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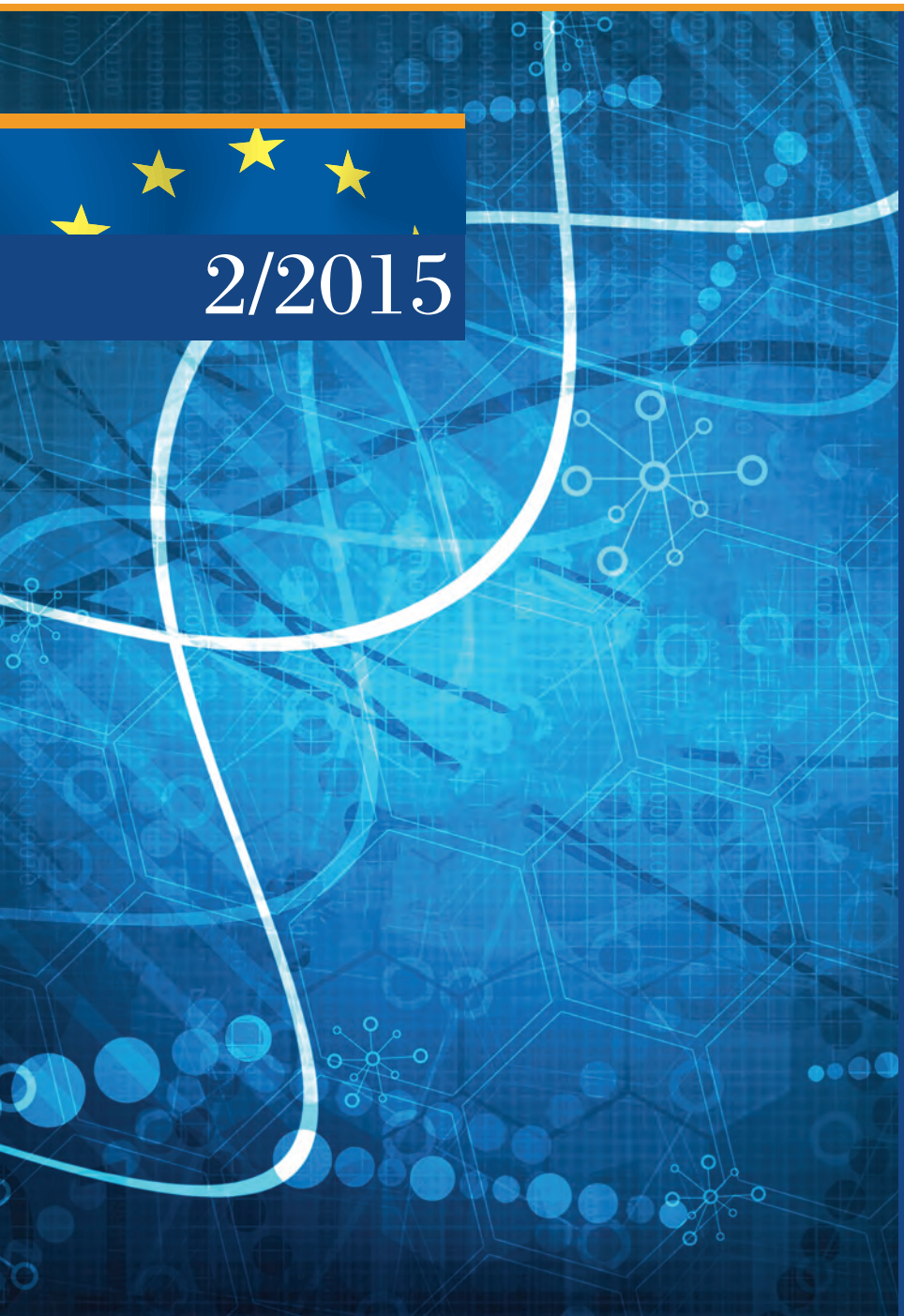

2/2015

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G. Maietta

Basophil activation test: do not lose control

Ambulatorio di Allergologia, ASL Lecce, Italy
Laboratorio di Immunologia, Istituto Pignatelli, Lecce, Italy

Key Words
Basophil Activation Test; Omalizumab; biomarkers of desentisization

Corresponding author
Gennaro Maietta
Istituto Pignatelli
Via Martiri d'Otranto 2
73100 Lecce, Italy
E-mail: gennaromaietta@pec.it

Basophils, as mastcells, represent the military arm of IgE-mediated immune response. Plasma cells-secreted IgE sensitize mast cells and basophils by binding to FcεRI. Subsequent exposure to the allergen leads to the activation of these cells by bridging/cross-linking of FcεRI receptors. The release of various mediators such as histamine, leukotrienes, prostaglandins and citokines is responsible of cutaneous symptoms (e.g., urticaria or angioedema), respiratory symptoms (e.g., asthma), and in some cases anaphylaxis.

Basophil Activation Test (BAT) is an amazing "*in vitro*" method, able to simulate the encounter between basophils and the allergen and to assess the subsequent cellular activation by analising the expression of activation markers on cell surface by flow cytometry. CD203c (a member of ectonucleotide pyrophosphatase/phosphodiesterase family) and CD63 (a protein associated with intracellular vesicles membranes) are the most reliable basophils activation markers presently available (1-3).

The test is performed using whole blood rather than isolated leukocytes, due both to the simpler and faster manipulation of the method, but also for the belief that leaving basophils in their natural environment ensures a better functionality (4).

Until ten years ago, BAT was used as a diagnostic method in drug allergy, with controversial results in terms of sensibility and specificity of different drugs evaluated.

During the last years, several scientists have shown the usefulnes of BAT as a functional assay, able to analyse the cellular activation threshold toward an allergen. In this way, BAT has been used to monitor the development of tolerance in children with food allergy before oral challenges (5,6). Other data showed the usefulness of BAT in the evaluation of tolerance induction in venom-allergic patients treated with specific immunotherapy (SIT), in order to predict the outcome of SIT and clinical sensitivity of the patient (7).

In the light of this novel use of BAT in allergy diagnosis and monitoring, the paper by Pereira Santos *et al.* about "the expres-

sion of FcεRI, IgE on basophils and dendritic cells in association with basophil function in two patients with severe allergic asthma treated with Omalizumab" appearing in this issue of European Annals of Allergy and Clinical Immunology, is very current and interesting. In this paper, the authors describe the evolution of IgE and FcεRI expression on different cell types, and changes in basophil activation following allergen stimulation before and during successful omalizumab treatment in two severe mite-allergic asthmatic patients.

After omalizumab treatment, the authors observed significant reductions of surface IgE and FcεRI expression on basophils, myeloid dendritic cells and plasmacytoid dentritic cells. By performing BAT, following mite stimulation, they observed a parallel trend with reduction in basophil reactivity in both patients during the first month, with additional reductions between months 1 and 12 of omalizumab treatment.

These data raise the possibility that BAT could be indicative of a complete, incomplete or non-response to omalizumab.

Whether BAT might also predict a possible relapse occurring after omalizumab discontinuation, represents a fascinating question.

In the present issue of European Annals of Allergy and Clinical Immunology, the paper by Pereira Santos *et al.* leads us to some technical considerations, particularly concerning the evaluation of basophil reactivity after allergen stimulation.

One of the crucial points in the sequential analyses performed to evaluate changes in basophil reactivity at different time steps (days or months) during a drug or SIT treatment, is a correct evaluation of the intrinsic cellular reactivity, which can vary over time. Basophil intrinsic reactivity may change from day to day and month to month. For this reason it is extremely important that a positive control able to check basophil specific immunologic intrinsic *IgE mediated* response is used, along with a negative control when BAT is performed. Monoclonal antibody anti FcεRI represents the best one, because it is able to induce the maximum FcεRI-mediated cellular activation (4).

Another crucial point is represented by the observation that basophils change their intrinsic reactivity over time. One can observe different values of anti-FcεRI-induced basophil activation if BAT is carried out in different times. For this reason, it is crucial to evaluate basophil activation after allergen stimulus by taking basophil intrinsic reactivity into account. The best evaluation of specific allergen basophil activation is performed by applying the following formula: [allergen basophil activation (%) / anti-FcεRI (%)] x100. This formula allows to relate BAT result after allergen stimulus with intrinsic basophil reactivity at the time when the test was performed, and to standardize the data. Clearly, a basophil activation of 45% after allergen stimulation in a patient showing a positive control of 50% has to be evaluated in a different way from the same percentage of activation if the same patient shows a positive control of 80% in another moment of his life.

In conclusion, BAT is a useful method to evaluate basophil reactivity and sensitivity to an allergen, and could be probably used as a biomarker in monitoring drug and/or SIT treatment in IgE-mediated diseases. However, even if you are struck by the charm of this test, remember… NOT TO LOSE CONTROL.

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M.C. Pereira Santos[1], A. Campos Melo[1], A. Caetano[1], J. Caiado[2], A. Mendes[2], M. Pereira Barbosa[2,3], M. Branco Ferreira[2,3]

Longitudinal study of the expression of FcεRI and IgE on basophils and dendritic cells in association with basophil function in two patients with severe allergic asthma treated with Omalizumab

[1]Unidade de Imunologia Clínica, Instituto de Medicina Molecular, Faculdade de Medicina da Universidade de Lisboa, Lisboa, Portugal
[2]Serviço de Imunoalergologia, Hospital Santa Maria, Centro Hospitalar de Lisboa Norte, Lisboa, Portugal
[3]Faculdade de Medicina da Universidade de Lisboa, Lisboa, Portugal

Key words

Asthma; basophils; dendritic cells; IgE; IgE receptor; omalizumab

Summary

Severe asthma is a challenging disease, and omalizumab has been an important tool to help clinicians address more efficiently this problem. Besides reduction of free and total serum IgE levels, there are a number of other immunologic effects of omalizumab that may be of relevance in its therapeutic action. We report two mite-allergic severe asthmatic patients successfully treated with omalizumab for one year.

Clinically, patients improved gradually, with no further need for systemic steroids or emergency department visits during that treatment period, and with Asthma Control Test (ACT) scores showing controlled disease, although pulmonary function didn't show any significant improvement.

Immunologically, we observed marked down-regulation of surface IgE and FcεRI on basophils, plasmacytoid and myeloid dendritic cells, as well as a reduction of basophil activation after specific allergen stimulation. These effects were clearly evident immediately after one month but were enhanced at 3, 6 and 12 months of omalizumab treatment, suggesting an advantage to continuing this therapy, and raising the hypothesis of some markers being useful to assess immunological responses to omalizumab, which could assist in the clinician's decision to stop or to restart this treatment.

Introduction

IgE play a central role in the pathogenesis of allergic asthma and chronic airway inflammation via high affinity (FcεRI) or low affinity (FcεRII-CD23) IgE receptors. FcεRI is predominantly expressed on mast cells, basophils and dendritic cells (DC). FcεRII-CD23 is expressed on a wide variety of inflammatory cells (1).

Omalizumab binds to circulating IgE antibodies, reducing serum IgE levels and preventing binding of IgE to high-affinity receptors (2,3). Accompanying the reduction of IgE levels, FcεRI expression is also decreased, leading to a reduction in allergen-mediated activation and degranulation of mast cells and basophils. Recent long-term clinical trials confirm that omalizumab reduces exacerbations and symptoms in adults and children with moderate-to-severe allergic asthma (2-4).

In this paper we describe the evolution of IgE and FcεRI expression on different cell types, and changes in basophil activation following allergen stimulation before and during successful

omalizumab treatment in two severe mite-allergic asthmatic patients.

Case reports

We present two non-related female patients, 25 and 26 years old, allergic to house-dust-mites. Both had long-standing moderate-to-severe asthma and rhinitis, receiving high-dose inhaled steroids, long-acting beta2-agonists, montelukast, nasal steroids and non-sedating H1-antihistamines. Both had uncontrolled disease with daily use of short-acting beta2-agonists and frequent emergency department visits and/or oral steroids (> 6/year). Total IgE levels were 252 and 294 kU/L, respectively. Both patients had been treated with allergen immunotherapy, stopped due to frequent asthma exacerbations.

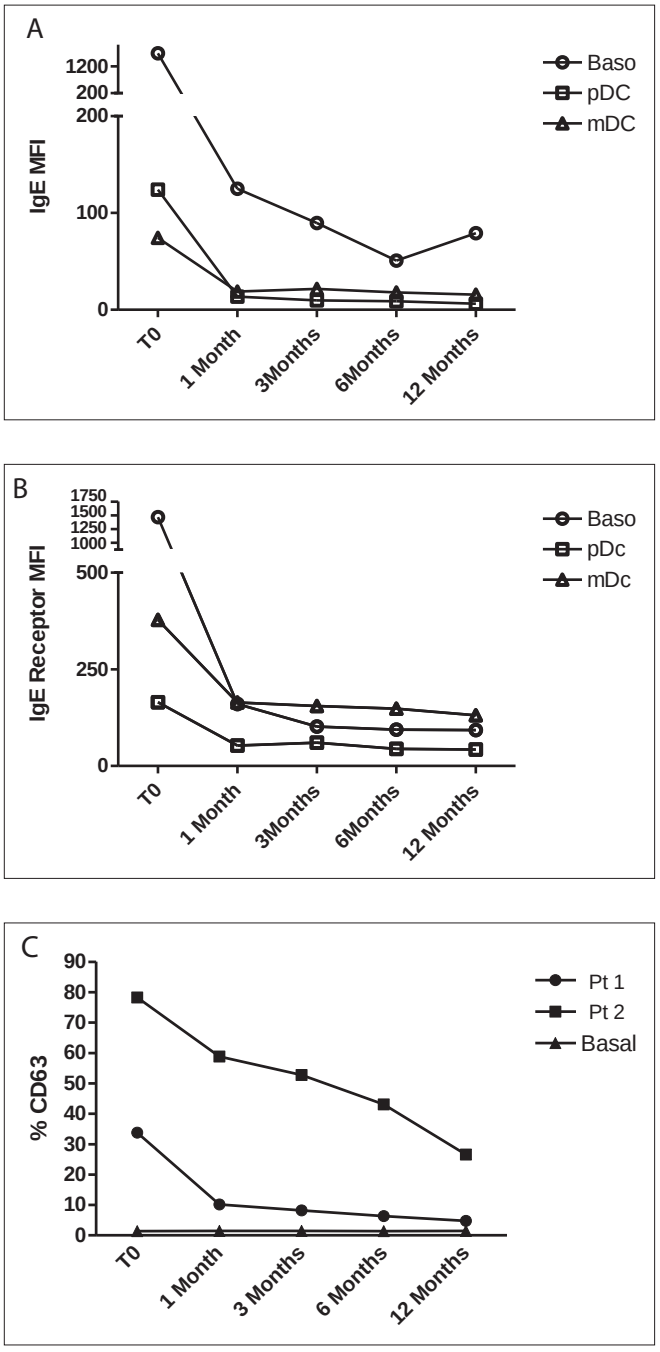
Pulmonary function showed reduced basal FEV1 values (50% and 78% of predicted, respectively) and mid-expiratory flow values (26% and 59%), with a 20-40% increase in all these values after 400 mcg salbutamol inhalation. Asthma control test (ACT) scores were 15 and 16, respectively.

Both patients started omalizumab 300 mg 2/2 weeks in January 2012, maintaining regularly this therapy. From a clinical perspective, both showed clinical improvements, without further need for systemic steroids or emergency department visits. ACT scores improved progressively to 23 and 24 at one-year. However, pulmonary function was only marginally improved.

For the immunologic evaluation blood samples were drawn before (T0) and at 1, 3, 6 and 12 months of omalizumab treatment. Flow cytometry (FACSCalibur, BD-Biosciences) was performed on 100 ul of whole blood using anti-IgE FITC; HLA-DR PerCP, CD123 APC (eBiosciences), FcεRI PE (eBiosciences). IgE and FcεRI expression (mean fluorescence intensity) was evaluated on basophils and on dendritic cells, either myeloid (mDC) or plasmacytoid (pDC), gated according to phenotype (CD123+/HLA-DR-; CD123-/HLA-DR+; CD123+/HLA-DR+, respectively). Additionally, basophil activation was determined according to CD63 expression, before and after allergen stimulation. Briefly, 100 ul of heparinised blood was incubated with a stimulation buffer (containing IL-3). Each sample was tested with negative control (PBS), positive control (N-formylmethionyl-leucil-phenylanine-FMLP) and allergen (*D. Pteronyssinus*). Analysis was performed using FlowJo software.

After the first two omalizumab injections (1 month) we observed significant reductions of surface IgE and FcεRI expression on basophils (93% and 89%, respectively), mDC (75% and 57%) and pDC (89% and 68%). These reductions were enhanced with continuation of therapy, albeit less pronounced (**figures 1a** and **1b**). This evolution was similar in both patients, despite different individual values (data not shown). Regarding basophil activation test (BAT) following mite stimulation, we observed a parallel trend with reductions in both patients in the first month (70% in patient 1 and 45% in patient 2), with additional reductions of 16% and 21% in patients 1 and 2 respectively, between 1 and 12 months (**figure 1c**). We did not find any significant correlations between timings of the immunologic changes and clinical improvement.

Figure 1



Discussion

During one year of omalizumab treatment surface IgE and FcεRI expression on basophils, mDC and pDC were consistently reduced in both asthmatic patients. Maximal reductions of FcεRI expression on basophils and DC were mostly achieved within one month of treatment, with further but smaller reductions during treatment. Substantial reductions of surface IgE and FcεRI expression on basophils and pDC have already been described after 6-52 weeks of omalizumab (2-5). Our study shows that omalizumab's immunologic effects are maintained and enhanced with continuation of therapy, a fact that parallels clinical evolution in most of our patients.

These case-reports highlight two less-studied effects of omalizumab: on mDC and on basophil activation following allergen stimulation. DC are antigen-presenting cells that play crucial roles in immune responses, whether innate or acquired. Some studies have shown in animal models of asthma that pDC play only a limited role in priming T cells in the OVA-allergic mouse model of asthma, while mDC are potent orchestrators of the asthmatic inflammatory response (6) Furthermore, in human lungs, mDC express CD80, CD86 and CD40 costimulatory molecules at higher levels than pDC, and stimulate more efficiently naïve T cell proliferation, suggesting a more important role of mDC in antigen presentation processes (7). Therefore, it is relevant to report that omalizumab reduces IgE and FcεRI expression on mDC, as already described for pDC.

Regarding omalizumab's effect on BAT following allergen stimulation, our study shows that both patients had very significant reductions; however in the patient with higher basal activation, reduction was not enough to render the test negative. This difference didn't correlate with any clinical data, both patients showing approximately the same degree of clinical improvement. Other authors have already described that amongst clinical responders to omalizumab, there are patients who achieve a negative BAT while others remain positive (8,9). In these cases, BAT intensity after omalizumab treatment seems to be more related to pre-treatment values than to the presence or absence of clinical improvement (8,10). Several studies have suggested that basophil response to allergen stimulation may reflect the underlying activity of allergic disease, being reduced after successful allergen immunotherapy (11). Our results suggest that this is also the case after successful omalizumab therapy.

In conclusion, these two case-reports of mite-allergic asthmatic patients show that omalizumab treatment induces reduction of different cellular activation mechanisms that can impact on effector mechanisms (basophil degranulation) but also on dendritic cell antigen-presentation mechanisms. These beneficial effects are evident immediately after the first two injections, and reinforced throughout the duration of therapy. These data raise the hypothesis that some laboratory cut-off values could be indicative of a complete, incomplete or non-response to omalizumab.

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E. Galli, L. Rocchi, R. Carello, P.G. Giampietro, P. Panei[1], P. Meglio

Serum Vitamin D levels and Vitamin D supplementation do not correlate with the severity of chronic eczema in children

Department of Pediatric Allergy, Research Centre, San Pietro Hospital - Fatebenefratelli, Rome, Italy
[1]Dipartimento del Farmaco e Terapie Sperimentali, Istituto Superiore di Sanità, Rome, Italy

Key Words
IgE chronic eczema; Not-IgE chronic eczema; Serum vitamin D levels; Vit D supplementation

Corresponding author
Elena Galli
Department of Pediatric Allergy
Research Centre, San Pietro Hospital - Fatebenefratelli
Via Cassia 600, Rome, Italy
Phone: +39 06 335 822 80
Fax: +39 06 332 512 78
E-mail: galli.elena@fbfrm.it

Summary
Background: *Eczema is one of the most common chronic inflammatory skin diseases, affecting about 20% of children. The pathogenic mechanisms of eczema are still not fully understood, and current treatment of moderate-severe eczema is often difficult. Recently, it has been suggested that Vitamin D plays a key role in this disease, even if mechanisms are only partially known.* ***Objective:*** *The purpose of our study was to assess the 25-Hydroxyvitamin D serum levels in a pediatric population suffering from chronic eczema (IgE-mediated and non-IgE-mediated), and to correlate these phenotypes with the SCORAD severity and selected clinical and biological parameters. Moreover, we aimed to evaluate whether a supplementation of Vitamin D3 could affect the same clinical and laboratory parameters.* ***Methods:*** *89 children with chronic eczema were enrolled in the study. Severity of eczema was assessed with the SCORAD index. Past and present history was taken, and patients were divided into two groups according to the state of sensitization. According to a randomization schedule, the enrolled children were assigned to the following groups: supplementation group, which received a daily oral Vitamin D3 supplementation (2000 IUs) for 3 months; control group which received no supplementation.* ***Results:*** *Vitamin D concentrations in patients with moderate and severe eczema were not statistically different from Vitamin D concentration detected in the serum of patients with mild eczema. Furthermore, we did not find any correlation between Vitamin D levels, total IgEs and SCORAD index, both in the Sensitized and in the Not-Sensitized group. The Vitamin D3 supplementation did not influence the SCORAD severity or the total IgEs concentration.* ***Conclusion:*** *To our knowledge, our study is the first one that shows no correlation between serum levels of Vitamin D, eczema severity and IgE sensitization in a pediatric population suffering from chronic eczema.*

Introduction

Eczema is one of the most common chronic inflammatory skin diseases, affecting about 20% of children and 3% of adults (1). It is a frustrating condition for both patients and caregivers, as intractable pruritus can cause sleep disturbance with important physical and psychological implications. Indeed, current treatment of moderate-severe eczema is often difficult (2,3).

Genetic and environmental factors, other than innate and adaptive immune defects, affect the development of eczema, although the pathogenic mechanisms are still not fully understood (4,5).

Recently, it has been shown that Vitamin D (Vit D) plays a key role in the innate and adaptive immunity (6). In the innate immune system, Vit D appears to improve antimicrobial

defences in general. Vit D induces endogenous expression of the antimicrobial peptide cathelicidin. This can be seen in the skin, in monocytes, and in the lung. Because cathelicidin has been found in multiple experimental systems to be essential for defence against a variety of microbial infections, it has been proposed that Vit D can enhance resistance to infections (7). Epithelial cells may express the Vit D receptor, and its activation implies a different expression of an array of target genes which, in turn, can interfere with the inflammation process and immune defence, possibly affecting those immune disorders characterized by an altered Th1/Th2 cytokines balance. The cutaneous production of cathelicidin can inhibit the production of IL-12. In this way the Th1 cell response is downregulated, and the Th2 cell response is upregulated with an IL-4 and IL-5 increase (6-8). This may explain the growing body of evidence connecting Vit D to the allergic disease, even if mechanisms are only partially known.

The purpose of our study was to assess the 25-Hydroxy Vit D (25-OH-D) serum levels in a pediatric population suffering from chronic eczema, both IgE-mediated and non-IgE-mediated type, and to correlate these two phenotypes with the SCORAD severity and selected clinical and biological parameters (allergic diseases, total and specific IgEs). Moreover, we aimed to evaluate whether, independently from baseline levels of Vit D, a daily 2000 IUs supplementation of Vit D3 (*cholecalciferol*) for 3 consecutive months could affect the same clinical and laboratory parameters.

Methods

Study design

We designed a randomized clinical trial. The power was set at 80%, confidence interval at 95%, the sample size was 78 patients for a risk/prevalence rate of approximately 5 (Fleiss).

This randomized open study was carried out in the Pediatric Allergy Unit, Research Centre, S. Peter Hospital, Fatebenefratelli, Roma, Italy, from January 2012 to March 2013.

Eighty-nine consecutive children with chronic eczema (48 boys) with a median age of 68 months (range 6-195 months), diagnosed according to the Hanifin and Rajka criteria (9), were enrolled in the study. Past and present medical history including food allergy, respiratory symptoms and cutaneous infections was taken from patients. Eczema was considered chronic if it lasted at least 6 months. The severity of eczema was evaluated by the same operator (EG) according to the SCORAD method (10). Patients with a SCORAD value less than 25 were considered as having a mild eczema, those with a score from 25 to 50 as having a moderate eczema and those with a SCORAD value greater than 50 as suffering from a severe eczema (**table 1**).

None of the enrolled children suffered from other chronic diseases or were taking topical or oral steroids, vitamins, minerals, fatty acids supplementation or immunosuppressive therapy at the time of investigation and in the last 6 months.

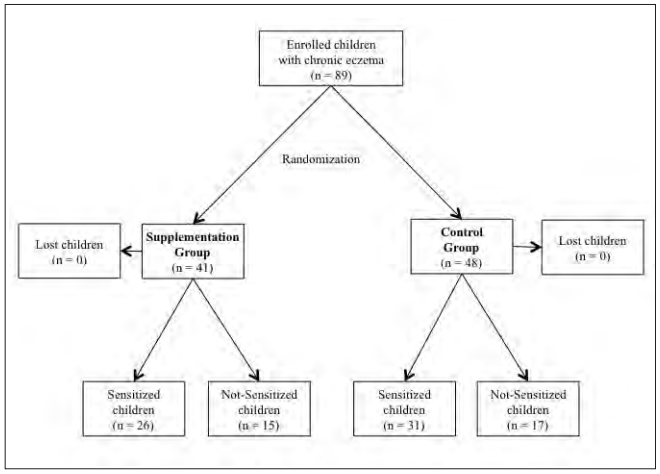
Seventy-one out of 89 patients did not have special dietary restrictions, and everyone enjoyed exposure to sun and ordinary outdoor activities.

After a descriptive analysis, the enrolled children were assigned to one of the 2 following groups according to a randomization schedule: supplementation group (SG) composed by 41 children (median age 91 months, range 11-195 months, 22 males) who were given a daily oral Vit D3 (*cholecalciferol*) supplementation (2000 IUs) for 3 months, in fat soluble form; and a control group (CG) composed by 48 children (median age 57.5 months, range 22-180 months, 26 males) who received no Vit D3 supplementation. The study design is depicted in **figure 1**.

Table 1 *- Characteristics of the 89 enrolled children with chronic eczema.*
RR: Relative Risk; NS: Not Significant; NA: Not Applicable

	BASIC ANALYSIS						
	Whole population	Enrolled children divided according to allergic sensitization			After randomization to Supplementation or Control group		
Variables	Total children (n = 89)	Not-Sensitized children (n =32)	Sensitized children (n =57)	p [RR]	Supplementation group (n = 41)	Control group (n = 48)	p
Demographic parameters							
Age, months, median (range)	68 (6-195)	62 (6-195)	74 (6-192)	NS	91 (11-195)	57.5 (22-180)	p = 0.003
Male, *n* (%)	48 (53.9%)	13	35	NS [RR=1.51 (0.95-2.41)]	22	26	NS
Clinical parameters							
SCORAD score, mean (±SD)	17.5 (±17.2)	16.5 (±16.5)	18.1 (±17.7)	NS	12.2 (±11.1)	22.1 (±20.1)	p = 0.006
Mild (< 25)	66 (74.2%)	22 (68.8%)	44 (77.2%)	NS	7.121 (+3.814)	9.879 (±5.952)	p = 0.029
Moderate (25-50)	17 (19.1%)	8 (25.0%)	9 (15.8%)	NS	29.29 (±1.89)	39.5 (+6.09)	p = 0.000
Severe (> 50)	6 (6.7%)	2 (6.3%)	4 (7.0%)	NS	0	3	NA
Duration of eczema, months, median (±SD)	63	58.41 (±34.89)	65.18 (±38.92)	NS	72.71 (±38.28)	54.23(±34.92)	p = 0.019
Staphylococcal infections	38 (42.7)	3 (9.4%)	35 (61.4%)	p < 0.0001 [RR = 6.55 (2.19-19.61)]	19 (46.3%)	19 (40.4%)	NS
Personal history of atopy	44 (49.4%)	13 (40.6%)	31 (54.4%)	NS [RR=1.34 (0.82-2.16)]	23 (56.1%)	21 (44.7%)	NS
Rhinitis	29 (32.6%)	7 (21.9%)	22 (38.6%)	NS [RR=1.76 (0.84-3.67)]	17 (41.5%)	12 (25.5%)	NS
Asthma/wheezing	34 (38.2%)	9 (28.1%)	25 (43.9%)	NS [RR=1.56 (0.83-2.92)]	16 (39.0)	18 (38.3%)	NS
Food allergy	18 (20.2%)	5 (15.6%)	13 (22.8%)	NS [RR=1.46 (0.57-3.72)]	11 (26.8)	7 (14.9%)	NS
Basic laboratory parameters							
Total IgE (IU/ml), mean (±SD)	376.3 (±837.5)	18.9 (±9.7)	577.0 (±994.0)	p = 0.002	547.5 (±1104.5)	230.2 (±477.2)	NS
25(OH)D$_3$ (ng/ml), mean (±SD)	48.3 (±40.6)	48.8 (±39.3)	48.0 (±41.6)	NS	56.0 (±53.5)	41.6 (±23.4)	NS
Vit D sufficiency (> 30 ng/ml)	48 (53.9%)	18 (56.3%)	30 (52.6%)	NS [RR=0.94 (0.63-1,38)	21 (51.2%)	2 (4.2%)	p = 0.0001
Vit D insufficiency (12-30 ng/ml)	31 (34.8%)	11 (34,4%)	20 (35.1%)	NS [RR=1.66 (0.95-2.91)	12 (29.3%)	19 (39.6%)	NS
Vit D deficiency (< 12 ng/ml)	10 (11.3%)	3 (9.3%)	7 (12.3%)	NS [RR=1.31 (0.36-4.72)	8 (19.5%)	27 (56.3%)	NS

Figure 1 *- Study design.*



Skin prick tests

Skin prick tests (SPTs) were performed on the volar aspect of the forearm for some foods (cow's milk, hen's egg white, wheat, peanut, soy and fish) and common aeroallergens (house dust mites, animal dander, molds and grass pollens). The reaction was read at 15 minutes and SPTs were considered positive if the wheal diameter was at least 3 mm greater than the negative control.

Isolation and identification of bacteria

Swabs were taken from the most severe skin lesion and from the non lesional skin only at the start of the trial. The swabs were plated on to blood agar and cultured. Colonies were grown for 24 h at 37°C. Staphylococcus aureus was identified by testing typical colonies for coagulase activity.

Serum 25-Hydroxy Vit D3, total and specific IgEs

In all the enrolled children 2 peripheral venous blood samples were collected: the first at the beginning of the protocol, and the second after 3 months. Blood samples were centrifuged and serum was stored at -20°C. For each serum sample total and specific IgEs (for the same allergens tested with SPTs) and serum 25-Hydroxy Vit D3 (25-OH VitD3) were determined. Total and specific serum IgEs were measured with a commercially available fluorometric enzyme-linked immunosorbent assay system ImmunoCAP (Thermo Scientific).
Circulating levels of 25-Hydroxy Vit D (25(OH)D) are considered to be the most reliable measure of overall Vit D status (11). Serum 25-OH VitD3 levels were measured by a competitive protein-binding assay with the 25 OH Vit D direct ELISA (DRG International, Inc. USA). Data were analysed by Manta MMaine5 software. For purposes of analyses, the prevalence of Vit D deficiency was based on the proposed definition of < 30 nmol/L (< 12 ng/ml), and cut-off values of 30-75 nmol/L (12-30 ng/ml) and > 75 nmol/L (> 30 ng/ml) were used to describe overt Vit D, insufficiency and sufficiency (12).
In our study, sensitized children have: total IgE > 40 UI/ml, at least 1 positive SPT and/or positive serum specific IgE. Not-sensitized children are those who did not have any positive SPT results and total IgE ≤ 40 UI/ml (**table 1**).

Ethical Concerns

The ethics committee of our hospital approved this study as a part of a registered protocol. The ethical procedures for the study protocol included informed consent of all parents or caregivers for scientific reporting of research findings based on the study protocol.

Statistical Analysis

The sample size was calculated using OpenEpi 3.01. Normality of variables has been tested with the Shapiro-Wilk test. The correlation between the variables examined was made with the Pearson test for continuous variables, and with Spearman test for non-parametric variables. Comparisons were one-tailed, and $p < 0.05$ was considered to be statistically significant. The Student's T test was used to compare the mean values of clinical and laboratory parameters. Statistical analysis was performed using SPSS (version 21.0).
Relative risk was computed using Epiinfo 7.1.2

Results

Descriptive analysis

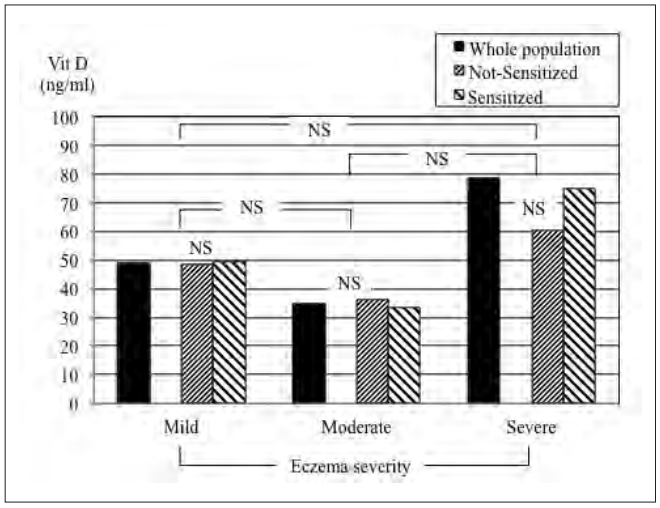
Eighty-nine children (48 males, 53.9%) with chronic eczema were enrolled in the study (**figure 1**). The demographic and clinical data of the whole enrolled population are depicted in **table 1**.

(Whole population)

The majority (53.9%) of the enrolled children had Vit D sufficiency (> 30 ng/ml) and 34,8% presented Vit D insufficiency (12-30 ng/ml). Only 11.3% of children had Vit D deficiency (< 12 ng/ml) (**table 1**).
The mean serum Vit D concentration (78.3 ng/ml ± 71,0) in the patients (77 out of 89) with severe eczema and the mean Vit D concentration (34.9 ng/ml ± 20,0) in the patients (17 out of 89) with moderate eczema were not statistically different from the mean Vit D concentration (49.06 ng/ml ± 40,2) detected in the serum of the patients (66 out of 89) with mild eczema (**figure 2**, black bars).

In the whole enrolled population there was no correlation between Vit D levels and total IgE, food allergy and the frequency of skin impetigo with positive cultures of Staphylococcus aureus (data not shown).

Figure 2 *- Correlation between eczema severity (SCORAD) and Vit D levels in the whole population and when children were divided in Not-Sensitized (total IgEs < 40UI/ml) and Sensitized (total IgEs ≥ 40UI/ml) groups. All correlations were not significant.*



(Not-Sensitized versus Sensitized children)

When the enrolled population was divided according to sensitization - sensitized children (n = 57, 64%) having at least one positive SPT and total IgE > 40 UI/ml - we found no significant differences between Not-Sensitized and Sensitized children for age, sex, SCORAD score, duration of eczema, personal history of atopy, allergic rhinitis, asthma and/or wheezing, food allergy and Vit D concentration. In particular, male sex, personal history of atopy, allergic rhinitis, asthma and/or wheezing and food allergy tended to be a relative risk for the eczema associated to sensitization, but they did not reach the statistical significance (**table 1**).
Moreover, eczema severity did not differ between Not-Sensitized and Sensitized children when they were sub grouped according to the SCORAD severity (mild, moderate or severe eczema) (**figure 3**).
No significant difference was found in Vit D serum levels when children were analysed according to sensitization and severity of eczema (**figure 2**, right bars).
In particular, Vit D levels were sufficient in 18/32 (56.3%) of the Not-Sensitized children and in 30/57 (52.6%) of the Sensitized children; insufficient in 11/32 (34.4%) versus 20/57 (35.1%) and deficient in 3/32 (9.3%) versus 7/57 (12.3%). Also this analysis did not reveal any statistical differences between Not-Sensitized and Sensitized children (**figure 4**).
Furthermore, we did not find any correlation between Vit D levels, total IgEs and SCORAD index, both in the Sensitized and in the Not-Sensitized group (**tables 2** and **3**).
The only parameter strongly associated to eczema in Sensitized children (3/32, 9.4%) with respect to Not-Sensitized (35/57, 61.4%) children were skin staphylococcal infections ($p < 0.0001$ and RR = 6.65 [2.19-19.61]) (**table 1**).

Table 2 *- Relationships between Vit D serum levels, SCORAD index and total IgE in Sensitized children with chronic eczema (57/89 children).*

	Mean (±SD)	p (one-tailed)	
Vit D	48.0 (±41.6)	0.41	0.94
Total IgEs	577.0 (±994.0)		
SCORAD	18.1 (±17.7)	0.55	

Table 3 *- Relationships between Vit D serum levels, SCORAD index and total IgE in Not-Sensitized children with chronic eczema (32/89 children).*

	Mean (±SD)	p (one-tailed)	
Vit D	48.8 (±39.3)	0.994	0.15
Total IgEs	18.9 (±9.7)		
SCORAD	16.5 (±16.5)	0.409	

Intervention analysis

After randomization, 41 children were assigned to the SG (that was given a daily oral Vit D3 supplementation for 3 months), and 48 to the CG. Apart from the enrolment visit, all children were visited again 3 months later and there were no dropouts (**figure 1**). At the second visit, the operator was not aware to which group the subject belonged.
The SG and CG were not homogeneous for either age, SCORAD score or duration of eczema. On the other hand, they were homogeneous for the other parameters considered (sex, secondary bacterial infections, personal history of atopy, asthma and/or wheezing, food allergy, total IgEs and Vit D concentrations) (**table 1**).
In table IV the Vit D serum level, the SCORAD score and total IgE before and after 3 months of cholecalciferol supplementation in the SG (41 children) are compared with data of the CG

Table 4 *- Vit D serum level, SCORAD score and total IgE in children with eczema before and after 3 months of Vit D supplementation (Supplementation Group) or without Vit D supplementation (Control Group).*

	Supplemetation Group (n = 41)		Control Group (n = 48)	
	Time 0	After 3 months (Vit D supplementation)	Time 0	After 3 months (NO Vit D supplementation)
	Mean (±SD)	Mean (±SD)	Mean (±SD)	Mean (±SD)
Vit D	56.0 (±53.5)	105.9 (±76.9)	41.6 (±23.4)	42.0 (±22.4)
	p < 0.001		NS	
SCORAD	12.2 (±11.1)	12.0 (±11.0)	22.1 (±20.1)	20.8 (±18.6)
	NS		NS	
Total IgE	547.5 (±1104.5)	416.9 (±694.9)	230.2 (±477.2)	239.7 (±538.4)
	NS		NS	

Figure 3 *- Mean eczema severity (SCORAD) in the whole population (left side) and % of mild, moderate and severe eczema in Not-Sensitized and Sensitized children with chronic eczema (right side).*

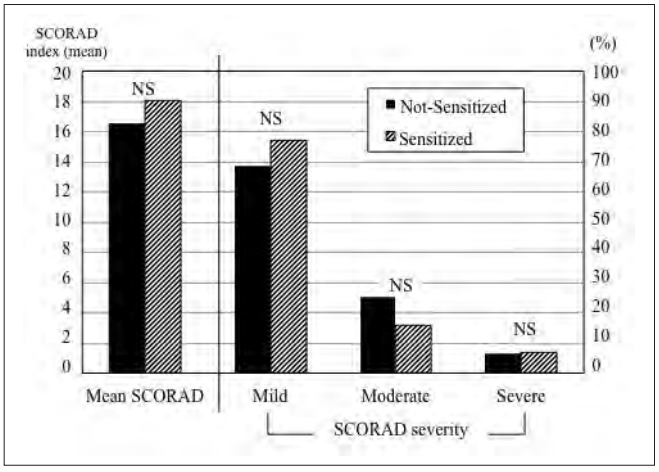
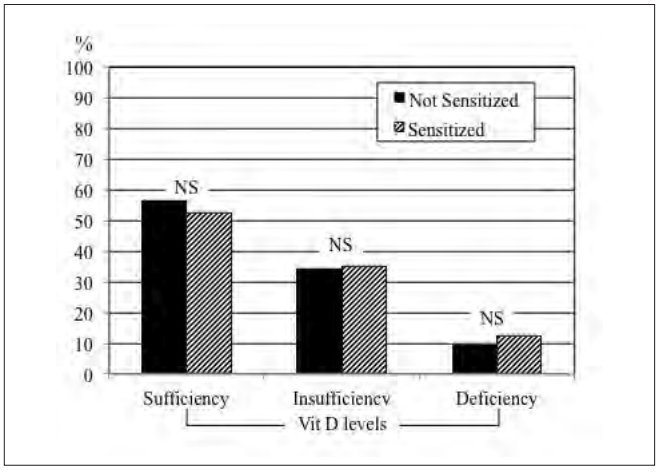


Figure 4 *- Vit D levels (classified as sufficient, insufficient and deficient in Not-Sensitized (total IgE < 40 UI/ml) and Sensitized (total IgE ≥ 40UI/ml) children with chronic eczema.*



(48 children). The daily cholecalciferol supplementation (2000 IUs for 3 months) significantly increased the Vit D levels in the SG with respect to the CG, as expected. Instead, it did not influence the severity SCORAD, or the total IgEs concentrations.

Discussion

In the past ten years, eczema research has focused on the barrier and the innate immune defects, especially in correlation to staphylococcal infections (13). Moreover, it has been suggested that Vit D plays a pivotal role in the innate and adaptive immunity (6). Furthermore, Liu et al. highlighted the connection between Vit D-mediated activation of Toll-like receptors, production of the antimicrobial peptide cathelicidin and human susceptibility to bacterial infections (14). The active form of Vit D - 1.25-$(OH)_2$D - induces the expression of antimicrobial peptides that help in preventing skin infection and possesses immunosuppressive properties in the skin. Moreover, some Authors have shown that it promotes immune tolerance (15,16). Thus, Vit D deficiency could contribute to the hallmark signs of eczema as altered barrier function, immune dysregulation and inadequate bacterial defence (17).

Recently, some of the studies aimed at assessing the impact of Vit D on allergic diseases with a focus on eczema (18,19), and some of them have assessed the prevalence and the severity of eczema in Vit D-deficient patients (20-22).

Furthermore, some Authors suggested the potential role of Vit D only in selected sub-groups of patients with eczema. Indeed, the results of Lee et al. (23) suggest that Vit D deficiency might be related to the severity of eczema only if accompanied by food sensitization, while Akan et al. (24) showed that Vit D might

affect the severity of eczema only in children with allergic sensitization. Finally, Chiu et al. (25) in a cross-sectional study of 94 children with eczema found instead no correlation between serum Vit D concentration and eczema severity.

To our knowledge, our study is the first one that shows no correlation between serum levels of Vit D and eczema severity, IgE sensitization and skin staphylococcal infections in a pediatric population suffering from chronic eczema.

In fact, Vit D levels and the SCORAD index were not correlated in the whole study group of 89 children (**figure 2**) and we did not find any correlation between serum Vit D concentration and eczema severity SCORAD when the whole population was sub grouped according to sensitization (**figure 2** and **tables 1, 2, 3**).

Moreover, our data show there was no correlation between Vit D serum levels and total IgEs neither in the whole study group, nor when the Sensitized and Not-Sensitized subgroups were evaluated separately (**figure 4, tables 1** and **2**).

As for Vit D levels in children with chronic eczema, our data differ from the results previously reported as 53.9% of the enrolled children had sufficient levels of Vit D (> 30 ng/ml) (**table 1**). Further studies are necessary to determine whether Vit D deficiency is really more prevalent in these children or whether other factors such race, geographic or diet can contribute.

Even if Sensitized and Not-Sensitized children may have identical clinical characteristics, they must have some different pathophysiologic mechanisms. Our series, however, shows that Vit D does not express a specific role in modulating the clinical presentation of eczema, despite its proven effects on the immune system (reducing and/or preventing inflammation) (6,16).

So far, there are only a few studies investigating the effects of Vit D supplementation in the treatment of eczema (26-31), and some of these reports found a beneficial effect on eczema although this improvement was not always statistically significant. For this, in our opinion, they should be regarded with caution.

In the present study, 41/89 children with chronic eczema were randomly given a daily Vit D(*cholecalciferol*) oral supplement of 2000 IUs for 3 consecutive months.

After supplementation, serum levels of Vit D have increased significantly ($p < 0.001$), but this had no influence on the severity of the disease measured by the SCORAD method (**table 4**), unlike other studies that have showed an improvement in the SCORAD index after the administration of lower doses of Vit D (27-29).

Even if the small number of participants is a limitation of this study, in our opinion it has the merit of being one of the few that dealt with pediatric only population. Nevertheless, it confirms that at present there are no certainties in this field due to differences in age, population geography, type of Vit D and schedules of administration. In particular, important questions need to be answered regarding the dosage of Vit D required, which may vary between sexes and between individuals, and the optimal timing and duration of such intervention. Randomized controlled trials adequately designed are still needed in order to establish optimal dosage and duration of treatment for the relative effect of Vit D supplementation.

In view of the phenotypic complexity of eczema, we believe that the current state of knowledge lacks large-scale prospective and randomized studies, which are needed to clarify the actual role of Vit D in this complex disease.

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R. Asero

Single NSAID hypersensitivity is associated with atopic status

Ambulatorio di Allergologia, Clinica San Carlo, Paderno Dugnano, Italy

Key words

Drug allergy; NSAID hypersensitivity; oral provocation testing; urticaria

Corresponding author
Dr. Riccardo Asero
Ambulatorio di Allergologia,
Clinica San Carlo
Via Ospedale 21
20037 Paderno Dugnano (MI), Italy
Phone: +39 02 990 38 470
Fax: +39 02 990 38 223;
E-mail: r.asero@libero.it

Summary

Background: *The relationship between hypersensitivity to NSAID and atopic status is still incompletely defined. Previous studies found a high prevalence of atopic diseases in multiple NSAID reactors. The present study aimed to investigate whether this is the case also in Italian adults hypersensitive to NSAIDs.* ***Methods:*** *Skin tests with a large panel of seasonal and perennial airborne allergens were carried out in 252 patients with a clear-cut history of acute urticaria induced by nonsteroidal anti-inflammatory drugs. Patients were classified as single or multiple NSAID reactors based on clinical history, presence/absence of chronic urticaria, re-challenge with the reported offending drug in case of doubt history, and oral challenges with aspirin or propionic acid derivatives.* ***Results:*** *Single NSAID reactors showed a much higher prevalence of atopic diseases than multiple NSAID reactors either with or without chronic urticaria (61% vs 19% and 19%, respectively; $p < 0.001$).* ***Conclusion:*** *As a difference from previous reports, in Italian patients hypersensitive to NSAID atopy is much more prevalent among single reactors, a finding that indirectly supports the possible IgE-mediated origin of this type of adverse drug reaction.*

Introduction

Non-steroidal anti-inflammatory drugs (NSAID) are the most frequent causes of hypersensitivity adverse drug reactions (1). Hypersensitivity reactions to NSAID have undergone several classifications over the years; the most recent of these (2) identifies five categories: a. NSAID-exacerbated rhinitis/asthma (also called NERD; NSAIDs exacerbated respiratory disease [3]); b. NSAID-exacerbated chronic urticaria (NECD; NSAIDs exacerbated cutaneous disease); c. Multiple NSAID-induced urticaria (NIUA; NSAIDs-induced urticaria-angioedema); d. Urticaria/angioedema or anaphylaxis induced by one single NSAID class (SNIUAA; single NSAID induced urticaria-angioedema or anaphylaxis); and e. Selective delayed-type hypersensitivity reaction (SNIRD; single NSAID-induced delayed hypersensitivity reaction). The first three phenotypes of adverse reactions are considered as non-immunologically-mediated, and are characterized by cross-reactivity between chemically unrelated NSAIDs. It is generally believed that in these adverse reactions the pathogenesis involves COX-1, as suggested by the fact that cross-reactions occur among COX-1 inhibiting drugs and by the reported protective effect exerted by leukotriene receptor antagonists. In fact, previous studies showed the existence of common eicosanoid alterations in aspirin reactors with underlying urticaria and asthma (4).

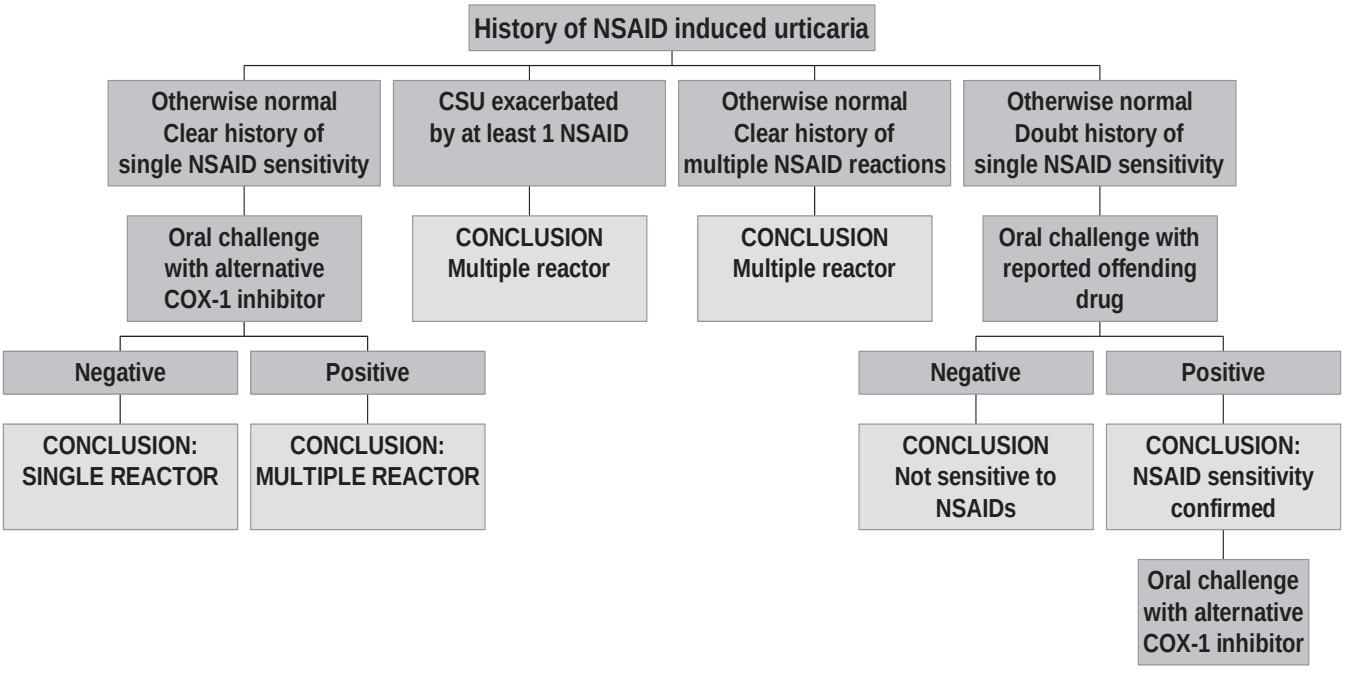
The relationships between atopic status and NSAID hypersensitivity are still incompletely defined. There have been several reports of an association between these two conditions in the past (5-7). Some studies found an association with hypersensitivity to specific airborne allergens, such as house dust mites, particularly in patients with multiple NSAID hypersensitivity without underlying chronic urticaria (1,8), but the association has been reported for other types of NSAID-induced hypersensitivity reactions as well (9). One study found that atopy represents a risk

factor for intolerance to substances, such as acetaminophen and nimesulide, which are generally well tolerated by NSAID hypersensitive subjects (10). Recently, NSAID hypersensitivity has been suspected to act as a co-factor in patients with food allergy caused by sensitization to the pan-allergen lipid transfer protein (10,11). The present study investigated the atopic status in a group of patients with cutaneous hypersensitivity to NSAID classified according to the criteria described above.

Patients and methods

Two-hundred-fifty-two subjects with a clear-cut history of acute urticaria with or without angioedema following the ingestion of NSAID, who were addressed at the Allergy Department of the Clinica San Carlo during the last 6 years, were studied. A clinical history was considered unequivocal if acute urticaria/angioedema followed by less than 2 hours the administration of a NSAID. Most adverse reactions were seen and recorded by doctors in Emergency Services or by the family doctors at patients' homes; patients frequently showed pictures taken by themselves showing the acute phases of their adverse reactions.
The diagnostic workup followed the one recently recommended by expert panel consensus papers and research articles. Briefly, patients with a history of chronic spontaneous urticaria exacerbated by NSAID were considered as multiple NSAID reactors even in the presence of a clinical history of single NSAID reactivity (13-16). Patients without a history of underlying chronic spontaneous urticaria, were diagnosed as having multiple NSAID hypersensitivity if they experienced multiple distinct episodes of acute urticaria occurring within 2 hours after the ingestion of two or more chemically distinct NSAIDs (13,17). Otherwise normal patients with a clear-cut history of acute urticaria following the ingestion of one single NSAID, were classified as single or multiple NSAID reactors based on oral open challenges with a therapeutic dose of a different COX-1 inhibitor. Aspirin was challenged in case of hypersensitivity to any NSAID other than ASA, and Ketoprofen was challenged in case of aspirin intolerance (13,18-20). Those who reacted to the alternative drug within 2 hours after the administration, were classified as multiple reactors; those who tolerated the alternative drug were eventually classified as single NSAID reactors. Four patients with a more doubtful history of hypersensitivity to one single NSAID (aspirin in 2 cases, ketoprofen in one case, and diclofenac in one case) were re-challenged with the offending drug in order to confirm their reactivity (21) before undergoing challenges with an alternative COX-1 inhibitor. Their hypersensitivity to the reportedly offending drug was confirmed in all 4 cases.
Oral challenges were carried out giving increasing doses of the drug under investigation one hour apart as previously described (19,20). Only the occurrence of unequivocal urticaria was considered as a positive response. For ethical reasons, in view of the potential risk of extremely severe reactions, patients with a

Figure 1 *- Diagnostic workup in patients intolerant to NSAID.*



clear-cut history of hypersensitivity to one single NSAID did not undergo oral confirmative challenges with the original offending drug (17,22) but underwent directly the challenge with either ASA or ketoprofen. The whole diagnostic algorithm is depicted in **figure 1**.

Patients gave an informed written consent before the start of the oral challenge procedures. Since this study is a report of one investigator's routine clinical practice no formal Ethical Committee approval was required. The study was approved by the Local Institutional Review Board.

Patients underwent skin prick tests (SPT) with a large panel of commercial extracts of seasonal and perennial airborne allergens (Allergopharma, Reinbeck, Germany), including grass, mugwort, ragweed, pellitory, plantain, birch, plane, olive and cypress pollen, as well as Alternaria, house dust mite, and dog & cat dander. In case of a negative result and of an unequivocal history of food allergy, patients underwent SPT with a panel of commercial food allergens (ALK-Abellò, Madrid, Spain) as well. Skin tests were carried out following established criteria using disposable 1 mm tip lancets, and were read at 15 min. Wheals exceeding 3 mm in their mean diameter were considered positive.

Proportions were compared by chi-square test with Yates' correction. Probability values less than 5% were considered statistically significant.

Results

A total of 99 oral challenges with alternative NSAIDs were carried out in subjects presenting with a history of single NSAID hypersensitivity in order to classify study participants. Eighty-nine challenges were carried out with aspirin in subjects with a clinical history of hypersensitivity to a single NSAID other than aspirin. In subjects with a history of hypersensitivity to aspirin only, challenges were carried out with ketoprofen (n = 10). Eighty-two subjects (M/F ratio 21/61; mean age 44.9 years, range 15-80 years) were eventually classified as single NSAID reactors (SNIUAA). Fifty-four (M/F 15/39; mean age 48.9 years, range 17-80) were diagnosed as having multiple NSAID hypersensitivity without underlying chronic spontaneous urticaria (NIUA); and 116 (M/F 25/91; mean age 47.9 years, range 10-79 years) were classified as having chronic spontaneous urticaria exacerbated by NSAIDs (NECD). The clinical features of study patients are shown in **table 1**. The 3 subgroups did not differ in terms of mean age and sex distribution. A marked female prevalence was present in all three subsets. The prevalence of atopy among the three subgroups was 61% (50/82) among single NSAID reactors, 19% (10/54) among multiple NSAID reactors without spontaneous urticaria, and 19% (22/116) among chronic urticaria patients. The statistical analysis showed that the prevalence of atopy among single NSAID reactors was significantly superior to that detected in the two other subgroups ($p < 0.001$). **Table 2** shows the offending NSAIDs in single drug reactors. Pyrazolones and propionic acid derivatives were by far the most frequent drug families involved in single NSAID reactions. Aryl acetic acid derivatives as well as aspirin were also well represented. Interestingly, a significant proportion of single drug reactions was associated with two drugs that have been long been used as alternative compounds in multiple NSAID reactors with and without underlying chronic urticaria, namely paracetamol and nimesulide. With the exception of the 2 patients hypersensitive to oxicams, all subsets of SNIUAA patients showed a prevalence of atopy that was more elevated than the one found in multiple reactors either with or without chronic spontaneous urticaria. Surprisingly enough, all 7 aspirin single reactors were atopic. The pattern of sensitization to airborne allergens in single NSAID reactors is shown in **table 3**. It largely mirrored the situation observed in the general population living in this geographic area. No statistically significant association between specific allergens and specific offending drugs was found. Six patients were found to have food allergy (not shown in table); 5 were sensitized to lipid transfer protein and 1 had peanut allergy.

Table 1 *- Comparison between the 3 subgroups of NSAID hypersensitive patients.*

	SNIUAA	NIUA	NECD	p
No.	82	54	116	
Mean age (range)	44.9 (15-80)	48.9 (17-80)	47.9 (10-79)	NS
M/F	21/61	15/39	25/91	NS
No. Atopic (%)	50 (61%)	10 (19%)	22 (19%)	P < 0,001

Table 2 *- Offending NSAIDs and atopic status in 82 single NSAID reactors.*

	No.	Atopic (%)
Propionic acid derivatives (Ibuprofen, Naproxen, Flurbiprofen, Ketoprofen)	16	12 (75%)
Aryl-acetic acid derivatives (Diclofenac, Ketorolac)	9	4 (44%)
Pyrazolones (Aminopyrine, Feprazone, Aminophenazone, etc)	27	18 (67%)
Oxicams	2	0 (0%)
Nimesulide	8	4 (50%)
Paracetamol	13	5 (38%)
Aspirin	7	7 (100%)

Table 3 *- Pattern of sensitization to airborne allergens among 50 atopic single NSAID reactors.*

	Grass	Mugwort	Ragweed	Pellitory	Plantain	Birch	Olive	Cypress	Mite	Cat	Alternaria
Total	30 (60%)	5 (10%)	32 (64%)	5 (10%)	6 (12%)	13 (26%)	8 (16%)	5 (10%)	11 (22%)	9 (18%)	1 (2%)
PAD	6	1	6	0	1	5	1	0	4	3	0
AAA	3	0	2	0	1	0	0	0	1	1	0
Pyr	10	1	12	2	3	5	4	4	4	2	0
Nim	3	1	4	1	0	1	1	1	0	0	0
Paracet	3	1	4	0	1	0	0	0	2	2	0
ASA	5	1	4	2	0	2	2	0	0	1	1

PAD: propionic acid derivatives; AAA: aryl acetic acid derivatives; Pyr: Pyrazolones; Nim: Nimesulide; Paracet: paracetamol; ASA: Aspirin.
15 patients were monosensitized to airborne allergens. Polisensitization to pollen (i.e., sensitization to > 3 seasonal allergen sources) was detected in 8 cases, all of which showed profilin sensitization.
No statistically significant association between specific allergens and specific offending drugs was found.

Discussion

Several groups reported an association between atopy and hypersensitivity to nonsteroidal anti-inflammatory drugs. Sanchez-Borges et al. found an impressively high prevalence of atopic diseases among patients with challenge-proven NSAID hypersensitivity; in most cases, these subjects were multiple NSAID reactors (5). In their study, Quiralte and co-workers noticed that atopy was significantly more frequent among patients with NSAID-induced isolated angioedema than in patients with other sorts of NSAID-dependent hypersensitivity reactions (6), whereas Szczeklik's group found and increased prevalence of atopy in both subjects with aspirin-induced asthma and isolated pyrazolone hypersensitivity by comparison with controls without NSAID intolerance (7). More recently, in a Spanish series, the prevalence of atopy was 25% among single NSAID reactors and 52% among those with cross-intolerance, a statistically significant difference (23). The present study found a marked prevalence of sensitization to airborne (and food) allergens in single NSAID reactors. This finding contrasts with previous observations both in adults and children (4,23,24), but it is all but illogical. In fact, several cases of IgE-mediated reactions to different NSAID have been reported so far, and it is generally believed that the proportion of IgE-mediated hypersensitivity reactions detected among single NSAID reactors would increase if we had better diagnostic tests (1,25-29). Thus, it is not surprising that patients genetically predisposed to mount IgE responses to common environmental allergens may show the highest propensity to produce (possibly) IgE-mediated responses against protein/drug complexes, or against some specific parts of either parental drugs or their metabolic derivatives. The prevalence of hypersensitivity to specific drug families among single NSAID reactors in this series mirrored those reported elsewhere (3). Pyrazolones have been detected as a major cause of drug-induced hypersensitivity ever since, and the high prevalence of intolerance to propionic acid derivatives among single NSAID reactors, is possibly associated with their widespread use as an OTC medication. Interestingly, all 7 subjects showing single reactivity to aspirin were atopic ones, a finding that clearly contrasts with that in patients showing multiple NSAID hypersensitivity with or without chronic urticaria. As a difference from previous observations (8), no association between specific airborne or food allergies and specific offending drugs was found, suggesting that it is the atopic status itself, and not the sensitization to specific allergens, the risk factor for single NSAID hypersensitivity. In effect, the prevalence of sensitization to some specific airborne allergens in the general population may show an extreme variability from a geographic area to another, and evidence that NSAID hypersensitivity is rare in a certain area due to a low prevalence of allergy to a certain allergen (e.g., mite) is missing.

One potential limitation of this study might be that in most single reactors NSAID hypersensitivity was not proven beyond any doubt, because these patients tolerated the alternative challenged drug (either ASA or Ketoprofen) but were not re-challenged with the reportedly offending drug. Although the possibility that some of these patients were in effect NSAID-tolerant cannot be ruled out, it must be considered that all but 4 fulfilled the criteria for an "unequivocal" clinical history established by a recent guideline (13) (i.e., the reaction occurred < 6 hours after the intake of one single drug, the patients recalled exactly the event, and in many cases the reaction was recorded by a physician or by a member of an emergen-

cy department), that all four patients with a doubt clinical history reacted to the reported offending drug on re-exposure (3), and that several patients reported more than one episode of urticaria induced by the same drug on separate occasions. Thus, although previous studies have found that clinical history alone may be unreliable (21), it seems unlikely that many of those who were eventually classified as single reactors in this study were NSAID-tolerant (17). Finally, since this study was based on routine practice, to carry out confirmative oral challenges with probable offending drugs on a regular basis irrespective of clinical history would have posed ethical problems due to the risk of potentially severe adverse reactions (22), let alone the fact that many patients would have refused to undergo a challenge with a drug that they considered as the one responsible for their previous reaction.

In conclusion, in this geographic area single NSAID hypersensitivity is often associated with atopic status, a finding that indirectly supports the possible IgE-mediated origin of at least part of this type of adverse drug reactions.

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V. Conti, M.C. Leone, M. Casato, M. Nicoli, G. Granata, M. Carlesimo

High prevalence of gluten sensitivity in a cohort of patients with undifferentiated connective tissue disease

Department of Clinical Immunology, Sapienza University of Rome, Rome, Italy

Key words

Undifferentiated connective tissue disease (UCTD); celiac disease (CD); gluten sensitivity; anti-nuclear antibody (ANA); hepatitis C virus (HCV) infection

Corresponding author
Maurizio Carlesimo
Department of Internal Medicine and Clinical Immunology
Viale dell'Università 37, 00185 Rome, Italy
Phone: +39 06 499 72 090
Fax: +39 06 444 08 06
E-mail: maurizio.carlesimo@uniroma1.it

Summary

Objectives: *The aim of this study was to investigate if co-morbid conditions as hepatitis C virus infection and celiac disease may be associated to undifferentiated connective tissue disease.* ***Methods:*** *We studied retrospectively and prospectively 52 patients with diagnosis of undifferentiated connective tissue disease, subdivided, according to Vaz criteria, in systemic lupus erythematosus, systemic sclerosis and Sjögren's syndrome-like subgroups. Serological markers of celiac disease as anti-gliadin, anti-endomysium and anti-tissue transglutaminase antibodies were investigated. An esophagogastroduodenoscopy with duodenal biopsy and histological examination was proposed to patients with positive celiac disease serology. In addition antibodies directed to hepatitis C virus and total IgA-antibodies were investigated.* ***Results****: Six patients (11,5%) were positive for celiac disease serological tests although two of them were asymptomatic. Four patients underwent an esophagogastroduodenoscopy, showing total or subtotal villous atrophy at duodenal biopsies. Hepatitis C virus serology was negative in all patients and none had IgA deficiency. 83% of celiac patients showed a scleroderma-like phenotype. We observed a statistically higher incidence of autoimmune symptoms in patients with gluten sensitivity. Fatigue and myalgia regressed early after the beginning of gluten-free diet.* ***Conclusions:*** *In our cohort of patients the prevalence of celiac disease was higher than that reported in the general population. We believe that all patients with diagnosis of undifferentiated connective tissue disease, especially those with a systemic sclerosis-like presentation, should be investigated for celiac disease, even in absence of gastrointestinal symptoms. Gluten-free diet should be early recommended to all patients having undifferentiated connective tissue disease and gluten sensitivity.*

Introduction

Undifferentiated connective tissue disease (UCTD) is an autoimmune disorder with positive antinuclear antibody (ANA) results, at least one clinical manifestation of connective tissue disease (CTD) for at least three years but not fulfilling the classification criteria for any defined CTD (1). In about 70% of cases UCTD remains stable over years, and rarely shows spontaneous or treatment-induced regression or, on the other hand, progression to a defined CTD, especially SLE (2). Vaz et al divided UCTD patients into three subgroups according with signs and symptoms of patients: a systemic lupus erythematosus (SLE)-like subgroup, a systemic sclerosis (SSc)-like subgroup and a Sjögren's syndrome (SS)-like subgroup (3).

The aim of this study was to investigate co-morbid conditions that may induce or modify clinical and immunological manifestations of UCTD. We focused on celiac disease (CD) and chronic hepatitis C virus (HCV) infection, two conditions that

frequently pass unrecognized and that are known to be associated with some autoimmune diseases (4-11).
The prevalence of CD in unselected populations in North America and Western Europe falls within the range of 0.5%-1.26%, while in Italy it is between 0.2% and 0.94% (12).
Although a definitive diagnosis of CD is usually based on the histological finding of villous atrophy in duodenal biopsy, the currently available serologic tests for the diagnosis of CD include anti-gliadin (AGA), anti-endomysium (EMA) and anti-tissue transglutaminase (tTG) antibodies, (13,14).
We show here that, while HCV infection is not more frequent in UCTD, the prevalence of CD is increased in patients with UCTD and SSc-like symptoms compared to the general population.

Patients and Methods

This study involved 52 UCTD patients (1 male and 51 females aged 21 to 69 years, median 44 years) referred to the Department of Internal Medicine and Clinical Immunology at Sapienza University of Rome. Classification of UCTD was done according to the criteria proposed by Mosca et al (1). Patients were further classified in subgroups according to the criteria of Vaz et al (3). Patients were interrogated about having a previous diagnosis of CD; five of them were known to be celiac, and three of them were on gluten-free diet from few months to four years at the moment of the study. Three patients having UCTD and CD showed, at capillaroscopy, major abnormalities resembling scleroderma-like pattern (i.e. haemorrhages, dilated capillaries, and giant capillaries), in other two patients with UCTD and CD we observed damages of medium entity (i.e. elongated and tortuous capillaries, haemorrhages); only one celiac patient showed non-specific capillaroscopic changes (i.e. dilated and tortuous capillaries). Patients showed these abnormalities in spite of gluten-free diet. The remaining 47 patients were prospectively investigated for serological markers of CD, irrespective of the presence or absence of signs and symptoms suggesting CD; non celiac patients showed, at capillaroscopy, non-specific abnormalities. Patients with UCTD and CD showed chronic diarrhea, weight loss, abdominal distention, iron deficiency or anemia and recurrent abdominal pain associated with RP, ANA positivity and other rheumatic symptoms, whereas in patients with UCTD only we did not observe significant gastrointestinal symptoms but rheumatic disease symptoms only. The diagnosis of CD was, in almost all patients, preceding with respect to UCTD diagnosis, and we knew it studying retrospectively patients' clinic history; we diagnosed UCTD observing signs and symptoms resembling rheumatic disease only. Serum IgA levels were measured in all patients by nephelometry (Nephelometer BN Prospec, Siemens) to exclude IgA deficiency; HCV serology was investigated in 31 of the 52 patients by third generation enzyme linked immunosorbent assay (Roche Diagnostics, Mannheim, Germany). IgA and IgG1 EMA were tested by indirect immunofluorescence, and IgA and IgG anti-tTG antibodies were measured using an enzyme-linked immunosorbent assay (ELISA) test, as described elsewhere (15,16). Conventional AGA were tested by a sandwich type enzyme immunoassay, used for the quantitative determination of IgA/IgG specific antibodies directed against the α-fraction of wheat gluten gliadin. The cut-off values, provided by the manufacturer, were 16.0 UA/ml and 50.0 U/ml for AGA IgA and IgG, respectively.
Esophagogastroduodenoscopy with duodenal biopsy was proposed to the patients with positive serological tests. In the course of esophagogastroduodenoscopy, three or four oriented duodenal biopsy were obtained, and histological examination was performed in accord to Marsh/Oberhuber classification (17). Results belonging to class III were considered compatible with CD diagnosis. A gluten free diet was recommended to all patients with a CD diagnosis. Unpaired data were analysed by the Mann-Whitney U-test, a P value of less than 0.05 was considered statistically significant.

Results

The most common clinical manifestations in 52 UCTD patients were arthralgia (58%), fatigue (35%), sicca syndrome (31%), Raynaud's phenomenon (RP) (25%) and myalgia (15%).
UCTD patients were sub-divided into 3 subgroups according to the criteria proposed by Vaz et al. (3): SLE-like, SSc-like and SS-like (**table 1**). 25 subjects (48%) were included in the SLE-like group, 14 (27%) in the SSc-like and 13 (25%) in the SS-like, on the basis, respectively, of the presence of fever, fatigue, arthralgia, myalgia for the SLE-like group; RP and capillary abnormalities on capillaroscopy for SSc-like group and sicca-syndrome for SS-like group. None of UCTD patients had IgA deficiency neither positive serology for HCV. Six of the patients (11.5%) were positive for serological markers of celiac disease. All six patients were positive for anti-tTG IgA and IgG, three of them were also positive for EMA IgA and IgG and only one was positive for AGA IgA and IgG. Four of these six subjects underwent esophagogastroduodenoscopy with multiple duodenal biopsies while two refused to perform this procedure. The histological evaluation of duodenal biopsies showed total or subtotal villous atrophy of bowel mucosa (IIIA type of the Marsh/Oberhuber classification). Only four subjects were symptomatic for celiac disease, presenting abdominal pain, diarrhea and iron deficiency anemia. In symptomatic celiac patients having UCTD almost all gastrointestinal symptoms regressed after the beginning of gluten-free diet whereas the majority of rheumatic disease signs and symptoms did not.
Five of the six subjects (83%) with gluten sensitivity belonged to SSc-like group (presence of RP and capillary abnormalities on capillaroscopy) and one to the SLE-like group (**table 2**). We also observed, in patients with UCTD and gluten sensitivity

compared to patients with only UCTD, a higher prevalence of autoimmune related symptoms such as arthralgia ($p < 0,027$), fatigue ($p < 0,008$), RP ($p < 0,0002$), myalgia ($p < 0,0002$) and thyroiditis ($p < 0,0076$). On the contrary, sicca syndrome had the same prevalence in the two groups ($p = 0.8$). Some of these autoimmune symptoms, particularly fatigue and myalgia, regressed after few months of gluten-free diet.

Discussion

Two of the six CD patients were asymptomatic for CD, and were characterized only by the autoimmune profile of UCTD. It is therefore important to consider a screening for CD in patients with a diagnosis of UCTD irrespective of intestinal symptoms or signs of malabsorption.

In the group of patients with CD, compared to the group with UCTD only, we observed a more severe clinical picture, characterized by an expanded spectrum of the typical symptoms of UCTD. In patients showing an SSc-like phenotype, anticentromere or anti-Scl70 antibodies were absent, suggesting that the induction of SSc-like manifestations by CD may be unrelated to that of specific autoantibodies. Because of the well-known association of HCV infection with autoimmune disorders (9), we investigated 31 of the 52 UCTD patients for HCV seropositivity, but we did not find a higher prevalence of HCV infection compared to the general population.

In conclusion, although in a small group of patients, we could observe a higher prevalence of CD in UCTD patients compared to general population. Therefore we suggest that serological screening for CD should be performed in all patients with UCTD, and especially in those with a scleroderma-like phenotype, even if without signs of malabsorption.

At present we don't know if a free gluten diet may contribute to regression or improvement of the clinical manifestations of UCTD, or may even prevent the clinical evolution to CTD. A longer follow-up study will give us this information and will further clarify the role of gluten sensitivity in autoimmune diseases.

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Table 1 *- Epidemiological and clinical features of 52 patients with UCTD diagnosis.*

UCTD pattern	N° (M/F)	Age (median, range)	Time from diagnosis (months; median range)	Previous IS therapy[1]	Diagnosis of CD[1]
SS-like	13 (0/13)	50 (68-30)	36 (1-60)	2 (15%)	0
SLE-like	25 (1/24)	40 (21-69)	36 (1-60)	6 (24%)	1 (4%)
SSc-like	14 (0/14)	39 (26-68)	12 (1-120)	6 (43%)	5 (36%)

IS: immunosuppressive

[1]number of patients (%)

Table 2 *- Clinical features of the 6 UCTD patients with gluten sensitivity.*

Case	UCTD pattern	Autoantibodies at diagnosis	CD-like symptoms	CD-associated antibodies	Intestinal biopsy	Previous IS therapy
1	SSc-like	ANA 1:160 speckled	Yes	EMA, tTG	Atrophy	Hydroxyurea
2	SSc-like	ANA 1:80 nucleolar	No	EMA, tTG, AGA	Atrophy	None
3	SSc-like	ANA 1:80 homogeneus	No	tTG	N.D.	Steroids
4	SSc-like	ANA 1:640 speckled	Yes	EMA, tTG	N.D.	None
5	SSc-like	ANA 1:640 speckled	Yes	tTG	Atrophy	Methotrexate, hydroxyurea, steroids, FANS
6	SLE-like	ANA 1:80 homogeneus	Yes	tTg	Atrophy	None

IS: immunosuppressive

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E.A. Pastorello[1], J.W. Schroeder[1], S.M. Veronese[2], V. Pravettoni[3], A. De Gasperi[4], S. Cantoni[5], L. Losappio[1], L. Farioli[6], R. Qualizza[1], B. Scarpati[7], A. Mascheri[1], J. Scibilia[1]

Two cases of elevated tryptase in abdominal aortic aneurysm

[1]Department of Allergology and Immunology, Niguarda Ca' Granda Hospital, Milan, Italy
[2]Department of Anatomy Histology and Cytogenetics, Niguarda Ca' Granda Hospital, Milan, Italy
[3]Clinical Allergy and Immunology Unit, Fondazione IRCCS Cà Granda Ospedale Maggiore Policlinico, Milan, Italy
[4]Department of Anaesthesia and Intensive Care, Niguarda Ca' Granda Hospital, Milan, Italy
[5]Department of Hematology, Niguarda Ca' Granda Hospital, Milan, Italy
[6]Department of Laboratory Medicine, Niguarda Ca' Granda Hospital, Milan, Italy
[7]Department of Immunohematology and Transfusion Medicine, Niguarda Ca' Granda Hospital, Milan, Italy

Key words

Abdominal aortic aneurysm; hymenoptera venom anaphylaxis; tryptase

Corresponding Author
Elide Anna Pastorello
Allergologia e Immunologia
Azienda Ospedaliera Ospedale Niguarda Ca' Granda
Piazza Ospedale Maggiore, 3
Milan 20162, Italy
Phone: +39 02 6444 27 51
Fax: +39 02 6444 20 82
E-mail: elide.pastorello@ospedaleniguarda.it

Summary

Introduction: *From the literature, patients with a history of anaphylaxis to hymenoptera venom and positive specific IgE have shown a correlation between elevated tryptase levels and two clinical situations: systemic mastocytosis and an increased risk of reactions to venom immunotherapy or hymenoptera sting. Other clinical scenarios could explain elevated tryptase levels.* ***Material and Methods:*** *A 67 year old male (P1) and a 77 year old male (P2) were evaluated for previous severe anaphylaxis to hymenoptera sting. They underwent standard diagnostic work-up for hymenoptera venom allergy. Having found elevated tryptase levels, these were followed by a bone marrow biopsy to rule out systemic mastocytosis.* ***Results:*** *P1: specific IgE and skin tests were positive for Vespula species; tryptase 52.8 ng/ml; P2: specific IgE and skin tests were positive for Vespa cabro and tryptase 153 ng/ml. Bone marrow biopsy results were negative for mastocytosis. We carried out magnetic resonance imaging, in P1 to better characterize the severe osteoporosis and in P2 because during physical examination a pulsating mass had been identified in the mesogastrium, and an aneurysm of the abdominal aorta which required surgical intervention in both patients was detected. Eight months after surgery, tryptase levels had diminished significantly (P1: 11.6 ng/ml and P2: 14.5 ng/ml).* ***Discussion:*** *The elevated tryptase levels were correlated to abdominal aneurysm in both patients. In fact, post-surgery tryptase levels dramatically decreased. These two cases demonstrate that high tryptase levels in subjects with a history of hymenoptera venom anaphylaxis can be associated to undiagnosed aneurysmatic disease.*

Abbreviations

HV: hymenoptera venom
SM: systemic mastocytosis
BM: bone marrow
AAA: abdominal aortic aneurysms
VIT: venom immunotherapy

Tryptase is a neutral serine protease secreted by mast cells during anaphylaxis, and invariably elevated in mastocytosis. Recently, an increase in baseline tryptase levels has been indicated in hymenoptera venom (HV) allergy in association with different clinical features. First of all, hymenoptera anaphylaxis has been frequently described in patients with cutaneous mastocytosis (1,2), and more recently in patients with indolent systemic mastocytosis

(SM) without cutaneous involvement (3). This association is comprehensible, given that the hymenoptera stings can cause anaphylaxis in mastocytosis patients even if not allergic. Secondly, an increase in baseline tryptase level (5.2 ng/mL) has been associated with a higher risk of severe anaphylaxis to hymenoptera stings or during venom immunotherapy (VIT) (4). Up to now, however, there has been no clear scientific explanation to justify the aforementioned relationship between venom allergy and tryptase increase in patients without mastocytosis. In some patients, however, a clonal mast cell disorder (5) has been recently described, putting, once more, the mast cell pathology at the basis of the tryptase behavior in HV allergic patients. Moreover, it has been at least a decade since mast cells have been recognized to be involved in cardiovascular diseases (6), and since then elevated tryptase titers have been associated with acute coronary syndrome (7), in some cases in association with allergic reactions in the so called Kounis syndrome (8). This is not surprising given that tryptase is directly involved in atherosclerotic plaque development, given its ability to activate protease zymogens (9). Moreover, different authors have recently demonstrated that the number of mast cells was increased in human AAA even more significantly than in atherosclerotic lesions, and that their number correlated with the diameters of the AAA (10), thus confirming a direct participation of mast cells in AAA formation, as recently demonstrated in several experimental studies on animal models (11). Furthermore, in a cohort study a significantly higher serum baseline tryptase level was found in AAA patients (12,13). All of these observations led us to infer that the meaning of the increased basal levels of tryptase in HV allergy could be due to associated pathologies that have yet to be fully elucidated. Herein we report the cases of two patients with HV anaphylaxis and very high serum baseline level of tryptase, in whom a suspicion of SM had been advanced. A complete diagnostic work up was applied in order to confirm this diagnosis. In both patients SM was excluded and an abdominal aortic aneurysm (AAA) was diagnosed. The patients were two males, one 67 year old (P1) and the other 77 year old (P2). Both patients were admitted to our Centre for a previous severe anaphylactic reactions to yellow jacket stings, that had occurred about one year before with cardiovascular, cutaneous and respiratory involvement (Muller grade III); both had been treated with epinephrine injection with complete regression of symptoms after a few hours. Both patients were not affected by chronic renal failure inasmuch as renal function was normal. Routine diagnostic work-up was performed for HV allergy. Skin prick tests and intradermal tests were positive for Vespula species (P1) and Vespa crabro (P2). About one year after the anaphylactic reaction the measurement of basal serum tryptase was 52.8 ng/mL (P1) and 153 ng/mL (P2), respectively (normal tryptase value < 5 ng/mL). Thus, bone marrow (BM) biopsy was performed and histologycal, immunohistochemical, phenotypic and morphologic BM examinations were carried out to ascertain the presence of SM. KIT and PDGFRα gene mutations were investigated in all of the BM samples. Sanger sequencing was used to detect the presence of potential mutations in exons 9, 11, 13 and 17 of the Kit gene and in exons 12, 14 and 18 of the PDGFRα gene. Moreover, p.D816V mutation research was performed using a mutant-enriched polymerase chain reaction that blocks the wild-type component and amplifies only the mutated component. Such an approach allows to reveal the presence of very low quantities of mutated DNA with an extremely high sensitivity (0.01% of mutant versus wild-type allele). International diagnostic criteria for the diagnosis of SM were employed, in particular we searched for the presence of multi-focal, dense mast cell infiltration (> 15 mast cells in aggregates) in BM samples and/or in extracutaneous organs (major criteria). As minor criteria we looked for: the presence of D816V KIT mutation in BM, blood, or extracutaneous tissues; the baseline serum tryptase concentration > 20 ng/mL; the expression of KIT plus CD2 and/or CD25 in mast cells from BM, and the presence > 25% of mast cells with atypical or spindle shape. The diagnosis of SM has to be made when either 1 major plus 1 minor or 3 minor criteria are present (14). Mast cell phenotypes were detected and analyzed by flow cytometry, using FACS Canto II flow cytometer (Becton Dickinson, san Jose, CA, USA). Acquisition and analysis were performed by means of Diva Software (Becton Dickinson). We excluded the diagnosis of SM for both patients on the basis of the negativity of the aforementioned criteria. Of note, BM biopsy of P2 revealed a 17 exon mutation of the c-Kit gene with a silent nucleotide substitution (c.2394 C > T; P.lle798lle) that, being not clinically significant, was considered negative and, using flow cytometry, a 0.15% of mast cells CD2/CD25 positive that, given the low percentage, was considered not significant. Subsequently, magnetic resonance imaging was performed in P1 to better characterize the severe osteoporosis detected by bone mineral density test, and in P2 because during physical examination a pulsating mass had been identified in the mesogastrium and the patient complained recurrent episodes of dyspnea. AAAs were detected in both patients with a diameter of 58 mm (P1) and of 54 mm (P2). Surgical treatment of the AAA was undertaken and a few months post-surgery, tryptase levels dramatically decreased to 11.6 ng/mL in P1 and to 14.5 ng/mL in P2. P1 is now in good health and has been undergoing VIT for 3 years without adverse reactions; during VIT he has been stung by hymenoptera without complaining any reaction. P2 is in good health and has been directed to undergo VIT, refusing it for practical reasons. **Table 1** shows demographic and clinical characteristics.

These two patients show that in some cases elevated basal tryptase levels may be related to AAAs inasmuch as post-surgery tryptase

Table 1 *- Demographic, clinical and diagnostic characteristics of P1 and P2.*

		P 1	**P 2**
Age (y) /Sex		67/M	77/M
Allergic reaction (Muller grade)		III	III
Polistes species sIgE (kU/L)		0.34	0.31
Vespula species sIgE (kU/L)		2.81	2.10
Vespa crabro sIgE (kU/L)		0.89	3.59
Basal serum tryptase (ng/mL)		52.8	153
Final diagnosis		Vespula venom anaphylaxis	Vespa crabro venom anaphylaxis
BM results	Multi-focal, dense mast cell infiltration (> 15 mast cells in aggregates) in samples of BM and/or in extracutaneous organs	Negative	Negative
	Presence of D816V KIT mutation in BM, blood, or extracutaneous tissues	Negative	Negative
	Baseline serum tryptase concentration of > 20 ng/mL	Positive	Positive
	Expression of KIT plus CD2 and/or CD25 in mast cells from BM	Negative	Positive
	Mast cells with atypical or spindle shape > 25%	Negative	Negative
MRI		Abdominal aneurysm (diameter = 58mm)	Abdominal aneurysm (diameter = 54mm)
VIT		Vespula species	Not performed
Post surgery tryptase level (ng/mL)		11.6	14.5

SPT: skin prick test; ID: intradermal test; BM: bone marrow; MRI: magnetic resonance imaging; VIT: venom immunotherapy.

levels decreased significantly. Interestingly, even though the patients did not fulfil the criteria for SM diagnosis, P2 had a 0.15% of mast cells expressing CD2/CD25 antigens. This finding, in the light of the high serum tryptase level, could suggest the presence of a mast cell clonal disorder as recently described in HV allergy. Anyway, the real origin of this phenomenon is unknown and we can only hypothesize that it represents the molecular basis of the mast cells invasion with tryptase release of the arterial wall, leading to AAA. As a consequence, the tryptase liberated from the aneurysmatic lesion could act as a risk factor for HV anaphylaxis in patients with a HV sensitization. Otherwise, one might hypothesize that in patients with HV anaphylaxis the high tryptase levels could alter the aortic wall on a preexisting lesion, facilitating the development of an AAA. Moreover, it is known that the role of mast cells in the pathogenesis of AAA formation consists in the degradation of extracellular matrix, apoptosis of smooth muscle cells, activity of the renin-angiotensin system and neovascularization. (15). The follow-up of our patients at 2 years after surgery showed stable low-range tryptase values and no AAA relapse, thus further confirming the probable relationship between the two events. Only future studies on larger study populations will allow to better address this issue.

In conclusion, the marked increase in basal tryptase levels in subjects with a history of HV anaphylaxis, may be associated with undiagnosed aneurysmatic disease. Being that the high levels of tryptase in patients with HV anaphylaxis occur in subjects who remain otherwise asymptomatic, on the light of the present observations we believe that in depth studies may give insight as to all the circumstances that can determine an accumulation of mastocytes that in turn give way to an increase in the reactivity of the HV allergic subjects.

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G. Ciprandi

Clinical bystander effect exerted by allergen immunotherapy: a hypothesis

IRCCS-Azienda Ospedaliera Universitaria San Martino, Genoa, Italy

Key words
Allergen immunotherapy; poly-allergy; bystander effect

Summary
Allergen Immunotherapy (AIT) is able to restore a physiological Th1 response and Tregs function. This effect is allergen-specific, even though it has been reported that it may also be non-specific, such as also extended to allergens not used in AIT. This immunological phenomenon may also be of clinical nature. This case report shows that a poly-allergic patient, successfully treated with Parietaria extract, also achieved a clinical tolerance towards other causal allergens, such as mites and cat. Of course, this was an anecdote, but it is reasonable to prospect the hypothesis that a bystander clinical effect may be observed during AIT in poly-allergic patients.

Corresponding Author
Giorgio Ciprandi
Viale Benedetto XV 6,
16132 Genova, Italy
Phone: +39 010 3533 18 20
Fax: +39 010 353 86 64
E-mail: gio.cip@libero.it

Allergy is characterized by a dysregulation of immune response sustained by a functional defect of T regulatory cells (Tregs), that induces a Th2 polarization and consequently a reduced production of Th1-dependent cytokines, namely IFN-g. In fact, it has been reported *in vitro* that allergic subjects have a diminished allergen-specific production of IFN-g, whereas non-specific stimuli, such as PHA, induce normal IFN-g production (1). Allergen immunotherapy (AIT) is the only cure of allergic disorders as it is able of: i) controlling allergic symptoms and inflammation, ii) modifying their natural course, and as iii) its effects last long time after discontinuation (2). Immunologic mechanisms of action include physiologic restoration of Tregs function, abolition of Th2 polarization, and increased production of IFN-g, such as a skewed Th1-response (the so called immune-deviation) (3,4). These immunologic effects are surprisingly fast. In this regard, it has been reported that sublingual immunotherapy (SLIT) significantly affected allergen-specific IFN-g production just after 3 months from starting (5). Very interestingly, it was also demonstrated that an *in vitro* bystander effect on IFN-g production, induced by AIT, could also occur (6). A group of allergic patients, sensitized to both pollens and mites, were treated with AIT only to pollens. AIT provided a significant increase of both pollen-induced as well as mites-induced IFN-g production. Therefore, this study provided evidence that though the defective IFN-g production is typically allergen-specific in allergic patients, the AIT effect on increased IFN-g synthesis may also be non-specific. This fact is not particularly surprising, as it is well known that antigen booster promoted by vaccinations generates both antigen-dependent and antigen-independent memory B cell response (7-9). Therefore, AIT is able of modulating immune response both via allergen-specific pathway and through non-specific effects (6). This phenomenon probably depends on a polyclonal activation of Th1 cells (6).

On the other hand, it is quite common to observe in the clinical practice that poly-allergic patients treated with AIT may develop a wide clinical immune tolerance also toward allergens not used in AIT. However, there is no formal demonstration of this issue. Therefore, a case representative of this issue is described here.

This case report was carried out in accordance with the ethical standards established in the *Declaration of Helsinki*, and a written informed consent was obtained at the first visit.

S.C. is a young man 18 years old suffering from allergic rhinitis and asthma since early childhood. He is poly-allergic to several allergens and symptoms occurrence is perennial, but with exacerbations during spring and fall. The exposure to dust often caused severe symptoms as well as cat exposure. In fact, he was not able to frequent the home of his best friend, as two cats lived there. Every time he moved to his home, severe breathlessness, wheezing, cough, sneezing, rhinorrhea, lacrimation, ocular itching, and swelling of eyelids sudden occurred.

He was treated with medications, including inhaled corticosteroids, antihistamines and antileukotrienes for long time, but allergic symptoms were not optimally controlled. Therefore, AIT was considered as new treatment option. Before AIT prescription, serum allergen-specific IgE were assayed: Der p 1 44 kU_A/L; Der p 2 35 kU_A/L; Par j 2 31 kU_A/L; Fel d 1 7.4 kU_A/L; Bet v 1 4.1 kU_A/L; Can f 1 3.4 kU_A/L; Ole e 1 2.5 kU_A/L. Despite poly-sensitization and perennial symptoms, it was decided to prescribe SLIT for Parietaria, as there was a periodicity of symptom exacerbations typically during Parietaria pollen peak.

He assumed a pre-co-seasonal SLIT course started in November 2013 and completed on March 2014. AIT was well tolerated. The symptom perception and medication use were assessed by visual analogue scale (VAS), comparing before- and after-AIT periods (10). Symptom VAS diminished from 8.1 to 3.5; drug VAS diminished from 6.2 to 0.4 (actually he did not assume any medication); and the VAS of perceived AIT effectiveness was 8.9. Very interestingly, he tolerated house dust, and overall he could move to the home of his best friend without relevant complaints.

This clinical case underlines the possibility of a clinical bystander effect induced by AIT. Indeed, Parietaria SLIT was able not only of improving pollen-dependent symptoms, but also of inducing an immunological tolerance, clinically relevant, also to other sensitizing allergens, such as mites and overall cat. Of course, this is an anecdotal report, thus there is the need of performing rigorous studies addressing this issue. In fact, even though this hypothesis of bystander effect of immunotherapy is interesting, it has been not formally demonstrated until now.

Anyway, the hypothesis that AIT might also exert non-specific mechanisms of action seems convincing. In fact, high allergen concentration may induce an immunological tolerance towards the used extract, responsible for the clinical improvement, as well known. In addition, AIT could provide non-specific effects that might explain these *in vitro*, such as increased IFN-g production, and in vivo, such as extended allergen tolerance, positive effects. However, one single patient's reported clinical benefit alone is not sufficient for formally supporting this hypothesis. If it is quite common to observe in the clinical practice that poly-allergic patients treated with AIT may develop a wide clinical immune tolerance also toward allergens not used in AIT, anyway, this impression requires at least a systematic observation of many cases for moving from impression to perception, and many experimental designs for moving from perception to formally demonstrated evidence.

In conclusion, it seems possible to hypothesize at present that a clinical bystander effect may be exerted by allergen immunotherapy.

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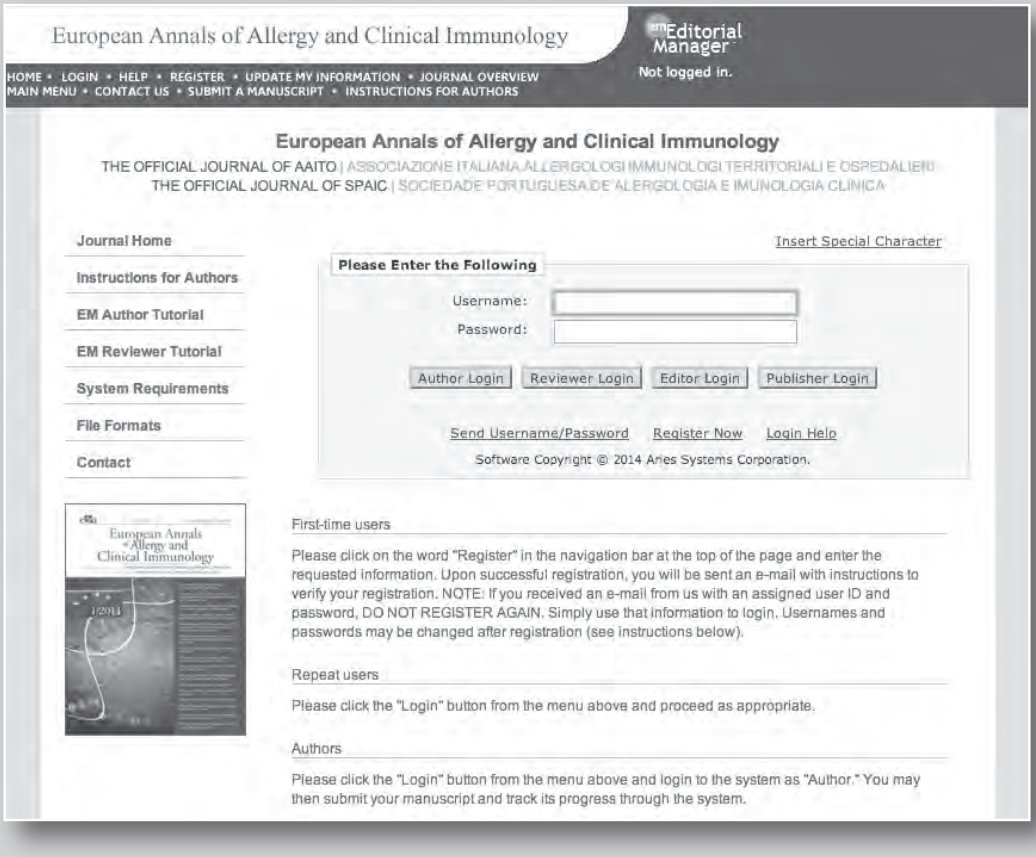

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