjectives scale ($r = - 0.17$, $p < 0.05$).
DFAα2 correlated with feeling of stress from adjectives scale ($r = 0.20$, $p < 0.02$) and occurrence of stressful events reported in subjective checklist ($r = 0.17$, $p < 0.05$).

Discussion

Naturalistic design proposed in this study was used to evaluate HR and HRV changes and subjective feelings of stress during normal day to day life. To make the design the least intrusive to the subjects and to collect reasonable amount of data, we used several repeated measurements, performed at the arranged dates by each subject.
Results from the repeated measurement design reported here showed lowered heart rate (HR) in both allergic and highly anxious subjects. From HRV measures, increases of overall HRV (measured via SDNN, Total power and SD2) as well as increases of HRV on respiratory frequencies (RMSSD, HF, SD1) were observed in both allergy and high trait anxiety group. While these HRV indices associated mostly with parasympathetic influences were increased, similar increases were found also in VLF-HRV, which corresponds to Tran et al. (36), where increases of VLF HRV were interpreted as sympathetic hyperactivation. On the other hand, DFAα1, which is an indicator of fractal attributes of healthy HR complexity, showed lowered values in both allergy and high anxiety group.

Allergy and HRV increase

Effect of increased HRV was expected in allergy, due to prior research (for a review, see 37). However, the mechanism of observed HRV changes in allergy is still not known. One hypothesis states that these effects may be attributed to the role of efferent vagus nerve in anti-inflammatory pathway (38). Increase in vagal activation may be a result of counter regulatory mechanism in response to allergic inflammation (22). On the other hand, HRV increase may also be due to sympathetic withdrawal associated with allergic inflammation itself and Th2 immune response, which increases local sympathetic activity in allergic reaction sites and decreases systemic sympathetic activity (39,40).

HRV changes in high trait anxiety

While there is more evidence for decrease in HR and increase in HRV for allergy, similar results in the high trait anxiety group are quite surprising. High trait anxiety was in most previous studies associated with an opposite effect of HR increase and HRV decrease (for a review, see 9).
However, findings of dysregulated stress response in highly anxious subjects were reported earlier. Duncko et al. (28) reported blunted cortisol as well as blunted noradrenaline and adrenaline responses, during acute stress in highly trait anxious individuals. These findings were interpreted in context of allostatic model, as inability to react with adaptive stress reaction. While allergy is often associated with increases in anxiety, depression and problems in emotional regulation (17,18), effects of blunted cortisol (41) as well as salivary alpha amylase, which is commonly associated with sympathetic activity (42) and aldosterone (24) were reported for both allergy as well as high trait anxiety.
Based on the mentioned effects we could speculate whether choosing only very high trait anxious subjects (STAI-T score < 49) and contrarily very low anxious subjects as controls (STAI-T < 39) could play a role in the observed effects.
While Bornas et al. (26) found differences between high trait anxiety subjects and controls in entropy measures, our results showed no differences in ApEn, however DFAα1 had lower values in both high trait anxiety and allergy group. These are consistent with significant negative correlation of DFAα1 and subjectively perceived stress (via PSS and adjective scales).

Sex differences and between group changes

Among the sex differences we recorded higher mean HR and lower overall HRV (SDNN, total spectral power, SD2 from Poincaré plot) in women, which is consistent with data from previously done meta-analysis of 172 HRV studies (43). In our data, lower VLF and LF variability in women were observed, while we detected no differences in fast beat-to-beat changes (HF, RMSSD). Differences in non-linear HRV showed higher entropy (ApEn) in women, while DFAα1 was lower consistently with meta-analysis results (43).

Interestingly, we found sex related differences in HRV changes attributable to allergy and high trait anxiety. All between group effects were more pronounced in women (see **Figure 1**), while effect of allergy and anxiety in respiratory frequencies HRV (RMSSD, HF, SD1) showed different trends between men and women. Increased vagal HRV parameters were not present in male subjects, though this effect was very strong in women (see **Figure 1**). These sex differences in vagal HRV related to allergy and anxiety are not clear, and they should be investigated more thoroughly with a larger number of subjects.

Differences between sexes in HRV, with both higher HR and vagally mediated HRV in women while men show higher overall variability, may be related to the effects of estrogen (44), which could attribute to more vagal activation. Moreover, higher levels of oxytocin were also associated with increased vagal tone and slowing of HR (43).

Changes in subjectively perceived stress and HRV

In intergroup comparison, both PSS score and score from stress related adjectives were significantly higher in high trait anxiety group compared to low anxious allergy and control group. An opposite trend was observable in positive affect adjectives, which were lower in high trait anxiety group. Differences between low anxious allergy and control group were small and statistically insignificant for all subjectively experienced stress measures. While stronger reported experience of stress and less positive emotion were expected in high trait anxiety group, an interesting finding is the indifference of allergy group to controls. We assume therefore that differences in subjectively reported stress are related to psychological trait of anxiety, not allergy itself. The association of allergy and higher subjective feelings of stress reported by numerous studies, might be explained by comorbidity of allergy and high trait anxiety described earlier (17,23).

Besides intergroup differences, correlation between HRV measures and subjective self-assessment were tested. Correlations were controlled for heart rate, which could partially eliminate strong individual variance in HRV. Positive relationship between VLF-HRV and PSS score was found, indicating higher VLF-HRV in psychological distress. Though VLF-HRV was previously interpreted in relation to sympathetic activity (36), it is also strongly correlated with all other spectral HRV parameters (Total power, LF, HF) indicating both sympathetic and vagal influences.

From non-linear parameters, DFAα1 correlated negatively with perceived stress, while DFAα2 showed opposite tendency. DFAα1, which is regarded as a marker of short term HRV complexity and healthy HRV was lowered when perceived stress was higher, as well as it was stably lower in allergy and high trait anxiety group. DFAα2, which should indicate long term complexity, showed exactly opposite manner. Positive correlation with PSS score, as well as feelings of stress from adjective scales and reported occurrence of stressful events on the day of the measurement, were reported. DFAα2 is normally correlated with long term variability measures from spectral analysis, VLF and LF HRV (33). Similar trends for VLF-HRV and DFAα2 were apparent in our data as well.

Naturalistic design specifics and limits

Main aim of the naturalistic design used in this study was to investigate more ecologically valid measurements of stress in follow up to our previously realized laboratory studies. HR, HRV and subjectively experienced stress were measured in response to normal day to day stressors, which were in our data mostly work related (50%) or related to personal situations, e.g. relationships etc. (40.2%).

Like Bornas et al. (26), we assume that investigating HRV in naturalistic setting may reveal more relevant information on subjects' reactivity during normal functioning. One of the most notable advantages of our design, was very little intrusiveness from the research team and therefore an insight on subject realistic functioning. The use of small and portable eMotion Faros ECG devices enabled us to design this HRV study in the least intrusive way.

On the other hand, there are limitations associated with this kind of design. Even though the cooperation with the subjects was mostly flawless, there were some who due to errors in ECG measurements, movement artifacts or problems in communication had to be excluded from the study, with their data not considered.

Possible confounding variables as movement, use of medication or other substances (caffeine, alcohol etc.) were monitored via self-assessment before each measurement. Possibly, subjects could fail to mention any conditions or medication, which might have been important. Furthermore, possible allergy symptoms in non-allergy group were not tested, only asked about in self-assessment. Therefore, there is a chance of some subjects being allergic despite not knowing about it.

Clinical relevance of the results

HRV changes in allergy and anxiety described here, as well as relationship between the two might lead to several clinically relevant consequences. Due to similar psychophysiological changes (24) and reported comorbidity of anxiety and allergy (16,17,18,23) as well as the role of stress in allergic process (15), psychological interventions aimed on better coping and reduction of anxiety could be beneficial to allergic patients (45). Similarly, interventions based on normalization of HRV such as HRV biofeedback might also be employed, which has already been demonstrated in a study with asthma (46). Future research is necessary to evaluate

the effectiveness of different psychological interventions and their contribution to the treatment of allergy.

Conclusion

This study investigated HRV differences in allergic and highly anxious subjects during their normal day to day functioning.
Complete analysis of HRV results showed decreased HR and increased overall HRV in both allergy and high trait anxiety group. While between group differences were most notable in overall HRV indices (SDNN, total spectral power, VLF, SD2) increases in vagal HRV indices (RMSSD, HF-HRV, SD1) were also present. From non-linear HRV indices, lowered DFAα1 in both allergy and high trait anxiety was observed. Interestingly, HRV differences related to allergy and anxiety interacted with subjects' sex showing notable increases in HRV in women, while no significant effects were observed in men.
Subjective measures of stress showed increases in high trait anxiety group, while low-anxious allergy group scored similarly to controls. Based on this, we assume that previously reported increase in subjectively experienced stress in allergy could be a consequence of difference in trait anxiety.
Correlations between HRV indices and subjectively reported feeling of stress were found for PSS and adjective scales and non-linear DFAα1 and α2, showing opposite tendencies.
Research design used in this study offered a naturalistic approach to monitoring of HRV in everyday life stressful situations.

Conflict of interest

The authors declare that they have no conflict of interest.

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Cypress pollen allergy is responsible for two distinct phenotypes of allergic rhinitis different from other pollinosis

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Key words

allergic rhinitis; bronchial hyperresponsiveness; cypress pollinosis; united airway disease; inflammatory cytokines

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Summary

Different phenotypes of allergic rhinitis have been identified based on the seasonality of the allergen involved. Within pollinosis, importance has to be paid to the responsible pollen species. Guidelines for clinical management are mostly based on studies performed in patients with grass pollen allergy. Only few data is available on tree pollen allergy and more specifically on cypress pollen allergy.

We focused on the clinical and biological features of cypress pollen allergy to determine whether it is associated with a specific phenotype of allergic rhinitis or not.

Our results suggest that cypress pollen can be responsible for two distinct phenotypes of rhinitis, both different from other pollinosis.

In the most common phenotype, cypress pollen was not responsible for bronchial hyperresponsiveness or systemic inflammation.

Close attention has to be paid to the allergen involved in allergic rhinitis. Different phenotypes leading to different pharmacological strategies may apply.

Introduction

Cypress pollen allergy is a winter pollinosis that may be caused by several *Cupressaceae* species around the Mediterranean basin, in North America and Asia (1,2). Its prevalence has increased over the last decades (1). The first cases of cypress pollinosis were described in South Africa in 1945 and in France in 1962 (3). During the following decades, cypress species have been extensively planted for ornamental purpose (1). This increased exposure (4,5) has been responsible for the increase in prevalence of sensitization and clinical manifestations of cypress pollen allergy (6-8).

Cypress pollinosis symptoms are often hard to control. Caimmi reported a 57.9% of cypress pollen allergic patients needing immunotherapy to control their symptoms (3). Most reported symptoms are rhinitis, conjunctivitis, asthma, cutaneous manifestations (1-3,9) and dry cough (1).

Several studies have highlighted the differences existing among allergic rhinitis phenotypes based on the seasonality of the al-

lergen involved (10-13). Within pollen-induced allergic rhinitis, importance has to be paid to the pollen species (14,15). Guidelines of clinical management have been built on available evidence, mostly based on grass pollen allergy studies. Only few data is available on tree pollen allergy and more specifically on cypress pollen allergy, although there is emerging evidence that clinical feature of cypress pollen allergy differs from other pollinosis. As an example, cypress pollinosis has been characterized by a lower prevalence of conjunctivitis and a higher prevalence of dry cough during pollen season than *Gramineae* pollinosis (1). In this study, we aim to focus on the clinical and biological features of cypress pollen allergy in order to determine whether cypress pollen-induced allergic rhinitis is associated with a specific phenotype of allergic rhinitis or not. This will help to rule on the clinical management of this pollinosis.

Material and methods

The study was conducted in Marseille, Southern France. This was a monocentric prospective study that aimed to enrol adults from 18 to 65 years with a diagnosis of allergic rhinitis to cypress pollen. The diagnosis was confirmed by positive skin prick test to at least one pollen from the *Cupressaceae* family.
Patients could be sensitized to other allergens, even perennial ones, but had to be clinically free from symptoms related to these allergens. Patients with diagnosed asthma were excluded from the study as well as patients that underwent allergenic immunotherapy during the past 5 years. Pregnant women, patients treated with beta-blockers, suffering of a clinically unstable disease during the past three month or a respiratory infection during the month preceding enrolment were not eligible to the study.
According to local legislation, all patients signed an informed consent form approved by an ethics committee (Comité de protection des personnes Sud Méditerranée I, project reference 2011-A00211-40). The study was conducted in accordance with the ethical standards established in the Declaration of Helsinki of 1946 and the international guidelines of Good Clinical Practice.
Thirty-six patients were enrolled into this protocol consisting in 2 visits. One visit outside the pollinisation and one during symptoms period. 32 patients attended both visits.
Outside the pollen season, patient profile of allergens sentitization was assessed using standardized skin prick tests with a battery of allergenic extracts consisting of *Juniperus ashei*, Mix of *Cupressaceae* (*Cupressus arizonica* and *sempervirens*), Grass, *Dermatophagoides pteronyssinus, Dermatophagoides farinae*, cat, dog, cockroach, *Parietaria*, *Olea* and Platane tree.
The skin reaction was recorded 20 minutes after the test by evaluating the skin response in comparison to the wheal induced by the histamine positive control test. A wheal diameter of at least half of the histamine wheal diameter was considered a positive reaction.
During and outside the allergic season, patients filled a symptom questionnaire, the Mini Rhinoconjunctivis Quality of Life Questionnaire (RQLQ) (16). It consists in 5 questions referring to allergic rhinitis symptoms and overall control. Each question consists in a 5 values scale. The total score range from 5 (uncontrolled rhinitis) to 25 (absence of symptoms).
Patients also reported personal and familial history of atopy, they underwent pulmonary function test and standardized methacholine challenge to assess bronchial hyperreactivity. Levels of IL10, IFNɣ, IL12p70, IL5, IL4, IL2, IL17a, IL1β, TNFα and CXCL8 were measured in serum sample with Cytometric Bead Array™ Flex Sets (CBA, Becton Dickinson, Le Pont de Claix, France) following the manufacturer's instructions. Briefly, specific antibody-coated beads detected target cytokines or chemokines, the signature of each type of bead being unique phycoerythrin-fluorescence intensity. Experiments were performed with a FACS Canto II flow cytometer using the FacsDIVA software (Becton Dickinson, Le Pont de Claix, France). Results are expressed as concentration (fg/ml).
Statistical analysis was performed using Graphpad Prism software. Depending on the data analysed we used different statistical tests. Patients' characteristics of coughing and non-coughing subgroups were compared by the Student test, excepted for percentages that were compared with the chi square test. The Wilcoxon test was used to compare data measured during and outside pollen season in the same patients (methacholine challenge results, symptom scores and blood cytokines levels). In all tests, we used a two-tailed hypothesis and a significance level of 0.5.

Results

As reported in **Table I**, the most frequent symptom in our cypress pollen induced allergic rhinitis population was rhinorrhea (94% of patients). Dry cough was present in 31% of patients. Mean age at onset of symptoms was 27 (± 17) years. Disease duration was 13.6 ± 9 years.

Table I *- Frequency of reported symptoms during the allergic season.*

rhinorrhea	94%
ocular itching	79%
sneezing	76%
nasal itching	44%
throat itching	44%
watery eyes	38%
nasal obstruction	33%
dry cough	31%

Table II *- Patients characteristics.*

	All patients	**Coughing patients**	**Non coughing patients**	**p-value**
men	42%	36%	44%	0.67
age	40 ± 17	42 ± 19	39 ± 17	0.69
age at symptoms onset	27 ± 17	28.8 ± 18	26.5 ± 17	0.73
disease duration	13.6 ± 9	13 ± 6.5	13.9 ± 10	0.83
symptom score during pollen season	17.7 ± 3.4	17.3 ± 3.9	17.8 ± 3.2	0.68
symptom score outside pollen season	24.1 ± 1.8	24.3 ± 1.5	24.1 ± 2	0.71
atopic disease history in mother	22% (allergic rhinitis) 5.6% (asthma) 5.6% (eczema)	18% (allergic rhinitis) 9% (asthma) 9% (eczema)	27% (allergic rhinitis) 4.5% (asthma) 4.5% (eczema)	0.66 0.5 0.5
atopic disease history in father	19.4% (allergic rhinitis) 2.8% (asthma)	9% (allergic rhinitis)	27% (allergic rhinitis) 4.5% (asthma)	0.27 0.49
atopic disease history in both parents	2.8% (allergic rhinitis in both parents)	0%	4.5%	0.49
atopic disease history in siblings	33% (allergic rhinitis) 14% (asthma)	20% (allergic rhinitis)	52% (allergic rhinitis) 16% (asthma)	0.09 0.21
number of positive skin prick tests	4.3 + /- 2,1	3.3 ± 2.3	4.7 ± 1.9	0.12
monosensitization to cypress pollen	22%	36%	16%	0.18
sensitization to cypress	100%	100%	100%	1
Juniperus ashei	89%	64%	100%	0.006
Cupressaseae mix (*arizonica and sempervirens*)	97%	91%	100%	0.3
both	86%	54%	100%	0.001
grass	47%	27%	56%	0.16
house dust mite	31%	36%	28%	0.70
Dermatophagoides pteronissinus	28%	36%	24%	0.45
Dermatophagoides farinae	19%	0%	28%	0.08
both	17%	0%	24%	0.15
cat	42%	27%	48%	0.29
dog	31%	27%	32%	1
cockroach	0%	0%	0%	NA
Parietaria	14%	18%	12%	0.63
Olea	44%	27%	52%	0.28
Platane tree	14%	9%	16%	1

Coughing patients had less personal and familiar history of atopy and were more likely to be monosensitized to cypress than non coughing patients: 36% and 16% of monosensitized patients in the coughing and non-coughing patients groups respectively. Nevertheless, these differences did not reach the statistical significance. Patients were considered to be monosensitized to cypress pollen if they had a positive SPT to one or both of the *Juniperus ashei* and *Cupressaceae* mix (*Cupressus arizonica and sempervirens*). The patients' profile of sensitization to other tested allergens is shown in **Table II**: we found no statistical difference between coughing and non-coughing patients profiles, excepted for cypress pollens species sensitiza-

***Figure 1** - Change in methacholine response during pollinisation.*

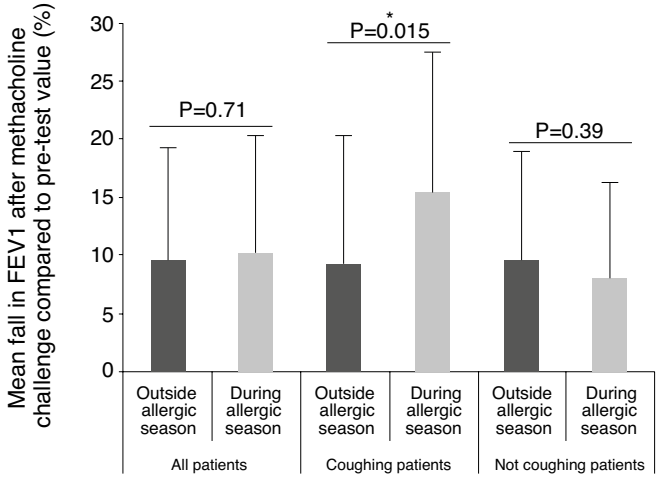
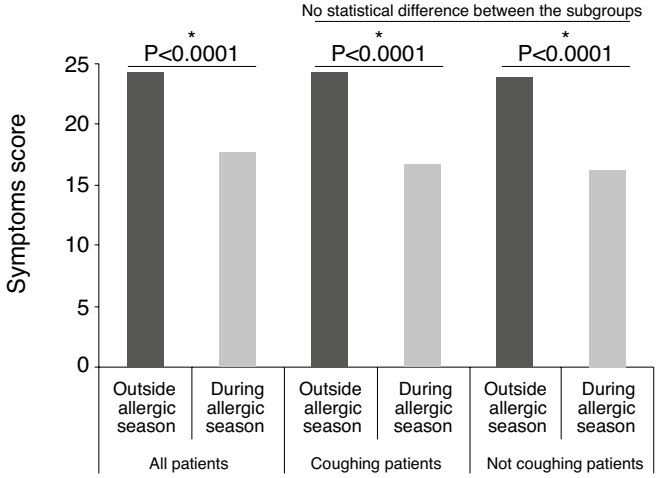


***Figure 2** - Change in symptoms score during pollinisation.*



tion. Non-coughing patients are more likely to be sensitized to *Juniperus ashei* in addition to other cypress pollen species than non coughing patients.

All patients had significantly more symptoms during season, as assessed by a decrease of 7 points in the mini rhinoconjunctivitis quality of life questionnaire score in all groups. There was no difference in symptoms score during or outside the pollen season based upon the coughing status (**Figure 2**). The mean RQLQ score was 17.7 ± 3.4 during pollen season and 24.1 ± 1.8 outside the pollen season.

In the majority of patients (69%) who have cypress pollen induced allergic rhinitis without dry cough, there was no change in the methacholine challenge results during and outside the pollen season. In non-coughing patients, FEV1 decrease was equal to 8.0% and 9.6% outside and during the pollen season, respectively.

An increase in methacholine challenge response does exist in the coughing patients group as compared with the non-coughing patients group (**Figure 1**). In coughing patients the mean decrease in FEV1 after methacholine challenge was 9.3% outside the pollen season and 13.9% during pollen season. However, in both groups, none of the patients having a negative methacholine challenge outside the pollen season reached the threshold of positivity during pollinisation.

Blood cytokines concentrations were stable outside and during the allergic season in the "all patients" analysis (**Figure 3**) and in both cough-related subgroups.

Discussion

Several limitations may apply to our findings. Firstly, only a small number of participants were experiencing cough during pollen season (31% corresponding to 11 patients). Secondly, as demonstrated by Polosa and colleagues, adenosine 5' monophosphate challenge may be a better way to assess bronchial hyperresponsiveness in these patients (17). This may underestimate the actual bronchial hyperresponsivness in this subgroup.

Nonetheless, our study succeeded in identifying two distinct phenotypes of cypress pollinosis. These phenotypes can be clinically discriminated by the presence or absence of dry cough during pollinisation.

Basically, the most frequent phenotype of cypress pollen induced allergic rhinitis does not include dry cough as a symptom (69% of patients). A subset of patients (31%) experience dry cough. This difference may lead to distinct therapeutic managements.

Severity of the disease

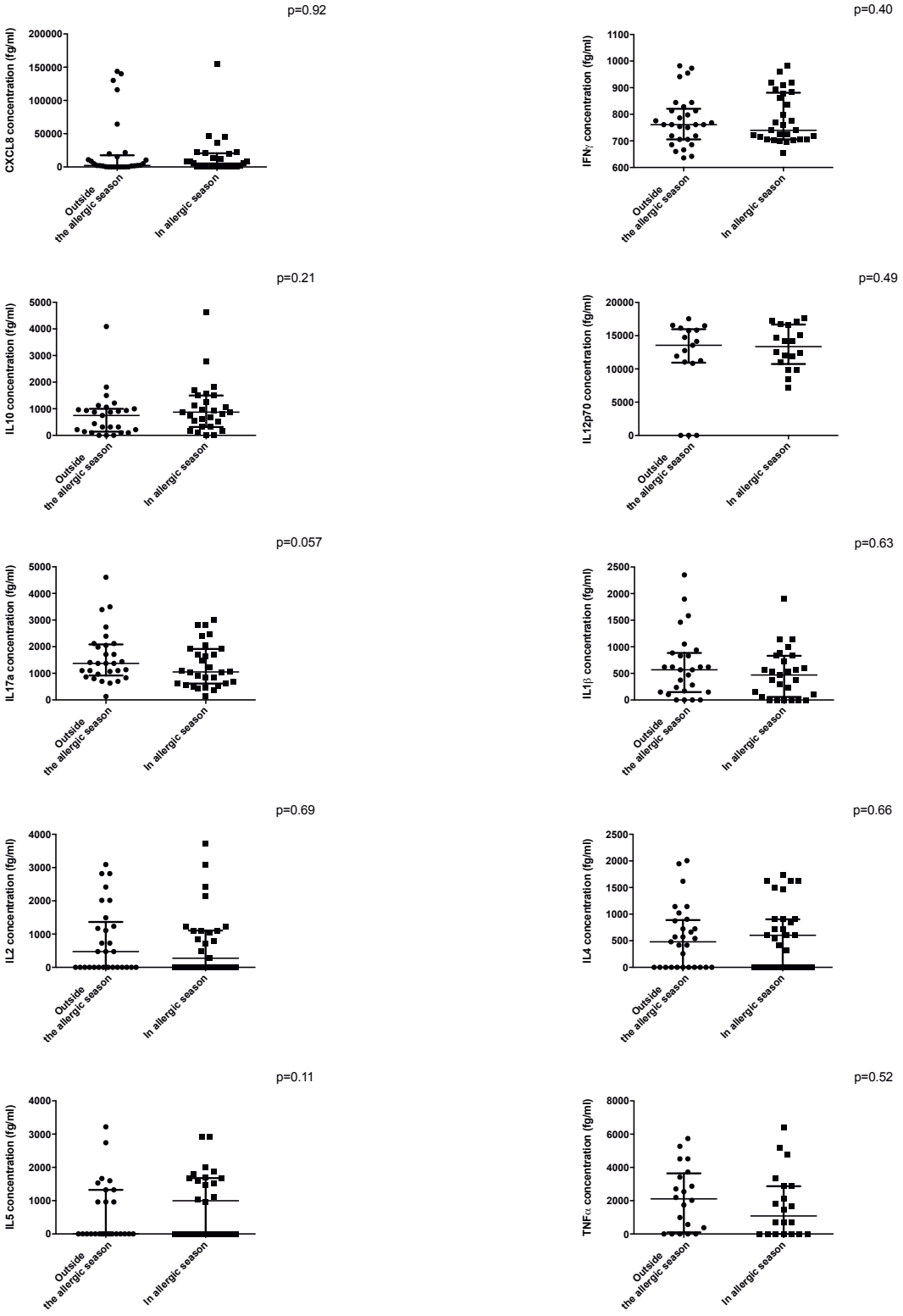
In our study, we failed to identify a difference in the disease severity between subgroups. The symptom score was similar in both groups, thus cough is not a marker a severity of the disease.

Bronchial hyperresponsiveness

Many studies highlight an increase in bronchial hyperresponsiveness during pollinisation in patients suffering from pollen induced allergic rhinitis. The large majority of these studies are addressing grass pollen allergy (15,18-20) and evidence of this finding is lacking in cypress pollen allergy.

Allergic rhinitis and asthma are airways diseases that often occur concomitantly. Epidemiological evidence from studies conducted in industrialized countries show that 60 to 98.9% of asthmatics patients have concomitant rhinitis and that 20 to 40% of rhinitics patients show clinical evidence of asthma (12,21-24).

Figure 3 *- Cytokines serum concentration during and outside the pollen season in all patients.*



This concomitant manifestation of symptoms in both upper and lower airways supports the concept of "united airways disease" (25). Because the association between asthma and rhinitis is observed in non-atopic subjects (24), the relationship cannot be explained by common risk factors, and it has been suggested that rhinitis might itself be a risk factor for asthma (26).
Both diseases are inflammatory, so biomarkers can overlap suggesting a common pathogenesis. The united airways disease theory relies on the idea that upper and lower inflammatory manifestations influence each other, and that therapeutic management of one or the other disease can help controlling symptoms at another bronchial level (27). Indeed, comorbid allergic rhinitis has been identified as a marker for more difficult to control asthma, and may worsen asthma outcomes (27). The Allergic Rhinitis and its Impact on Asthma (ARIA) initiative recognizes the interactions between these two entities and supports a global therapeutic and diagnostic management described in evidence-based guidelines (28).
Triggers of allergic rhinitis may play a role in the concomitant existence or onset of symptoms in the lower airways.
Firstly, it may depend on the seasonality of the sensitization. An interesting finding supporting this point comes from the work of Ricconi and colleagues in 2002. They highlighted the fact that perennial allergy is associated with greater bronchial hyperresponsiveness than seasonal allergy (10). This was confirmed in 2014 by Ciprandi and colleagues, stating that patients showing bronchial hyperresponsiveness had significantly more frequent mite allergy (11). In the same way, Linneberg and coworkers reported asthma in 25% of patients with allergic rhinitis who were pollen-sensitive and in 50% of those patients who were mite-sensitive or animal-sensitive (12). Ciprandi demonstrated that bronchial hyperresponsiveness was present in 50% of seasonal allergic rhinitis patients and in 70% of patients experiencing perennial symptoms (13).
Secondly, in pollen induced rhinitis, lower airways impairment may depend on the pollen species. Di Lorenzo and colleagues brought out that bronchial hyperresponsiveness incidence among allergic rhinitis patients during pollen season and off season depend on the kind of pollen responsible for the allergy (14). In this study conducted on 49 patients with Parietaria, Gramineae and Olea pollen induced rhinitis, the authors conclude that Parietaria pollen allergy is more important than Gramineae or Olea pollen allergy as a risk of developing nonspecific bronchial hyperresponsiveness, measured as response to inhaled methacholine. Indeed, 16 patients showed bronchial hyperresponsiveness during pollen season (100% were parietaria sensitized) and 8 patients off pollen season (7/8 were parietaria sensitized). In grass pollen induced rhinitis, Kurt has demonstrated that 50% of patients experience bronchial hyperresponsiveness during the pollen season (15).
In our study, the majority of patients (69% i.e. non-coughing patients) showed no change in their methacholine challenge results during and outside the cypress pollen season. Thus, there is no allergen induced bronchial hyperresponsiveness in these patients. A significant increase in methacholine challenge response does exist in the coughing patients group as compared with the non-coughing patients group. Therefore, none of the patients having a negative methacholine challenge outside the pollen season reached the threshold of positivity during the season (decrease of at least 20% in FEV1). This may assess an increase of bronchial reactivity in patients coughing during cypress pollinisation, but cough cannot be considered a marker of bronchial hyperresponsiveness in cypress pollen induced allergy.
A limitation of our study is the lack of FeNO dosage. This is indeed considered an important parameter for the early characterisation of bronchial hyperresponsiveness, and this could have been complementary to methacholine challenge (29-33).

Systemic inflammation

Blood cytokines dosages failed to demonstrate a systemic inflammation as all concentrations are stable outside and during the allergic season.
In grass pollen induced allergic rhinitis, several inflammatory biomarkers have been identified to increase during pollen season and to correlate to bronchial hyperresponsiveness. IL5, IL18 blood levels, blood and sputum count of eosinophils have been shown to increase during pollen season and to parallel bronchial hyperresponsiveness (14,15,18-20).
Given these observations, the stability of proinflammatory cytokines during and outside the pollen season in our study are consistent with the absence of cypress pollen induced bronchial hyperresponsiveness in the majority of patients.
As mentioned previously, the small number of coughing patients does not allow us to extrapolate this result to this specific subgroup and a systemic inflammation may exist in coughing patients.
Nonetheless, in the majority of cypress pollen allergic patients, the absence of systemic inflammation is in favour of an ear-nose-throat local inflammatory reaction.

Implication for diagnosis and therapeutic management

Two distinct phenotypes of cypress pollen induced allergic rhinitis have been identified in our study. Both phenotypes differentiate from other pollinosis by their late onset.
The "coughing phenotype", which is the less common, has similarities with other pollinosis, as it may be characterized by an increase in bronchial hyperresponsiveness.
Nonetheless, these patients have less personal and familiar history of atopy and are more likely to be monosensitized to cypress

pollen than non coughing patients. This differentiates this first phenotype of cypress pollen induced allergic rhinitis from other pollinosis. In these patients, treatment of both nose and bronchi may improve disease control.

The most frequent phenotype, the "non-coughing" phenotype, also differs from other pollinosis and contradicts the united airway disease theory. Cypress pollen is in most of the cases responsible for a local reaction that does not affect lower airways. In those patients, a local treatment of symptoms should be preferred.

Conclusion

Overall, we would like to stress the importance to pay close attention to the allergen involved in allergic rhinitis. Depending on the allergen involved, different phenotypes leading to a different pharmacological strategy may apply, and more surprisingly one allergen may be responsible for different phenotypes. As mentioned, due to a modest number of patients enrolled, this study remains a preliminary work, and further investigations are required to strengthen these findings.

Conflict of interest

The authors declare that they have no conflict of interest.

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Cutaneous drug reactions to antiepileptic drugs and relation with HLA alleles in the Turkish population

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Key words

antiepileptics; carbamazepine; HLA-A allele; HLA-B allele; drug hypersensitivity; delayed reactions; SJS; TEN; MPE

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Summary

Background and objective. *Many studies have shown associations between HLAB*15:02, HLA-A*31:01 and carbamazepine (CBZ)-induced delayed cutaneous hypersensitivity reactions. The aim of this study is to evaluate a possible association between delayed cutaneous reactions to antiepileptic drugs (AEDs) and certain HLA-A and HLA-B alleles in the Turkish population.* ***Methods.*** *The study consisted of 3 groups: Group I (reactive group) included the patients who had documented delayed cutaneous reactions to any antiepileptic drug. Group II (non-reactive group) included the patients who have been on antiepileptic treatment at least for three months without any adverse reactions. Group III consisted of healthy subjects. The HLA-A and B alleles were analyzed in all groups.* ***Results.*** *Forty patients (29 female) had experienced different hypersensitivity reactions due to AEDs: maculopapular exanthema (26 patients), Stevens-Johnson syndrome (6 patients), drug rash with eosinophilia and systemic symptoms (7 patients), toxic epidermal necrolysis (1 patient). Lamotrigine (11) and CBZ (10) were the most common culprit drugs involved in the reactions. The HLA-B*15:02 was not present in any of the study groups. However, HLA-B*35:02 was found in 4 patients from the reactive group, while it was not observed in non-reactive patients and was detected in only one healthy subject (p = 0.021).* ***Conclusion.*** *Although our preliminary results did not indicate a strong allele association with AED hypersensitivity, HLA-B*35:02 appears to be a candidate allele for MPE / DRESS / DIHSS induced by AED's in Turkish population. Further studies with a larger sample size may result in more comprehensive data about the genetic tendency for AED hypersensitivity in the Turkish population.*

Introduction

Drug hypersensitivity entails an important clinical morbidity. It consists of a variety of phenotypes, mainly the cutaneous adverse reactions that range from milder skin reactions (e.g., exanthem, urticaria, and angioedema) to severe cutaneous adverse reactions (SCARs) (1). SCARs are life-threatening, and include Stevens-Johnson syndrome (SJS), toxic epidermal necrolysis (TEN), and drug reaction with eosinophilia and systemic symptoms (DRESS) or drug-induced hypersensitivity syndrome (DIHS) (2). These reactions are differed from other drug reactions by their high morbidity and mortality rates. At this point, any factors that can predict the development of these particular reactions are highly warranted. So far, a limited number of drugs are associated with a high risk for SJS and TEN: several antiepileptic agents, especially CBZ, phenytoin, phenobarbital and lamotrigine, antibacterial sulfonamides, anti-inflammatory drugs of the oxicam family, allopurinol and the antiretroviral agent nevirapine (3).

Over the last decade, a large number of studies have shown associations between various HLA alleles and different delayed drug hypersensitivities (4). To date, the best characterized HLA-associated drug related hypersensitivity reactions are documented by abacavir, nevirapine, CBZ and allopurinol (5). Among these drugs, abacavir has the strongest association with HLA-B*57:01 allele. Individuals with this allele have approximately a 50% chance of developing abacavir hypersensitivity syndrome, while no one without this allele is predicted to develop an immunologically confirmed hypersensitivity reaction, whereas CBZ hypersensitivity has been shown to be strongly associated with the HLA-B*15:02 allele (6). The latter association was first demonstrated in Han Chinese patients, in which 44 with CBZ-induced SJS/TEN were positive for HLA-B*15:02 compared to 3% in the CBZ-tolerant cohort (odds ratio 2504) (7). Subsequent studies have also confirmed this association in Thai, Korean, Malaysian and Indian populations (8). As a result of this suggestive results, the US Food and Drug Administration has recommended prescreening testing for HLA-B*15:02 allele before prescribing CBZ in the ethnic groups at risk (9).

Later on another allele, HLA-A*31:01 was reported to be associated with all phenotypes of CBZ-induced cutaneous reactions, including maculopapular exanthema (MPE), DRESS and SJS/TEN in European and Japanese populations (10,11).

So far, an association of certain HLA types with delayed drug hypersensitivity reactions has not been studied in Turkish population. So, we aimed to investigate whether any HLA A or B based genetic tendency exists for AED induced hypersensitivity reactions in Turkish population.

Methods

This was a multi-centered, case-control study in which the Neurology and Allergy Immunology Departments of 5 Universities from 4 different regions of Turkey, Istanbul, Ankara (two centers), Izmir and Rize have participated. The study population involved three groups as below:

Group 1, patients with documented delayed cutaneous drug reactions (CDRs) due to any AED (CBZ, oxcarbazepine, phenytoin or lamotrigine) determined by file screenings, and the patients who experienced delayed CDRs during the follow up period of three months after the starting of a newly prescribed AED.

Group 2, the patients who have been receiving treatment with any AED at least for three months without any drug related reactions (drug taking controls).

Group 3, healthy subjects with no drug hypersensitivity reaction and with no use of any AED.

The clinical features of the past drug reactions recorded on patient files were evaluated by expert dermatologists. Newly emerging CDRs were classified by the same experts according to the following criteria (14). SJS was diagnosed if a rapidly developing blistering exanthema of purpuric macules and target-like lesions accompanied by mucosal involvement with skin detachment of up to 10% of the body surface area were present. Patients with skin detachment from 10 to 29% of the body surface area were categorized as SJS-TEN overlap, whereas TEN was defined by widespread macules or blisters with skin detachment of up to 30% of the body surface area. The inclusion criteria of DRESS were a suspected drug reaction with MPE, plus the involvement of at least one internal organ (e.g., hepatitis, pneumonitis, myocarditis, pericarditis, nephritis), one of either lymphadenopathy or hematologic abnormality (e.g., eosinophilia, atypical lymphocytosis) and a fever above 38 °C. Patients who met three or more of these criteria were considered as DRESS (14). The patients who were exposed to another "high-risk" drug or had any viral or bacterial infection in the same period when drug hypersensitivity symptoms arose, were excluded from the study.

The patients determined by file screenings were called and invited to participate to the study. Twenty-eight patients accepted to be involved and give blood samples. Also, twelve patients diagnosed as AED related CDR during their follow-up visits volunteered to be study subjects.

The ethical approval was obtained from Ethical Committee of Istanbul Medical Faculty, Istanbul University. All volunteer participants gave written informed consents prior to enrollment on the study.

HLA analysis

10 ml blood samples were collected in EDTA tubes from all groups. All the samples were genotyped at the Department of Medical Biology of Istanbul Medical Faculty, which has accreditation to perform clinical HLA typing by the European Federation of Immunogenetics.

The genomic DNAs were obtained from peripheral venous blood samples by using the Bio-robot EZ1 (Qiagen N.V., Venlo,

The Netherlands). Genotyping of HLA-B allele was performed by the PCR-SSOP method (polymerase chain reaction with sequence specific oligonucleotide probe) using Lambda One subtype kits (PCR-SSP, InGen, Technopolis, Chilly Mazarin, France) and confirmed by Olerup SSP high resolution HLA-B kits (PCR-SSP, Geno-Vision Inc, West Chester, Pennsylvania, USA). HLA-A alleles were typed by SBT method by Invitrogen kits (Thermo Fisher Scientific, Waltham, Mass, USA).

Statistical analysis

Statistical analyses were performed by Statistical Package for Social Sciences (SPSS 21.0) for Windows. Numeric values were expressed as mean ± SEM, whereas nominal values were given as n (%). Comparisons of the frequencies of HLA-A and HLA-B alleles between the subgroups were performed using Fisher's exact tests. All P-values were two-tailed. The statistical significance was defined as $P < 0.05$.

Results

The age and sex distribution were comparable in all study groups except Group II, in which the rates of female and male were equal (**Table I**). In the reactive group, the most common drugs causing CDR were lamotrigine (n = 11), CBZ (n: 10), and different combinations of these drugs (11). Other drugs were phenytoin (n = 3), valproate (n = 1), prymidone (1), gababentin (1) pregabalin (1), topiramate (1). Most of the reactions were MPE (n = 26) (**Table II**). The numbers of serious AED reactions were 6 for SJS, 1 for TEN and 7 for DRESS / DIHSS. Lamotrigine was the sole responsible drug (in 3 patients) or as a combination with another antiepileptic drug (in 3 additional patients) in patients presenting with SJS (**Table III**). On the other hand, CBZ was more frequent in DRESS / DIHSS (**Table IV**). Allele frequencies were similar in both AED reactive and tolerant patients (**Table V** and **Table VI**). However, the HLA-B*35:02 was found in 4 of 40 AED-reactive patients (in two patients with MPE and two patients with DRESS

Table I *- Age and gender characteristics of the study groups.*

	Group 1 (n = 40)	**Group 2 (n = 60)**	**Group 3 (n = 118)**
age (mean ± SEM) (min - max)	32.6 ± 13.8 (18 - 74)	36.2 ± 12.6 (20 - 64)	34.3 ± 11.7 (21 - 70)
sex (female / male)	29 / 11	30 / 30	85 / 33

Table II *- The characteristics of the patients with antiepileptic drug induced hypersensitivity reactions.*

Phenotypes	**SJS / TEN**	**DRESS / DIHSS**	**MPE**	**FDE**
N	6 (1 TEN)	7	26	1
sex (female / male)	6 / 0	3 / 4	20 / 6	M
age (mean ± SEM)	37.7 ± 19.9	28 ± 8.9	32.9 ± 13.1	54
(min - max)	(21 - 74)	(19 - 44)	(16 - 62)	

Abbreviations: SJS, Stevens-Johnson syndrome; TEN, toxic epidermal necrolysis, DRESS, drug rush with eosinophilia and systemic symptoms; DIHSS, drug induced hypersensitivity syndrome; MPE; maculopapular exanthema; FDE; fixed drug eruption.

Table III *- The HLA-B and HLA-A alleles of 5 patients with SJS and 1 TEN induced by antiepileptic drugs (DNA sampling could not be done in one SJS patient because the blood sample was clotted).*

Patients	**HLA-B alleles**		**HLA-A alleles**		**Culprit antiepileptic**
SB	07:05	44:02	02:01	24:02	lamotrigine + valproate
MV	15:01	35:01	02:01	24:02	lamotrigine
ÇK	40:02	51:01	11:01	32:01	lamotrigine + topiramate
GY	38:01	49:01	01:01	03:01	lamotrigine
MG	35:03	35:03	24:02	32:01	lamotrigine + oxcarbazepine
PB (TEN)	27:07	51:01	24:02	24:03	carbamazepine + phenytoin

Abbreviations: SJS, Stevens-Johnson syndrome; TEN, toxic epidermal necrolysis.

Table IV *- The HLA-B and HLA-A alleles of 7 patients with DRESS / DIHSS induced by antiepileptic drugs. B*35:02 allele was present in two more patients with MPE.*

Patients	HLA-B alleles		HLA-A alleles		Culprit Antiepileptics
FY	18:01	35:08	24:02	32:01	pyrimidone
FK	15:17	53:01	02:01	68:02	lamotrigine
NÇ	07:02	51:01	11:01	24:02	carbamazepine
PÇ	08:01	51:01	01:01	26:01	carbamazepine
NÖ	27:04	**35:02**	03:01	24:02	pregabaline
SB	**35:02**	51:01	31:01	68:01	carbamazepine
ŞB	44:03	51:01	02:01	03:01	carbamazepine

DRESS, drug rush with eosinophilia and systemic symptoms; DIHSS, drug induced hypersensitivity syndrome; MPE; maculopapular exanthema.

/ DIHSS) while it did not exist in the AED tolerant group, and it was found in only one subject in the healthy control group (**Table IV**). This difference was statistically significant (p = 0.021, OR 10.48, 95% CI = 1.205 - 95.336).

In HLA-A evaluation, no predominant allele frequency was found in any of the groups. HLA-A* 31:01 existed in 3 of the AED reactants (one DRESS, two MPE) while it was found in 2 of the non-reactants (p = 0.26). The HLA B*15:02 was not detected in any of the samples.

Discussion

This study is the first one to evaluate an association between AED hypersensitivity and HLA alleles in the Turkish population. Briefly, we did not find any specific HLA A or B alleles in delayed cutaneous reactions to AEDs. The results related to HLA-B*15:02 allele association in SJS to CBZ were in accordance with European studies. However, HLA-B*35:02, which has not been described as an associated marker with AED hypersensitivity in previous trials, was detected in our study group. HLA-B*15:02 allele is accepted as a strong marker for CBZ induced SJS / TEN in the patients with Asian descent. However, previous studies conducted in European countries showed that HLA-B*15:02 was a very rare allele in *Caucasians*, indicating that this marker cannot be used universally for CBZ related SJS (12,13). Lonjou et al. also did not find the existence of an alternative single HLA-B allele with a very strong association in Europe, except for four patients with Asian ancestry who presented HLA-B*15:02. They proposed that different alleles at the same locus could be responsible for increased disease risk in different populations (13). Our country is located in the middle of Asia and Europe continents and the majority of the people living in Turkey are *Caucasians*; expectedly, our results were in accordance with European results.

Another genetic association proposed for AED-induced severe cutaneous reactions is HLA*31:01, which was first shown in CBZ related MPE and DRESS in Han Chinese (14). Later, HLA-A*31:01 was reported to associate with all phenotypes of CBZ-induced cutaneous reactions, including MPE, DRESS and SJS / TEN in Europeans and Japanese (10,11). More recently, it was demonstrated that HLA-A*31:01 was only associated with CBZ-DRESS but not with CBZ-SJS / TEN in Europeans or Chinese (15). The authors found that HLA-A*31:01 had a sensitivity of 70% and a specificity of 96% as a predictor for CBZ-DRESS in Europeans, yet had a sensitivity of 50% and a specificity of 96% in Chinese. In our series, we observed HLA*31:01 in 3 of 40 AED reactive patients (one of 7 DRESS / DHSS and 2 of 26 MPE) and 2 of 60 tolerant patients (not-significant). Accordingly, HLA*31:01 does not seem to be related with DRESS / DHSS or SJS in our study patients. However, this result needs to be cautiously evaluated as the number of the patients presenting with DRESS / DHSS in our group is very limited.

As similar to our results, in another study conducted in patients of European origin, no single major HLA-related genetic risk factor was identified for lamotrigine-induced SCARs (15). The authors obtained only suggestive evidence for B*58:01, A*68:01, Cw*07:18, DQB1*06:09, and DRB1*13:01. We detected B*58:01 in only two patients with MPE (one with lamotrigine, the second with CBZ). Although 4 reactive and 2 non-reactive patients presented HLA-A*68:01, this difference was not significant. But interestingly, one patient with DRESS caused by CBZ displayed A*68:01 allele together with A*31:01. The possibility that this special haplotype may reflect a risk for DRESS needs further exploration in a larger sample series.

Apart from the previously reported HLA alleles in these particular reactions, we found a possible new HLA allele association in delayed type cutaneous reactions to AEDs. In this sense HLA-B*35:02, which has not been described as an associated marker with AED hypersensitivity in previous trials, was detected in four patients of the AED reactive group, while it was absent in AED tolerant patients, and was present in only one of healthy subjects. Although

Table V *- HLA alleles of antiepileptic reactive patients.*

	HLA-B	HLA-B	HLA-A	HLA-A
FK	15:17	53:01	02:01	68:02
FA	55:01	55:01	03:01	24:02
VA	38:01	53:01	01:01	24:02
FY	18:01	35:08	24:02	32:01
TK	51:01	57:01	01:01	01:01
SB	07:05	44:02	02:01	24:02
NÇ	07:02	51:01	11:01	24:02
ABK	18:01	35:03	01:01	02:01
PÇ	08:01	51:01	01:01	26:01
NÖ	27:04	**35:02**	03:01	24:02
DE	35:01	50:01	02:02	03:01
EŞT	08:01	47:01	01:01	01:01
MV	15:01	35:01	02:01	24:02
AT	40:02	51:01	03:01	24:02
ÇK	44:02	55:01	11:01	32:01
SB	**35:02**	51:01	31:01	68:01
MÜ	38:01	58:01	26:01	33:03
HG	44:02	47:01	02:01	03:xx
HL	50:01	51:01	02:01	24:02
EŞ	15:01	51:01	02:01	24:02
NA	35:01	35:03	02:01	68:01
GY	38:01	49:01	01:01	03:01
MG	35:03	35:03	24:02	32:01
KK	14:02	**35:02**	02:01	03:01
SK	15:01	15:01	02:01	03:02
AD	35:03	50:01	02:05	31:01
BM	07:05	58:01	03:01	03:02
SY	**35:02**	44:02	24:02	31:01
YS	40:02	51:01	02:01	68:01
AV	51:01	55:01	02:01	24:02
PB	27:07	51:01	24:02	24:03
AK	07:02	57:01	30:01	30:01
SK	53:01	55:02	02:01	03:01
HT	18:01	35:01	02:17	03:02
ŞB	44:03	51:01	02:01	03:01
MK	40:01	44:02	02:01	24:02
SD	35:03	35:08	01:01	24:02
ND	15:03	41:01	01:01	02:05
DH	15:18	35:01	03:01	68:01
ES	13:01	55:01	11:01	30:01

Table VI *- HLA alleles of antiepileptic tolerant patients.*

Case	HLA-B	HLA-B	HLA-A	HLA-A
MY	38:01	44:02	03:02	26:01
EE	08:01	38:01	01:01	11:01
NZ	35:01	58:01	11:01	31:01
GÇ	08:01	13:02	01:01	30:01
ÜG	08:01	35:01	01:01	03:01
ÜA	07:05	55:01	02:01	26:01
GÜ	41:01	51:01	24:02	32:01
KT	35:01	55:01	03:01	24:02
ŞÇ	15:01	53:01	30:02	68:01
KA	07:02	08:01	03:01	30:01
BAT	27:03	44:03	02:01	33:01
KES	40:01	41:01	02:01	02:02
CE	27:02	55:01	01:01	24:02
ME	07:02	51:01	02:17	24:02
SY	14:02	55:01	24:02	33:01
ST	51:01	51:01	01:01	11:01
NU	50:01	51:01	01:01	02:01
ÖK	35:03	49:01	02:01	24:02
HS	13:01	51:09	32:01	68:01
Mİ	07:02	35:01	03:01	11:01
EY	44:03	51:01	02:01	02:01
MÖ	13:02	35:03	11:01	11:01
HBB	38:01	40:01	25:01	32:01
MY	08:01	18:01	03:02	32:01
AA	14:02	58:01	11:01	33:01
CU	40:01	55:01	02:01	02:01
ET	13:02	27:03	02:01	30:01
AKA	37:01	39:01	01:01	26:01
HSE	44:03	51:01	01:01	29:02
LU	44:03	58:01	01:01	02:01
FU	35:01	38:01	01:01	03:02
MA	07:02	51:01	02:01	24:02
GG	18:01	44:02	11:01	24:02
KD	13:01	51:01	02:01	30:01
MA	27:02	51:01	02:01	26:01
SK	07:02	44:03	02:01	33:03
ÖK	51:01	57:01	01:01	24:02
ÖE	51:01	55:01	26:01	26:01
YB	35:01	35:01	03:01	03:01
GT	35:03	55:01	03:01	24:02
SÇ	08:01	40:01	26:01	26:01
SA	07:02	14:02	11:01	33:01

Table VI *- HLA alleles of antiepileptic tolerant patients.*

FZÇ	44:02	57:01	02:01	24:02
NG	35:01	44:02	02:01	03:01
MD	50:01	51:01	02:01	26:01
BM	35:03	35:03	24:02	33:03
YD	48:01	52:01	11:01	24:02
GY	35:03	39:06	03:01	31:01
HY	08:01	18:01	24:02	30:02
İS	35:01	51:01	02:01	24:02
AFM	38:01	38:01	03:01	33:03
İA	38:01	38:01	26:01	26:01
EB	44:02	48:01	24:02	26:01
EG	08:01	14:02	30:02	33:01
EA	51:01	58:01	02:01	02:01
EÇ	35:01	40:01	02:01	24:02
KK	15:01	38:01	02:01	11:01
PK	14:02	18:01	33:01	66:01
MMÖ	51:01	55:01	01:01	02:01
HP	07:02	35:01	02:01	02:01

this association seems to be inspiring, the results to be derived from more patients are needed to draw more definite conclusion and whether or not this HLA allele is really associated with certain type of reaction with certain AEDs in Turkish population.

We have some limitations in this study. Both small sample size for each SCAR and the heterogeneity of the responsible AEDs were the main limiting factors. The many previous studies on CBZ hypersensitivity reactions suffered from the low incidences, difficulty of patient's enrollment, and small sample sizes (17). Focusing on a specific drug and analyzing HLA genotype-phenotype correlations in the clinical spectrum, could provide more comprehensive pharmacogenetic data in AED-induced hypersensitivity reactions. So far, there is no information about the frequency of severe drug reactions to antiepileptic drugs in the Turkish population. Accordingly, in this preliminary study the target drug and the number of centers needed to provide a sufficient sample size could not be predicted.

In conclusion, the results of this preliminary study demonstrated that any HLA-A or B allele was not strongly associated with cutaneous adverse reactions to AEDs in the study patients. The weak HLA*35:02 association needs to be clarified. Therefore, further studies with a larger sample size would be helpful in analyzing genetic tendency for AED hypersensitivity in the Turkish population.

Conflict of interest

The authors declare that they have no conflict of interest.

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Bodybuilding protein supplements and cow's milk allergy in adult

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Key words

adult-onset cow's milk allergy; cow's milk proteins; gastrointestinal symptoms; protein supplements; bodybuilding

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Summary

We report a case of a previously healthy 24-year-old man with a 3-month history of gastrointestinal symptoms during exercise and also few minutes after the ingestion of cow's milk (CM) without exercise. He reported the ingestion of a blend of hydrolyzed whey and casein proteins for bodybuilding for the last 2 years. The in vivo tests showed positivity to CM, α-lactalbumin, β-lactoglobulin and casein extracts, and also to the protein supplement. The serum specific IgE was positive for CM, β-lactoglobulin and α-lactalbumin. The in vivo and in vitro tests results suggested an IgE-mediated CMA. Adult-onset CMA has been rarely reported, and to our knowledge this is the first case possibly related to bodybuilding supplements. The authors theorize that the presentation of large amounts of proteins in the gastrointestinal tract may favor sensitization.

Previous presentation

The data reported in this study was presented by Maria João Sousa (co-authors: Ferreira AR, Moreira Silva JP) at the EAACI Congress 2015 in Barcelona (Spain) 6-10 June 2015, as a poster entitled "Adult-onset cow's milk allergy and Bodybuilding: is there a connection?".

It was awarded the Poster Prize (Thematic Poster Session 54) and was published in the abstract form in Allergy 2015; 70(Suppl. S101):611; at the 36a Reunião Anual da Sociedade Portuguesa de Alergologia e Imunologia Clínica in Coimbra (Portugal) 9-11 October 2015, as a poster entitled "Alergia às proteínas do leite de vaca: uma apresentação incomum".

It was awarded the 1st Poster Prize (Poster Session IV) and was published in the abstract form in Rev Port Imunoalergologia 2015; 23(Suppl. 1):64.

Introduction

Food allergy is characterized by a specific immune response that reproducibly occurs upon exposure to a given food (1). Cow's milk (CM) is one of the main causes of food allergy in the first years of life, but adult-onset cow's milk allergy (CMA) is rare, with an estimated prevalence of 0.49-0.6% (2). The mechanisms underlying sensitization after the first years of life are unknown. Diagnosis of food allergies should be based on a thorough patient history, skin tests, specific IgE and confirmed by double-blind, placebo-controlled food challenge (DBPCFC) (1).

Case report

The authors report the case of a 24-year-old man referred to our Allergology Department for suspected CMA. He reported a 3-month history of recurrent episodes of abdominal discomfort

and nausea during physical exercise, with progressive worsening to abdominal pain and vomiting. These reactions occurred exclusively during exercise at the gym, and the symptoms improved without medication in a few hours. These complaints were never associated with mucocutaneous, respiratory or cardiovascular manifestations; the patient had never attended the emergency department, nor previously sought medical care for those complaints. Later, the patient noticed that reproducible symptoms were also elicited few minutes after the ingestion of CM, which he previously tolerated, without association with exercise. The patient had no previous history of allergy or intolerance to foods or drugs, nor other atopic manifestations.

When questioned about food ingestion prior to exercise, he reported the regular ingestion of protein supplements for muscle building before and during exercise at the gym for the last 2 years, and for the last 2 months he noticed moderate rhinoconjunctivitis symptoms while preparing it. These protein blends are used as a food supplement for improving athletic performance and muscular mass, and the one the patient ingested consisted of a protein blend of high concentration of hydrolyzed whey protein isolates (a mixture of mostly β-lactoglobulin and α-lactoalbumin) with 24 grams of whey protein per dose (dose: 30.4 g of supplement). Previously, he changed the supplement for another one containing mostly casein (25 grams of protein per dose; dose: 31 g of supplement) but the abdominal symptoms remained during exercise, as well as rhinoconjuntivitis during preparation. We stress that one dose of these supplements has a protein content about 8 times the amount of protein in cow's milk (3-3.5 g).

Later, he started a 100% vegetable protein supplement, with tolerance, and continued physical activity uneventful. Few weeks later, after the development of oral allergy symptoms with yogurt and cheese, he began avoidance of dairy products, maintaining ingestion of baked products with milk, as cookies and cakes.

On admission to our department, physical examination was normal. Skin prick tests (SPT) using commercial extracts of aeroallergens, milk and milk proteins (Bial-Aristegui, Bilbao, Spain) were performed and interpreted as previously described (3). Skin prick-to-prick tests (SPPT) with CM, casein and vegetable supplements were also performed. SPT were positive to cow's milk (12 mm), α-lactalbumin (16 mm), β-lactoglobulin (9.5 mm) and casein (8.5 mm) extracts, and negative for all other allergens tested (including mammalian meats). SPPT were positive to CM and the casein supplement aforementioned, and negative for the vegetable supplement. The results of laboratory studies (full blood count, erythrocyte sedimentation rate, serum electrolytes, and liver and renal function tests) were within normal range. Total and specific IgE (sIgE) to cow's milk and cow's milk proteins were measured using ImmunoCAP FEIA system® (Thermo Fisher Scientific, Uppsala, Sweden). Total serum IgE was 37.5 KU/L; sIgE was positive for CM (18.20 KU/L), for β-lactoglobulin (12.80 KU/L) and for α-lactalbumin (10.50 KU/L), and negative for casein (0.08 KU/L). Lactose intolerance was excluded by a negative Hydrogen Breath Test. The patient refused to perform oral food challenge with milk.

Patient education included a written emergency plan and a prescription of anti-histamine and systemic corticosteroids, to be used in case of allergic reaction upon accidental exposure, maintaining the ingestion of tolerated foods with baked milk. A reevaluation appointment after 4 months was scheduled, but the patient did not attend. After telephone contact, the patient mentioned to keep avoidance of cow's milk and dairy products without history of adverse reactions with accidental exposure.

Discussion

Adult-onset CMA has been rarely reported, and to our knowledge this is the first case possibly related to bodybuilding protein supplements. This patient never had a previous history of food allergy, which makes us assume that this is an adult-onset CMA. Data on CMA in adults are scarce (4). CMA in adults is more likely to be severe and persistent in adults (4,5). Previous studies have shown that spontaneous tolerance in adult CMA rarely occurs (4,6), and during follow-up DBPCFC should be used to evaluate tolerance acquisition (4).

The immunological mechanisms that lead to the development of CMA are still not clarified. To prevent an indiscriminate immunization, secondary to the absorption of foreign antigens through the gastrointestinal barrier, the gut has developed non-specific, non-immunological mechanisms, and specific, immunological factors, such as the production of secretory IgA and antigen interaction with the Gut Associated Lymphoid Tissue (GALT). In normal individuals, antigen presenting cells, mostly dendritic cells (in GALT), process food antigens and present them on a major histocompatibility complex class II receptor to T cells, resulting in a status of immunologic homeostasis known as oral tolerance, characterized by the deletion of antigen-specific T cells and production of regulatory T cells (Treg) that suppress inflammatory responses to antigens (7).

A defect in oral tolerance is thought to be the underlying cause of food allergy (8). A decreased immune response towards foreign antigens, resulting in dysfunction on Treg cellular activity, seems to be the necessary background for both IgE- and non IgE-mediated CMA (9). The increased antigenic load combined with factors such as atopic predisposition, may initiate an abnormal mucosal immune response resulting in chronic enteropathy (10). Gastrointestinal symptoms in food allergy have been explained by alterations in transport across the intestinal wall (increased secretory and/or decreased absorptive functions), increased permeability and mobility of the intestine (10).

The authors theorize that, in this patient, a temporary dysfunction of the protective mechanisms with loss of tolerance may have occurred. The presentation of large amounts of CM proteins in the gastrointestinal tract (increased antigenic load), associated with exercise (increased intestinal permeability), may have favored sensitization and allergy. The widespread use of protein supplements may contribute to adult-onset CMA prevalence increase.

Conflict of interest

The authors declare that they have no conflict of interest

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An unusual case of positive sIgE to Galactose-alpha-1,3-galactose from South Italy

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Key words

sIgE to α-Gal; delayed food anaphylaxis; tick bit; basophil activation test; hunter

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Summary

We report the case of a 38-year-old man who was bitten several times during his life by a tick. He didn't report any previous history of anaphylaxis after the ingestion of red meat. The serum specific IgE showed positivity to α-Gal. The proximity of the bits didn't increase the titer of IgE antibodies to alpha-gal. We could hypothesize that the frequency of the exposure to the tick bites and the amount of tick bites during his lifetime induced a sort of tolerance in this patient.

Introduction

A new form of delayed anaphylaxis has been observed in the United States since 2009 (1), due to IgE antibody directed at a mammalian oligosaccharide epitope, galactose-alpha-1,3-galactose (α-Gal).
This antibody was recently identified in two subsets of patients: a, patients who had developed severe anaphylactic reactions at the first treatment with cetuximab (2); and b, patients who had developed delayed-onset anaphylaxis 3-6 hours after the ingestion of red meat.
Further studies strongly suggested that tick bites were a cause, if not the only significant cause, of IgE Ab responses to α-Gal in the United States (3). Subsequently, in Australia (4) and in Europe (5) cases of delayed anaphylaxis related to IgE-mediates alpha-gal sensitisation have been described, although the tick responsible seems to be *Ixodes* in Europe, while *Amblyoma americanum* seems to be the vector in the USA.
Recently, in Italy both allergy (6) and a high prevalence of sIgE to Galactose-alpha-1,3-galactose have been described in rural areas (7). Therefore, we've decided to investigate if this oligosaccharide could be detected in South Italy.

Material and methods

We report a case of a 38-years-old man who was bitten by a tick on December 2016 and went to the Zooprophylactic institute of Palermo to control if Rickettsia infection developed.

A serum sample and an interview were obtained from this man, that was a veterinarian from Bisacquino, a little town placed at 710 meter above the sea, in West Sicily. He told us that while he was hunting, he was bitten by an insect that he recognized as a tick. He brought the insect to the Zooprophylactic institute for identification, and they confirmed that the insect was a tick from the *Ixodes ricinus* species. This type of tick is frequently found in the hunting area of Bisacquino.

Immediately, they analyzed the serum for antibodies to Rickettsia, and the result was negative.

A detailed clinical history was collected. The patient used to hunt 3 times a year. He reported that he was bitten by a tick 3 times / year minimum for almost 20 years during his lifetime. As usually, last year he was bitten 3 times (once in January, twice in December with an interval of 20 days) and once in January 2017.

Surprisingly, he had been eating every kind of meat (pork, beef, lamb, chicken, rabbit, liver meat, etc) after all episodes of tick bit, without developing any early or delayed allergic symptoms. No previous history of anaphylaxis after the ingestion of red meat was reported. No history of malignancy in therapy with cetuximab or other similar was reported. Only a positive history of seasonal rhinitis was reported in the last year.

ImmunoCap technique (Thermofisher, Sweden) was used to measured sIgE antibodies to α-Gal in the sera of the patient collected on December 2016 (20 days after the first tick bite in that month) and 1 day after the last tick bit (January 2017). For sIgE, the cut-off used for a positive reaction was ≥ 0.1 kU_A/L as suggested by the manufacturer. Surprisingly, the results were 1.08 kU_A/L and 0.58 kU_A/L, respectively. sIgE for pork, beef, lamb and milk were negative. sIgE for inhaled allergens were positive only for *Dermatophagoides pteronyssinus* 1.73 kU_A/L, *Parietaria* 16.7 kU_A/L and *Cupressus* 1.82 kU_A/L. *Dermatophagoides farinae*, *Graminacea mix*, olive, cat, dog and *Alternaria*, were negative. Total IgE was 53.8 kU_A/L. The day after the last tick bit, basophil activation test with beef and cetuximab in different dilution was performed. Interesting, the results of the basophil activation with both allergens were below the cut-off suggested by the manufacturer (≥ 15% of activated basophils for food, and ≥ 5% for drugs). (**Figure 1a, 1b**)

Discussion

To our knowledge, this is the first time that a case of positive α-Gal patient without symptoms has been reported in Italy.

It has been described that the IgE Ab to α-Gal appears to decrease over time, but this trend can be reversed by additional tick bites (8). Thus, patients can be led to believe that they are no longer allergic to mammalian meat because they have eaten small amounts of meat without reactions (likely, their IgE Ab to α-Gal has fallen quite low). Overall, the factors which feed into the equation to produce a reaction are clearly complex and variable, especially in the setting of an IgE Ab to α-Gal that may 'naturally' decrease over time (9).

In this patients, the risk factors like number of tick bites during the lifetime and recent tick bites did not show a higher value of positivity, as it has been described by Villalta et al. (7). On the other hand, we agree with this author with respect to the risk factors associated with increased sIgE to α-Gal, like atopy (this patient suffer from a poly-sensitized allergic rhinitis), male gender and hunting.

Interesting, the results of the basophil activation test (below the cut-off for beef and cetuximab) correlate with the absence of clinical symptoms in this patient.

Until today, this patient hasn't developed any type of allergic reaction after eating any type of red meat. Very interestingly, the proximity of the bits hasn't increased the titer of IgE antibodies to alpha-gal.

Allergy is not a disease in itself, but a mechanism leading to disease that not always is clinically manifested. Sensitization is a complex interplay among the individual exposed (inherited risk of becoming allergic), the timing of exposure, the dose, the context of exposure and endogenous properties of the allergen (10). Therefore, we could hypothesize that the frequency (minimum 3 times / year) of the exposure to the tick bites and the amount of tick bites during his lifetime have induced a sort of tolerance in this patient.

Figure 1a *- % of CD 63 activated basophils with beef.*

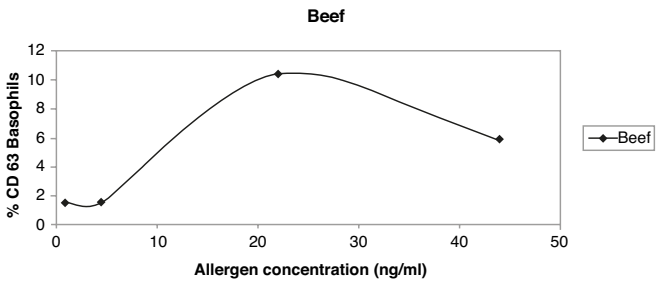
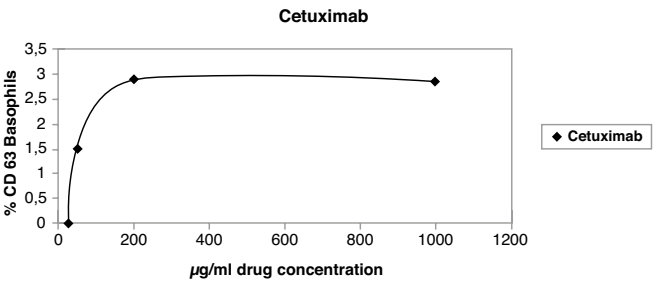


Figure 1b *- % of CD 63 activated basophils with cetuximab.*



Conclusion

The evidence about ticks as a cause of the IgE response is good, but certainly it does not exclude a role for other parasitic exposures (11).

Why some positive α-Gal patients develop delayed-onset anaphylaxis after the ingestion of red meat and others do not, is a question that needs to be answered. Immunological studies need to be done to clarify this.

Conflict of interest

The authors declare that they have no conflict of interest.

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