

Increased Osteopontin Levels in Children Undergoing Venom Immunotherapy May Serve as a Marker of Clinical Efficacy

Süleyman Tolga Yavuz Ozge U. Soyer Bulent E. Sekerel Betul Buyuktiryaki
Ozlem Cavkaytar Umit Murat Sahiner Cansin Sackesen Ayfer Tuncer

Department of Pediatric Allergy and Asthma Unit, Hacettepe University School of Medicine, Ankara, Turkey

Key Words

Insect venom allergy · Plasma osteopontin · Serum basal tryptase · Venom immunotherapy

Abstract

Background: Venom immunotherapy (VIT) has its effect by modulating various mediators resulting in immune tolerance. The aim of this study was to measure changes in plasma osteopontin (OPN) and serum basal tryptase (sBT) levels over the course of 1 year of VIT in children with venom allergy. **Methods:** Children who suffered from a large local reaction (LLR) or a systemic reaction (SR) after insect stings were included along with control subjects. Measurements were performed before the initiation of VIT and 6 and 12 months after it had been started. **Results:** A total of 58 children (24 with SR, 18 with LLR and 16 control subjects) with a median age of 9.5 years (range 6.7–12.8) were enrolled. The plasma OPN levels of patients with LLR [median 1,477 ng/ml, interquartile range (IQR) 1,123–1,772] were significantly higher than patients with SR (882 ng/ml, 579–1,086; $p < 0.001$) and healthy control subjects (1,015 ng/ml, 815–1,203; $p = 0.002$). A significant increase in plasma OPN levels in children was determined after the 1-year VIT. The sBT levels of children with SR (4.1 ng/ml, 3.6–5.8) were significantly higher than children with LLR (3.1 ng/ml, 2.5–4.0) and control subjects (3.0 ng/ml, 2.9–3.8; $p = 0.001$). There was no significant change in the sBT levels of the patients after the 1-year

VIT. **Conclusions:** The results of our study showed higher baseline levels of OPN in children with LLR compared to control subjects and children with SR. In children with SR, OPN levels were increased after the 1-year VIT. Our results may suggest a possible association between OPN and successful VIT in children.

© 2014 S. Karger AG, Basel

Introduction

Venom immunotherapy (VIT) is a well-established and effective treatment to avoid life-threatening systemic reactions (SR) in patients with venom allergy [1]. It has its effect by modulating various immunologic mediators, resulting in immune tolerance. Peripheral tolerance is induced by the generation of allergen-specific regulatory T (Treg) cells, which suppress proliferative and cytokine responses against venom allergens during VIT. Treg cells are characterized by IL-10 secretion, which directly or indirectly influences effector cells of allergic inflammation such as mast cells, basophils and eosinophils [2].

Along with several changes in cytokines, antibodies and mediators, recent studies suggest a possible role of an extracellular matrix protein, osteopontin (OPN), in IgE-mediated immune responses during VIT [3]. The result of a study on adults showed a significant increase in serum OPN levels and suggested that OPN could be a candidate

biomarker related to successful VIT [4]. The only method for testing the effectiveness of VIT is to perform a risky sting challenge, so the identification of a biomarker associated with successful VIT is crucial, particularly for children.

Recent studies on adults and children have established the role of high serum basal tryptase (sBT) levels as a risk factor for severe SR in patients with venom allergy [5, 6]. A retrospective study conducted on an adult population determined a significant decrease of sBT levels, reported to be associated with successful immunotherapy [7]. However, there are limited data regarding the effect of VIT on sBT levels in the pediatric literature [8, 9].

The aim of this study was to measure changes in plasma OPN and sBT levels over the course of 1 year of VIT in children with venom allergy.

Materials and Methods

Patients

Children referred from January 2010 to October 2011 to the outpatient clinic of the Department of Pediatric Allergy, Hacettepe University, with a history of large local reaction (LLR) or SR after a hymenoptera sting, were consecutively enrolled and analyzed along with control subjects who were admitted to the outpatient clinic with various symptoms of suspected atopic diseases, such as asthma and/or rhinitis. Children in the control group had been stung at least once and had no documented history of any local reaction or SR. All children and their parents were interviewed and a questionnaire including information about insect sting history, medication and demographic data was performed. The children with a clinical history of an LLR after hymenoptera stings without any evidence of previous SR were enrolled in the LLR group. The index sting, to which all other information was referred, was defined as the field sting that had resulted in the hitherto most severe SR (in the SR group) or LLR (in the LLR group) according to the individual patient's history. Picture cards of insects (wasps and bees) were displayed to children and parents who had noticed the sting in order to determine the culprit insect. For all sting reactions, the presence of detailed systemic symptoms were queried and recorded. VIT was initiated in children with SR.

Twenty-four children with venom allergy who had encountered SR after insect stings were enrolled, along with 18 children with a history of LLR after hymenoptera stings and 16 age- and sex-matched control subjects (i.e. with no reaction, NR).

The study had approval from the Institutional Review Board of the Hacettepe University and written informed consent was obtained from the children and their parents.

Diagnosis of Venom Allergy

Diagnosis of Hymenoptera venom allergy was made using guidelines published by the European Academy of Allergy and Clinical Immunology and the American Academy of Allergy, Asthma and Immunology [10, 11]. Children with a history of previous systemic sting reactions were diagnosed as having venom allergy after confirming sensitization by a skin test reaction, i.e.

prick and intradermal tests with *Vespula* (wasp) and *Apis mellifera* (bee) venom at least 2 weeks after the reaction, and/or the detection of specific immunoglobulin E (sIgE) to the culprit type of insect, shown by elevated titers of sIgE (>0.35 kU/l) [10]. The skin tests were not performed in children with a history of LLRs and children without any reaction after stings [12, 13].

LLR is defined as edema and erythema at the site of the sting that exceeds a diameter of 10 cm that lasts longer than 24 h [10].

Study Measurements

Skin Tests

Skin tests were performed with bee and wasp venom (*A. mellifera* and *Vespula*, Stallergenes, Antony, France) in children with SR, as defined previously [14]. Briefly, skin-prick tests were performed with a venom concentration of 1 µg/ml; if negative, then intradermal injections with a concentration of 10-fold dilutions ranging from 0.001–1 µg/ml with 0.02 ml of venom injected into the volar surface of the forearm.

All participants were also skin-prick tested for common aeroallergens for our region and 'aeroallergen sensitization' was defined as 'having at least one positive skin test response' to one of the aeroallergens tested [15].

Tests in vitro

Baseline levels of venom sIgE, total IgE (tIgE), plasma OPN and sBT were measured in all groups of patients. The molecular changes induced by VIT in children with SRs were assessed by measuring serum venom sIgE and tIgE, serum IL-10, plasma OPN and sBT. Venom sIgE, tIgE, IL-10 and OPN were measured in samples obtained before the initiation of VIT and 6 and 12 months after it was started, while sBT levels were measured before and 12 months after the initiation of VIT.

Serum Venom sIgE and tIgE Levels

Serum allergen sIgE levels to the implicated insect (bee or wasp) and tIgE levels were measured with the ImmunoCAP system (PhadiaAB, Uppsala, Sweden) according to the manufacturer's instructions. Venom sIgE measurements were performed at least 2 weeks after the sting reaction [10]. Results >0.35 kU/l were considered positive.

Serum IL-10 Levels

Serum IL-10 concentrations were measured using enzyme-linked immunosorbent assay (IL-10 ELISA Bender Med Systems, Vienna, Austria) according to the manufacturer's instructions.

Plasma OPN Levels

Plasma OPN levels were measured using an Osteopontin EIA kit (IBL Co. Ltd., Gunma, Japan).

Serum Tryptase Levels

The method for sBT level measurement was defined previously [6]. The levels were estimated by UniCAP (System ImmunoCAP Tryptase®, Pharmacia & Upjohn, Uppsala, Sweden).

Venom Immunotherapy

Children with SR underwent VIT with the culprit insect venom. VIT was performed using aluminium hydroxide-adsorbed venom extract preparations (Alutard SQ® 100% *A. mellifera* or *Vespula* spp. ALK, Horsholm, Denmark). A modified cluster outpatient

Table 1. VIT modified cluster protocol

Day	Venom concentration, µg/ml	Volume, ml	Cumulative daily dose, µg
1	0.1	0.1	0.01
		0.2	0.02
4		0.4	0.04
		0.8	0.08
8	1	0.1	0.1
		0.2	0.2
11		0.4	0.4
		0.8	0.8
15	10	0.1	1
18		0.2	2
22		0.4	4
25		0.8	8
29	100	0.1	10
36		0.2	20
42		0.3	30
49		0.4	40
56		0.5	50
63		0.6	60
70		0.7	70
77		0.8	80
84		0.9	90
91		1	100

protocol up to a maintenance dose of 100 µg was followed during the induction phase (table 1). After the maintenance dose of 100 µg was achieved, injection intervals were scheduled for every 4 weeks throughout the maintenance phase. Injections were administered subcutaneously at the distal lateral aspect of the upper arm.

Statistical Analysis

The PASW Statistics software package, version 18.0 (SPSS Inc., Chicago, Ill., USA) was used for all calculations. Descriptive data for categorical and numerical variables were expressed as medians and interquartile range (IQR) for nonnormally distributed variables. Group comparisons were established using the Mann-Whitney U test or ANOVA on ranks as appropriate, and the χ^2 test or the Fisher test for categorical variables. The Friedman test was conducted to test whether there was a significant change in variables and the Wilcoxon test was performed to test the significance of pairwise differences, using the Bonferroni correction to adjust for multiple comparisons. A p value of <0.05 was considered significant.

Results

General Demographics and Clinical Characteristics of Study Groups

Forty-two children (24 with SR and 18 with LLR) with insect venom allergy were enrolled along with 16 age- and sex-matched control subjects. There was no significant difference between groups in terms of aeroallergen sensitization status and total IgE levels. Demographic and laboratory data of the study groups are presented in table 2.

The plasma baseline OPN levels of patients with LLRs (median 1,477 ng/ml, IQR 1,123–1,772) were significantly higher than patients with SR (882 ng/ml, 579–1,086; $p < 0.001$) and healthy control subjects (1,015 ng/ml, 815–1,203; $p = 0.002$; fig. 1a).

Baseline sBT levels in children with SR (4.1 ng/ml, 3.6–5.8) were significantly higher than in children with LLR (3.1 ng/ml, 2.5–4.0) and control subjects (3.0 ng/ml, (2.9–3.8; $p = 0.001$).

Effects of Venom Immunotherapy

VIT with *Vespa* venom was initiated in 22 patients (91.7%) and with *A. mellifera* venom in 2 patients (8.3%). There were no significant changes in the venom sIgE levels of children with SR during the 1-year VIT. The total IgE levels across the 12-month period showed no change overall ($p = 0.648$), with a significant increase between baseline and 6 months ($p = 0.011$) followed by a significant decrease between 6 and 12 months ($p < 0.001$; fig. 2).

A significant increase in the plasma OPN levels of children with SR was determined after 1 year of VIT compared to at baseline and 6 months ($p < 0.001$ and $p = 0.015$, respectively; fig. 1b).

The serum IL-10 levels in children with SR who underwent VIT increased significantly after 1 year of VIT [0.7 (0.5–1.2) vs. 0.2 pg/ml (0.1–0.3); $p < 0.001$] when compared to baseline levels (fig. 3). There was a significant increase after 6 months of VIT [0.5 (0.3–0.6) vs. 0.2 pg/ml (0.1–0.3); $p < 0.001$] and a further increase was also observed after 1 year of VIT [0.7 (0.5–1.2) vs. 0.5 pg/ml (0.3–0.6); $p < 0.001$].

There was no statistically significant change in the sBT levels of patients with SR after 1 year of VIT ($p = 0.466$).

Six patients (25%) in the SR group were restung during the VIT (during the first 3 months after initiation) and none of them had any local reaction or SR after the sting.

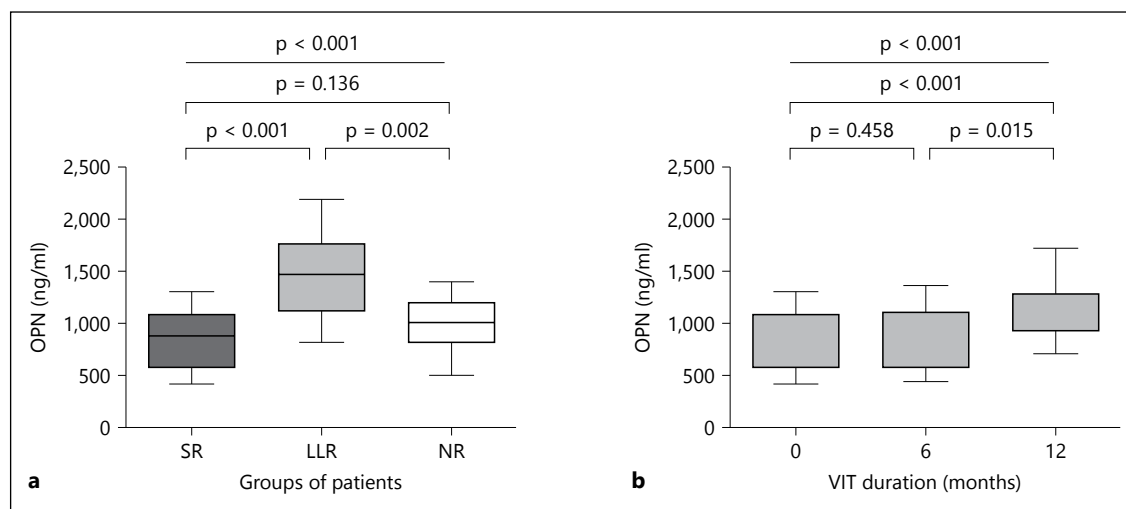


Fig. 1. **a** Baseline plasma OPN levels of the study population. **b** Plasma OPN levels in patients with SR during VIT.

Table 2. Demographic and laboratory data of the study population

	SR	LLR	NR
Total number of patients	24	18	16
Age, years	9.6 (6.8–12.3)	8.4 (6.0–13.0)	10.1 (7.9–14.0)
Gender, M/F	19/5	16/2	10/6
Aeroallergen sensitization, n	7	4	3
Level of venom sIgE, kU/l			
Implicated insect	5.6 (1.7–24.8)	5.3 (0.5–11.2)	–
<i>A. mellifera</i>	0.3 (0.04–0.6)	0.1 (0.04–0.4)	0.03 (0.03–0.03)
<i>Vespula</i>	4.2 (1.5–19.2)	5.2 (0.5–11.2)	0.03 (0.02–0.04)
tIgE, kU/l	130 (63–346)	91 (28–321)	70 (16–167)
sBT, ng/ml	4.1 (3.6–5.8)*	3.1 (2.5–4.0)	3.0 (2.9–3.8)
Plasma OPN, ng/ml	882 (579–1,086)	1,477 (1,123–1,772) [†]	1,015 (815–1,203)

Data are presented as number or median (IQR). * $p = 0.001$; [†] $p < 0.001$.

Discussion

In this study, we found that the plasma OPN levels in children with LLR were higher than those in children with SR or children with NR to insect stings. We demonstrated an increase of plasma OPN levels in children, which started after 6 months of VIT. We also determined a significant increase in serum IL-10 levels during the year of VIT. However, we could not determine any significant changes in tIgE or venom sIgE levels, eosinophil counts or sBT levels after 1 year of VIT.

The role of OPN in the pathogenesis and severity of allergic diseases including allergic conjunctivitis [16], al-

lergic rhinitis [17, 18] and asthma [19–21], in particular, have been investigated in several studies performed predominantly in adult patients. OPN had initially emerged as a key cytokine involved in Th1 immune responses; however, recent findings also established a role of OPN in Th2-related diseases, including asthma. Furthermore, OPN is reported to be associated with the regulation of allergic and fibrotic responses [22, 23]. The possible association between OPN and asthma pathogenesis has also been determined in 2 studies on children [24, 25]. To sum up, the data obtained from previous studies support the idea that OPN plays a key role as an effector cytokine involved in allergic inflammation. Therefore,

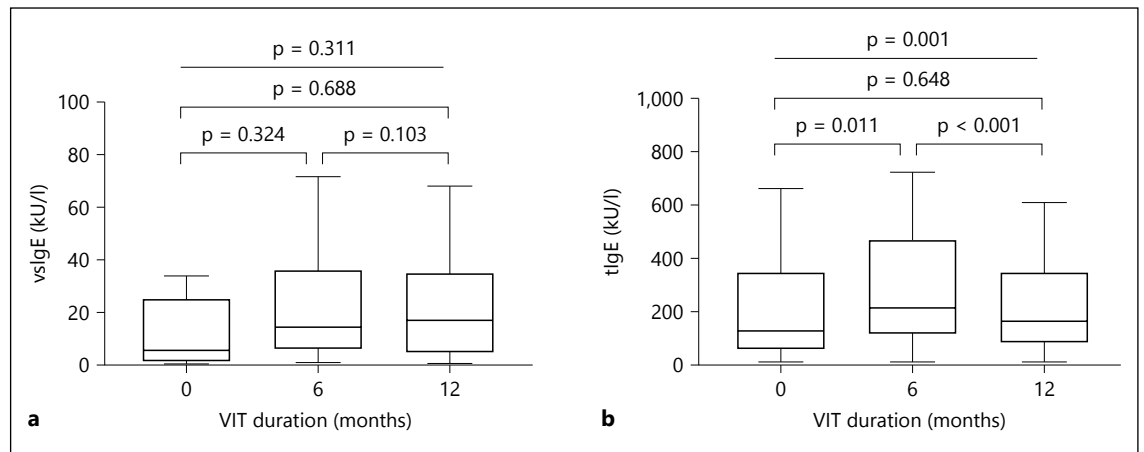


Fig. 2. **a** Venom sIgE levels of patients with SR during VIT. **b** tIgE levels of patients with SR during VIT.

modulation of OPN expression has been highlighted as an important mechanism that maintains homeostasis and limits the extent of allergic and inflammatory responses [26].

Contradictory and/or dual roles of OPN in allergic diseases suggest that OPN has a multifunctional nature in allergic responses. Xanthou et al. [27] demonstrated opposing effects of OPN in mouse models with allergic airway disease. They established that OPN plays a proinflammatory role during the primary sensitization phase but has an anti-inflammatory and protective effect during further antigenic challenges. They have attributed this dual role of OPN to differences in the recruitment of different dendritic cell (DC) subsets, particularly plasmacytoid DCs, which have been previously reported to have a suppressive potential on Th2 cell responses [28–30].

Apart from the studies regarding various Th2-mediated allergic diseases, 2 studies by the same group have been conducted on the potential role of OPN in tolerance induction as a result of VIT in patients with venom allergy [3, 4]. Konno et al. [3] investigated potential markers associated with successful VIT by cDNA microarray analysis and determined the relationship of genes that play a role in the expression of OPN with successful VIT in adults. In order to test this hypothesis, they compared serum OPN levels in patients with and without VIT and found that, in patients who underwent 5–6 years of VIT, these levels were significantly higher, even 3–6 years after the completion of the therapy [3]. They also reported a significant increase in the serum OPN levels of 8 adult patients with no history of SR who experienced an LLR to insect stings, particularly after

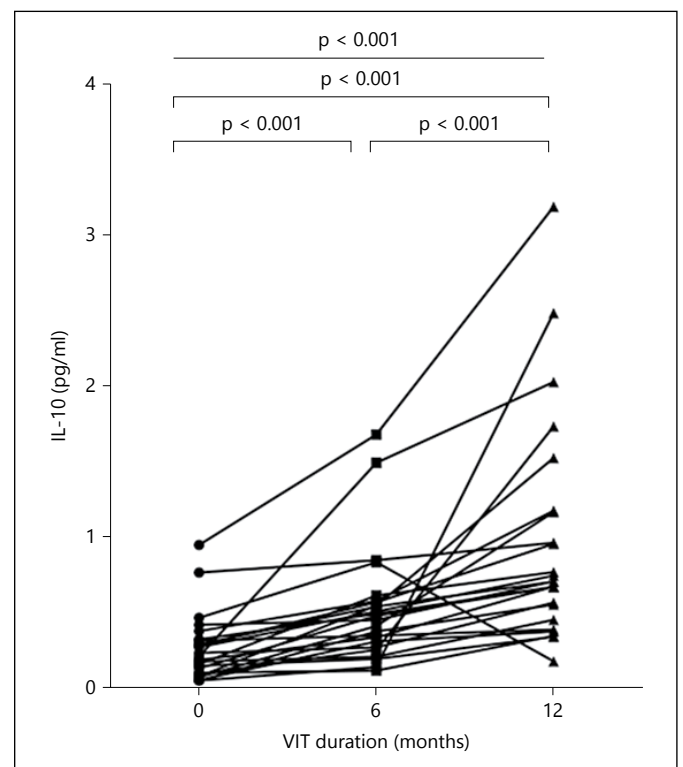


Fig. 3. IL-10 levels of patients with SR before and after the 1-year VIT.

6 months of VIT, along with a reverse relationship with venom sIgE levels [4].

Frenzel and Weiss [26] have proposed a model for the possible role of OPN in the mechanism of specific immunotherapy (SIT). They claimed that conventional DCs

(cDCs) produce high levels of OPN and IL-12 when they encounter the antigen. IL-12 secreted from cDCs not only promotes Th1 proliferation, causing the secretion of high levels of OPN and IFN- γ , but also suppresses Th2 inflammation, leading to reduced production of IL-4/IL-13. Eventually, Treg cells are induced by special DC subsets that regulate plasma cell secretion to switch from IgE antibodies to IgG4. They have also indicated that Treg cells may suppress the effector functions of Th2 effector cells like mast cells with the help of immunosuppressive cytokines such as IL-10 and TGF- β .

In our study, the plasma OPN levels of children who experienced LLR without any history of SR after insect stings were significantly higher than in children with SR. Furthermore, in parallel to the findings of Konno et al. [3], we also demonstrated a significant increase of plasma OPN levels in children who underwent VIT, particularly after 6 months. This increase may support the findings of Xanthou et al. [27] and the proposed model of Frenzel and Weiss [26] regarding the anti-inflammatory and protective effect of OPN after recurring antigenic challenges like SIT.

The major role played by IL-10 in tolerance induction related to SIT is a well-established phenomenon [31–33]. Several investigators have confirmed this association, particularly in VIT studies. Nasser et al. [34] have demonstrated a significant increase in IL-10-positive cells, with a trend towards an increase in IL-10 mRNA, in cutaneous biopsies performed before and 3 months after wasp VIT. Bussmann et al. [35] have reported that IL-10 serum levels increase from the second day of the build-up phase during bee VIT. Moreover, Pereira-Santos et al. [36] have remarked that a nonresponsive state is established in allergen-specific T cells due to the direct suppressive effects of IL-10 after 28 days of VIT. Two forms of OPN, i.e. secreted OPN and intracellular OPN, have been described in immune cells that convey complex functions in immunity [37]. Both forms can variously affect different functions of cells in allergic inflammation. Different types of secreted OPN modified by proteolytic cleavage or posttranslational modifications can bind to different OPN receptors, specifically modulating cell functions. An IL-10-inducing effect of secreted OPN from monocytes/macrophages has been established [26].

We have also demonstrated a significant increase in IL-10 levels in children during a 1-year course of VIT, which may be considered as an indication of a successful immunotherapy mechanism in our study population.

Immune tolerance usually implies a permanent change in the immune response with ablation of the clinical re-

sponse, whereas clinical protection can be achieved in days or weeks without necessarily inducing true tolerance. These data may therefore be important for identifying the possible role of OPN expression as a protective mediator for SR and/or the induction of tolerance by means of VIT in children with venom allergy.

Vadas et al. [38] established a relationship between serum platelet-activating factor (PAF) levels and PAF acetylhydrolase activity with the severity of anaphylaxis in patients with peanut allergy. Recently, Pravettoni et al. [39] have established a significant correlation between basal PAF acetylhydrolase activity and the grade of anaphylaxis in patients with venom allergy. These results may provide a plausible background to make an assertion that 'low' OPN levels or a possible metabolic pathway related to OPN could be a predictor of the risk for severe sting anaphylaxis, analogous to PAF and PAF acetylhydrolase.

Previous studies investigating the immunological changes during allergen SIT determined an early increase followed by a decrease in total and specific IgE levels [40, 41]. Although the duration of the VIT was shorter in our study, our results regarding IgE levels were similar to the data obtained previously.

Changes in sBT levels due to VIT were initially investigated by Dugas-Breit et al. [7]. In this retrospective study, a slight but significant decrease of sBT levels (2.5%/year) over time was determined. In previous studies, sBT levels were reported to increase with age [42, 43]. Investigators attributed a decrease in sBT levels, which is in contrast to the physiological process, to the effect of VIT. They have speculated that such a decrease is linked to the inhibition of the number and function of mast cells by IL-10 and other cytokines, the production of which is known to be increased during the VIT process. Cichocka-Jarosz et al. [8] prospectively analyzed data on 5 children who underwent 5 days of rush initial immunotherapy and 2 subsequent maintenance doses. They reported that sBT values were increased during the rush phase and subsequently returned to initial values with no significant change due to VIT being noted. We were also unable to demonstrate a significant change in sBT levels after 1-year conventional VIT. However, as in the study by Cichocka-Jarosz et al. [8], the relatively short follow-up and duration of VIT in our study (compared to that of Dugas-Breit et al. [7]) hindered the demonstration of a possible decline in sBT levels due to VIT. Long-term follow-up of our patients and the analysis of their future data may contribute to a better evaluation of the effect of VIT on sBT levels, a well-established risk factor for severe SR.

There are several strengths to our study. First of all, it is the first to investigate the potential role of OPN in the induction of immune tolerance due to VIT in children. In addition, our study groups consisted of children with reactions of varying type and severity along with control subjects with no venom allergy. However, this diversity might have led to a relatively small number of subjects in each group and provided inadequate data, particularly for the patients allergic to bee venom. Another study limitation was the lack of sting challenges in patients who underwent VIT, as this is not an appropriate option in children. However, a quarter of our patients were stung naturally during the VIT schedule and they experienced no LLR or SR, indicating the efficacy of the treatment. The prospective, controlled design is a strength of the study.

In conclusion, children without venom allergy and children with an LLR exhibited higher baseline levels of

OPN than children with an SR. Furthermore, increased OPN levels were observed after 1-year VIT in children with SR. Our results may suggest a possible role of OPN as a surrogate marker for response to VIT. Further studies may help to better identify the pathophysiological mechanisms related to OPN and evaluate the utility of OPN as a functional marker of VIT.

Acknowledgement

This study was supported by the Scientific Research Unit of Hacettepe University (010 D09 101 006).

Disclosure Statement

We report that there is no potential conflict of interest.

References

- ▶ 1 Bonifazi F, Jutel M, Bilo BM, Birnbaum J, Muller U: Prevention and treatment of hymenoptera venom allergy: guidelines for clinical practice. *Allergy* 2005;60:1459–1470.
- ▶ 2 Ozdemir C, Kucuksezer UC, Akdis M, Akdis CA: Mechanisms of immunotherapy to wasp and bee venom. *Clin Exp Allergy* 2011;41:1226–1234.
- ▶ 3 Konno S, Golden DB, Schroeder J, Hamilton RG, Lichtenstein LM, Huang SK: Increased expression of osteopontin is associated with long-term bee venom immunotherapy. *J Allergy Clin Immunol* 2005;115:1063–1067.
- ▶ 4 Konno S, Golden DB, Schroeder J, Hamilton RG, Lichtenstein LM, Huang SK: Level of osteopontin is increased after bee venom immunotherapy. *J Allergy Clin Immunol* 2005;115:1317–1318.
- ▶ 5 Rueff F, Przybilla B, Bilo MB, Muller U, Scheipl F, Aberer W, Birnbaum J, Bodzenta-Lukaszyk A, Bonifazi F, Bucher C, Campi P, Darsow U, Egger C, Haeblerli G, Hawranek T, Korner M, Kucharewicz I, Kuchenhoff H, Lang R, Quercia O, Reider N, Severino M, Sticherling M, Sturm GJ, Wuthrich B: Predictors of severe systemic anaphylactic reactions in patients with hymenoptera venom allergy: importance of baseline serum tryptase – a study of the European Academy of Allergology and Clinical Immunology Interest Group on insect venom hypersensitivity. *J Allergy Clin Immunol* 2009;124:1047–1054.
- ▶ 6 Yavuz ST, Sackesen C, Sahiner UM, Buyuktiryaki B, Arik Yilmaz E, Sekerel BE, Soyer OU, Tuncer A: Importance of serum basal tryptase levels in children with insect venom allergy. *Allergy* 2013;68:386–391.
- ▶ 7 Dugas-Breit S, Przybilla B, Dugas M, Arnold A, Pfundstein G, Kuchenhoff H, Rueff F: Serum concentration of baseline mast cell tryptase: evidence for a decline during long-term immunotherapy for hymenoptera venom allergy. *Clin Exp Allergy* 2010;40:643–649.
- ▶ 8 Cichocka-Jaros E, Dorynska A, Pietrzyk JJ, Spiewak R: Laboratory markers of mast cell and basophil activation in monitoring rush immunotherapy in bee venom-allergic children. *Immunotherapy* 2011;3:1013–1017.
- ▶ 9 Cichocka-Jaros E, Sanak M, Szczeklik A, Brzyski P, Gielicz A, Pietrzyk JJ: Serum tryptase level is a better predictor of systemic side effects than prostaglandin d2 metabolites during venom immunotherapy in children. *J Invest Allergol Clin Immunol* 2011;21:260–269.
- ▶ 10 Bilo BM, Rueff F, Mosbech H, Bonifazi F, Oude-Elberink JN: Diagnosis of hymenoptera venom allergy. *Allergy* 2005;60:1339–1349.
- ▶ 11 Cox L, Nelson H, Lockett R, Calabria C, Chacko T, Finegold I, Nelson M, Weber R, Bernstein DI, Blessing-Moore J, Khan DA, Lang DM, Nicklas RA, Oppenheimer J, Portnoy JM, Randolph C, Schuller DE, Spector SL, Tilles S, Wallace D: Allergen immunotherapy: a practice parameter third update. *J Allergy Clin Immunol* 2011;127:S1–S55.
- ▶ 12 Ellis AK, Day JH: Clinical reactivity to insect stings. *Curr Opin Allergy Clin Immunol* 2005;5:349–354.
- ▶ 13 Severino M, Bonadonna P, Passalacqua G: Large local reactions from stinging insects: from epidemiology to management. *Curr Opin Allergy Clin Immunol* 2009;9:334–337.
- ▶ 14 Yavuz ST, Sahiner UM, Buyuktiryaki B, Soyer OU, Sackesen C, Sekerel BE, Tuncer A: Clinical features of children with venom allergy and risk factors for severe systemic reactions. *Int Arch Allergy Immunol* 2012;160:313–321.
- ▶ 15 Sahiner UM, Civelek E, Yavuz ST, Buyuktiryaki AB, Tuncer A, Sekerel BE: Skin prick testing to aeroallergen extracts: what is the optimal panel in children and adolescents in Turkey? *Int Arch Allergy Immunol* 2012;157:391–398.
- ▶ 16 Uchio E, Matsuura N, Kadonosono K, Ohno S, Ueda T: Tear osteopontin levels in patients with allergic conjunctival diseases. *Graefes Arch Clin Exp Ophthalmol* 2002;240:924–928.
- ▶ 17 O'Neil SE, Malmhall C, Samitas K, Pullerits T, Bossios A, Lotvall J: Quantitative expression of osteopontin in nasal mucosa of patients with allergic rhinitis: effects of pollen exposure and nasal glucocorticoid treatment. *Allergy Asthma Clin Immunol* 2010;6:28.
- ▶ 18 Liu Y, Lu X, Yu HJ, Hua XY, Cui YH, Huang SK, Liu Z: The expression of osteopontin and its association with Clara cell 10 kDa protein in allergic rhinitis. *Clin Exp Allergy* 2010;40:1632–1641.
- ▶ 19 Hillas G, Loukides S, Kostikas K, Simoes D, Petta V, Konstantellou E, Emmanouil P, Papiris S, Koulouris N, Bakakos P: Increased levels of osteopontin in sputum supernatant of smoking asthmatics. *Cytokine* 2013;61:251–255.
- ▶ 20 Delimpoura V, Bakakos P, Tselioli E, Bessa V, Hillas G, Simoes DC, Papiris S, Loukides S: Increased levels of osteopontin in sputum supernatant in severe refractory asthma. *Thorax* 2010;65:782–786.
- ▶ 21 Samitas K, Zervas E, Vittorakis S, Semitekolou M, Alissafi T, Bossios A, Gogos H, Economidou E, Lotvall J, Xanthou G, Panoutsakopoulou V, Gaga M: Osteopontin expression and relation to disease severity in human asthma. *Eur Respir J* 2011;37:331–341.

- ▶ 22 Puxeddu I, Berkman N, Ribatti D, Bader R, Haitchi HM, Davies DE, Howarth PH, Levi-Schaffer F: Osteopontin is expressed and functional in human eosinophils. *Allergy* 2010;65:168–174.
- ▶ 23 Simoes DC, Xanthou G, Petrochilou K, Panoutsakopoulou V, Roussos C, Gratziau C: Osteopontin deficiency protects against airway remodeling and hyperresponsiveness in chronic asthma. *Am J Respir Crit Care Med* 2009;179:894–902.
- ▶ 24 Arjomandi M, Galanter JM, Choudhry S, Eng C, Hu D, Beckman K, Chapela R, Rodriguez-Santana JR, Rodriguez-Cintron W, Ford J, Avila PC, Burchard EG: Polymorphism in osteopontin gene (SPP1) is associated with asthma and related phenotypes in a Puerto Rican population. *Pediatr Allergy Immunol Pulmonol* 2011;24:207–214.
- ▶ 25 Akelma AZ, Cizmeci MN, Kanburoglu MK, Bozkaya D, Catal F, Mete E, Kutukoglu I, Namuslu M: Elevated level of serum osteopontin in school-age children with asthma. *Allergol Immunopathol (Madr)* 2014;42:275–281.
- ▶ 26 Frenzel DF, Weiss JM: Osteopontin and allergic disease: pathophysiology and implications for diagnostics and therapy. *Expert Rev Clin Immunol* 2011;7:93–109.
- ▶ 27 Xanthou G, Alissafi T, Semitekolou M, Simoes DC, Economidou E, Gaga M, Lambrecht BN, Lloyd CM, Panoutsakopoulou V: Osteopontin has a crucial role in allergic airway disease through regulation of dendritic cell subsets. *Nat Med* 2007;13:570–578.
- ▶ 28 de Heer HJ, Hammad H, Soullie T, Hijdra D, Vos N, Willart MA, Hoogsteden HC, Lambrecht BN: Essential role of lung plasmacytoid dendritic cells in preventing asthmatic reactions to harmless inhaled antigen. *J Exp Med* 2004;200:89–98.
- ▶ 29 Kohl J, Baelder R, Lewkowich IP, Pandey MK, Hawlich H, Wang L, Best J, Herman NS, Sproles AA, Zwirner J, Whitsett JA, Gerard C, Sfyroera G, Lambris JD, Wills-Karp M: A regulatory role for the C5a anaphylatoxin in type 2 immunity in asthma. *J Clin Invest* 2006;116:783–796.
- ▶ 30 Idzko M, Hammad H, van Nimwegen M, Kool M, Muller T, Soullie T, Willart MA, Hijdra D, Hoogsteden HC, Lambrecht BN: Local application of FTY720 to the lung abrogates experimental asthma by altering dendritic cell function. *J Clin Invest* 2006;116:2935–2944.
- ▶ 31 Akdis CA, Blesken T, Akdis M, Wuthrich B, Blaser K: Role of interleukin 10 in specific immunotherapy. *J Clin Invest* 1998;102:98–106.
- ▶ 32 Jutel M, Akdis M, Budak F, Aebischer-Casaltula C, Wrzyszc M, Blaser K, Akdis CA: IL-10 and TGF-beta cooperate in the regulatory T cell response to mucosal allergens in normal immunity and specific immunotherapy. *Eur J Immunol* 2003;33:1205–1214.
- ▶ 33 Akdis CA, Blaser K: IL-10-induced anergy in peripheral T cell and reactivation by microenvironmental cytokines: two key steps in specific immunotherapy. *FASEB J* 1999;13:603–609.
- ▶ 34 Nasser SM, Ying S, Meng Q, Kay AB, Ewan PW: Interleukin-10 levels increase in cutaneous biopsies of patients undergoing wasp venom immunotherapy. *Eur J Immunol* 2001;31:3704–3713.
- ▶ 35 Bussmann C, Xia J, Allam JP, Maintz L, Bieber T, Novak N: Early markers for protective mechanisms during rush venom immunotherapy. *Allergy* 2010;65:1558–1565.
- ▶ 36 Pereira-Santos MC, Baptista AP, Melo A, Alves RR, Soares RS, Pedro E, Pereira-Barbosa M, Victorino RM, Sousa AE: Expansion of circulating Foxp3+D25^{bright} CD4⁺ T cells during specific venom immunotherapy. *Clin Exp Allergy* 2008;38:291–297.
- ▶ 37 Cantor H, Shinohara ML: Regulation of T-helper-cell lineage development by osteopontin: the inside story. *Nat Rev Immunol* 2009;9:137–141.
- ▶ 38 Vadas P, Gold M, Perelman B, Liss GM, Lack G, Blyth T, Simons FE, Simons KJ, Cass D, Yeung J: Platelet-activating factor, PAF acetylhydrolase, and severe anaphylaxis. *N Engl J Med* 2008;358:28–35.
- ▶ 39 Pravettoni V, Piantanida M, Primavesi L, Forti S, Pastorello EA: Basal platelet-activating factor acetylhydrolase: prognostic marker of severe hymenoptera venom anaphylaxis. *J Allergy Clin Immunol* 2014;133:1218–1220.
- ▶ 40 Van Ree R, Van Leeuwen WA, Dieges PH, Van Wijk RG, De Jong N, Brewczynski PZ, Kroon AM, Schilte PP, Tan KY, Simon-Licht IF, Roberts AM, Stapel SO, Aalberse RC: Measurement of IgE antibodies against purified grass pollen allergens (Lol p 1, 2, 3 and 5) during immunotherapy. *Clin Exp Allergy* 1997;27:68–74.
- ▶ 41 Gleich GJ, Zimmermann EM, Henderson LL, Yunginger JW: Effect of immunotherapy on immunoglobulin E and immunoglobulin G antibodies to ragweed antigens: a six-year prospective study. *J Allergy Clin Immunol* 1982;70:261–271.
- ▶ 42 Farage MA, Miller KW, Berardesca E, Maibach HI: Clinical implications of aging skin: cutaneous disorders in the elderly. *Am J Clin Dermatol* 2009;10:73–86.
- ▶ 43 Gonzalez-Quintela A, Vizcaino L, Gude F, Rey J, Meijide L, Fernandez-Merino C, Linneberg A, Vidal C: Factors influencing serum total tryptase concentrations in a general adult population. *Clin Chem Lab Med* 2010;48:701–706.