

ORIGINAL ARTICLE

High-mobility group box-1 (HMGB-1) and serum soluble receptor for advanced glycation end products (sRAGE) in children affected by vernal keratoconjunctivitis

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Keywords

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Abstract

Background: Vernal keratoconjunctivitis (VKC) is a chronic disease affecting conjunctiva even though the immunopathogenetic mechanisms underlying this inflammation are unclear. The aim of our study is to investigate serum levels of HMGB1 and circulating sRAGE in children affected by VKC before and after treatment with cyclosporine A (CsA) eye drops and in a group of healthy children.

Methods: Twenty-four children affected by VKC aged between 5 and 12 yrs of life were enrolled at the Department of Pediatrics, Division of Allergy and Immunology, 'Sapienza' University of Rome. Twenty-four healthy children without atopy, ocular, and systemic disease, cross-matched for sex and age to patients affected by VKC, represented the controls. All children affected by VKC were treated with CsA 1% eye drops for 4 wks, and blood samples were collected before and 2 wks after the end of treatment while the controls underwent to a single blood sample at the time of enrollment.

Results: Serum basal levels of HMGB1 and sRAGE were higher in children with VKC when compared with controls while, in patients affected by VKC, no difference was detected between atopic and non-atopic, and between ANA-positive and ANA-negative children. A significant reduction in serum HMGB1 and sRAGE levels was detected after the therapy while CsA serum levels were negative.

Conclusions: Our study gives a support to the definition of VKC as a systemic inflammation in which HMGB1 and its soluble receptors could play a role.

Vernal keratoconjunctivitis (VKC) is a chronic and often severe form of bilateral tarsal and/or bulbar conjunctivitis. It is more common in children and young adults with a predilection for males. VKC usually occurs between 5 and 10 yrs of life, and it tends to regress during or soon after puberty (1). Although it is a self-limiting disease, patients may often present exacerbations of inflammatory symptoms with possible permanent ocular damages and serious consequences on the quality of life. The diagnosis is based on the classical symptoms of conjunctivitis (hyperemia, itching, and tearing) and on some specific ocular signs such as cobblestone papillae visible with the eversion of the upper lid or the presence of papillae in the limbus. Three forms of VKC have been described: the tarsal form with papillae on the tarsal conjunctiva, the limbal form with limbal papillae often covered with Horner-Trantas dots and the mixed phenotype with intermediate characteristics. Moreover, the corneal

involvement is known as an important sign as it is associated with more severe disease (2).

Many risk factors were investigated and described in the literature (2, 3), but the role played by endocrine, genetic, neurogenic, environmental, and socioeconomic factors remains still unclear.

Previous studies investigating the immunopathogenesis of VKC suggested that both IgE-dependent (type-I allergic) and IgE-independent (type-IV allergic) mechanisms may be involved (2).

Although many clinical and laboratory findings support the presence of an IgE-dependent immediate hypersensitivity reaction in VKC (2), the conflicting results concerning the association between VKC and atopy suggest that an IgE-independent mechanisms could also be involved (1).

High-mobility group box protein 1 (HMGB1) has been recently identified as an important proinflammatory mediator

with many characteristics similar to classic proinflammatory cytokines. HMGB1, like other proinflammatory mediators that participate in feed-forward regulation, induces its own release both *in vivo* and *in vitro*. HMGB1, well known as an intranuclear protein, can be released by several types of cells in two different ways: active secretion from living inflammatory cells or passive release from necrotic cells acting as an alarmine and a Th1-type cytokine (4, 5). The active secretion of HMGB1 into the extracellular milieu begins 8–12 h after ligation to specific receptors and continues to increase for 18–36 h: a time frame significantly delayed in comparison to that of tumor necrosis factor α (TNF α) and of interleukin-1 (IL-1) that are the prototypical early proinflammatory cytokines (6). A distinctive feature of HMGB1 compared with other proinflammatory cytokines is that HMGB1 elicits its biological response by signal transduction through receptors that can also interact with other foreign molecules both exogenous (TLR4-TLR9) and endogenous receptor for advanced glycation end products (RAGE).

RAGE belongs to the immunoglobulin superfamily of transmembrane proteins and it is a multiligand receptor expressed on the surface of a wide variety of cells including endothelial cells, vascular smooth muscle cells, neurons, and monocytes/macrophages (7). Its main ligands include advanced glycation end products (AGEs), S100/calgranulins (such as S100P and S100A4), and HMGB1. The engagement of full-length RAGE with its ligands triggers rapid generation of intracellular reactive oxygen species and activates an array of cell signaling pathways that lead to the activation of the transcription factor NF- κ B (8). Structurally, RAGE is composed of three extracellular immunoglobulin-like domains (one V variable and two C constant domains C1 and C2), a single transmembranous domain and short cytoplasmic tail that is essential for RAGE-mediated signaling. Soluble RAGE (sRAGE) is a splice variant that lacks the transmembranous and intracellular signaling domains of the full-length receptor and therefore circulates in plasma. By competing with cell surface RAGE for ligand binding, sRAGE may contribute to the removal/neutralization of circulating ligands functioning as a decoy (9).

HMGB1 signaling through RAGE mediates chemotaxis and stimulation of cell growth, differentiation of immune cells, the migration of immune and smooth muscle cells, and the upregulation of cell surface receptors. Another fundamental role of sRAGE is the amplification of the inflammatory response. Moreover, several studies have provided evidence that extracellular HMGB1 is involved in a variety of pathophysiological events including asthma and allergic rhinitis (10–13).

Topical corticosteroids represent an effective treatment of patients affected by VKC (14), even though their prolonged use is often associated with important complications such as glaucoma, cataracts, and bacterial infections. On the other hand, the effectiveness of antihistamines, inhibitors of mast cell degranulation, and non-steroidal anti-inflammatory drugs was reported only in a few cases (15). Topical cyclosporine A (CsA) is a safe and effective treatment in both moderate and severe forms of VKC (16), with an immunomodulatory effect on the

conjunctiva leading to a reduction in inflammatory cell particularly T cells, HLA-DR⁺ cells, and plasma cells.

In this study, we investigate whether HMGB1 and sRAGE could be involved in the pathogenesis of VKC. We analyzed the serum levels of these proteins in children affected by VKC before and after treatment with CsA eye drops and in a group of healthy children.

Patients and methods

Between November 2011 and November 2012, a total of 24 children (16 males; 67%) affected by VKC aged between 5 and 12 yrs of life were enrolled at the Department of Pediatrics, Division of Allergy and Immunology, 'Sapienza' University of Rome. Twenty-four healthy children with negative skin prick test (SPT), without allergic, ocular, and systemic disease, cross-matched for sex and age to patients affected by VKC, were used as controls. The diagnosis of VKC was made by an ophthalmologist investigating ocular signs (OS) (conjunctival hyperemia, tarsal and/or limbal papillae, giant papillae) and subjective ocular symptoms (itching, photophobia, tearing, foreign body sensation, and burning sensation) according to two disease severity scales graded from '0' absent to '2' severe, as previously described (3). Children were classified as having severe VKC if the score was >3 points for one eye for each scale. Signs and symptoms were recorded at entry into the study and after 4 wks of treatment with CsA eye drops.

Three forms of VKC were identified: the tarsal form with papillae on the tarsal conjunctiva, the limbal form with limbal papillae, and the mixed phenotype with intermediate characteristics.

Atopic status was assessed by SPT to airborne and food allergens (Lofarma, Milan, Italy) and/or elevated specific (>0.35 kU/l) and total IgE (>100 kU/l). Panels included the following allergens: *Dermatophagoides pteronissinus*, *Dermatophagoides farinae*, dog/cat dander, *Olea europea*, *Lolium perenne*, *Alternaria tenuis*, *Parietaria officinalis*, lactalbumin, β -lactoglobulin, casein, egg white and yolk, soy, codfish. A positive SPT was defined by the presence of a wheal more than 3 mm respect to the wheal size of control (saline solution).

Antihistamines and/or corticosteroids were not included in the treatment regimen and they had been suspended at least from 2 month before the beginning of the treatment with CsA. Children affected by VKC were treated with CsA 1% eye drops suspension (Sandimmun_ galenical collyrium Pharmacy Umberto I hospital, Rome, Italy) (1 drop/eye twice daily) for 4 weeks, prepared strictly respecting sterility criteria, by the Chemistry Service Institute at the University of Rome, following a formulation including one part of commercially available CsA solution for intravenous infusion (Sandimmun_Novartis Farma S.p.A.S.S., Varese, Italy), diluted in aqueous vehicle (Vismed Light_TRB Chemedica, Haar/Munich, Germany). Parents were asked to keep refrigerated, shielded from light, the vial with the eye drops and to use it within 15 days from the opening.

Blood samples were collected for children with VKC before and after treatment. The controls underwent a blood sample at enrollment.

This study was approved by the International Review Board of 'Sapienza' University of Rome and performed with the written informed consent of the parents of all children.

Samples collection

Five milliliters of peripheral blood was collected and centrifuged at 1000 g for 10 min to obtain serum samples. All sera were aliquoted in small amounts and stored frozen in plastic vials at -20°C until analysis.

Antinuclear antibodies (ANA) were detected with indirect immunofluorescence (Menarini, Florence, Italy), and their value was considered positive if fluorescence had a titer of 1:40/1:80.

Serum level of CsA was measured after treatment with CsA eye drops in all the children with VKC.

Western blot analysis

Human serum samples were separated, respectively, on 15% and 10% SDS-polyacrylamide electrophoresis gel to detect HMGB1 and sRAGE.

Samples were heat-denatured for 5 min, loaded on standard Tris-HCl polyacrylamide gel, and run on ice at 40 V for the stacking gel and 80 V for the running gel. Proteins were transferred into a PVDF membrane (Bio-Rad, Hercules), previously activated in 100% methanol for 15 s. The membrane was blocked in TBS-T and 5% albumin for 1 h, probed overnight at 4°C by the specific antibody (monoclonal anti-HMGB1 by Sigma and monoclonal anti-sRAGE by Millipore). At the end of the incubation time, the membrane was washed and incubated with anti-mouse IgG peroxidase-conjugated secondary antibodies (Sigma) for 1 h at room temperature. The signal was detected by autoradiography (Kodak Biomax light film; Sigma-Aldrich) using the chemiluminescent peroxidase substrate kit (Sigma-Aldrich) then quantified by densitometric analysis using software (Quantity-One, Bio-Rad).

Statistical analysis

Statistical analyses were performed using SPSS (Statistical Package of Social Sciences, Chicago, IL, USA) software version 19. Descriptive statistics were performed expressing continuous data as means with SDs, and categorical data were expressed by frequency and percentage. Comparisons of categorical data were evaluated using a chi-square test while a *t*-test or a Mann-Whitney *U*-test was used, after assessing for normality with the Shapiro-Wilk test, to compare serum levels of HMGB1 and sRAGE, before and after treatment, in VKC subjects, between children affected by VKC and healthy controls and between all the subgroups. A *p*-value less than 0.05 was considered statistically significant.

Results

Twelve children (50%) with VKC had a positive SPT: 8 children (67%) to house-dust mites, 3 (25%) to *Lolium*

perenne, 3 (25%) to *Cynodon*, 3 (25%) to *Olea europea*, 3 (25%) to *Parietaria officinalis*, 3 (25%) to *Alternaria tenuis*, 3 (25%) to cat dander (Table 1). The tarsal form was diagnosed in 12 children (50%); the mixed phenotype in 12 children while no patient had the limbal form. The cornea was involved in 15 children (62%): 9 patients (60%) with corneal abrasion, 3 (20%) with punctate keratitis, 3 (20%) with shield ulcer. Nine children (37.5%) were ANA positive. All patients presented a worsening of symptoms during spring and summer.

Serum levels of HMGB1 and sRAGE were higher in children with VKC compared with the controls (9.53 ± 2.42 vs. 8.22 ± 1.29 , $p = 0.026$; 7.66 ± 2.24 vs. 6.53 ± 1.35 , $p = 0.041$, respectively) (Fig. 1).

In children affected by VKC, serum levels of HMGB1 and sRAGE showed no significant difference between atopic and non-atopic, ANA-positive and ANA-negative children, and between the two forms of VKC detected in our study sample. On the other hand, they were markedly higher in children with corneal involvement (Table 1).

A significant reduction in both HMGB1 and sRAGE levels (Fig. 2) (9.5 ± 2.4 vs. 8.1 ± 2.8 , $p < 0.001$; and 7.6 ± 2.2 vs. 4.2 ± 1.9 , $p < 0.001$, respectively) and of scores severity scale (OS from 5.75 ± 1.75 to 1.96 ± 0.95 , $p < 0.001$; SS from 7.63 ± 1.83 to 1.67 ± 0.64 , $p < 0.001$) were detected after topical CsA treatment whereas serum levels of CsA remain unmodified.

Discussion

Vernal keratoconjunctivitis is a chronic disease affecting the conjunctiva even though the immunopathogenetic mechanisms underlying this chronic inflammation are still matter of debate.

In the past, VKC was considered a type-I hypersensitivity reaction but the hypothesis of an IgE-mediated process is not sufficient at all to explain the clinical and histopathological

Table 1 HMGB1 and sRAGE levels in our study samples

	Nr	HMGB-1	p-value	RAGE	p-value
Healthy controls	24	8.20 ± 1.43	0.02	6.53 ± 1.44	0.04
Children with VKC	24	9.53 ± 2.42		7.66 ± 2.24	
Atopic	12	9.17 ± 1.60	0.48	8.27 ± 2.59	0.19
Non-atopic	12	9.88 ± 3.07		7.06 ± 1.74	
ANA positive	9	9.63 ± 1.54	0.88	8.16 ± 2.97	0.41
ANA negative	15	9.47 ± 2.88		7.37 ± 1.72	
Tarsal form	12	10.22 ± 2.73	0.16	7.51 ± 1.91	0.74
Mixed phenotype	12	8.83 ± 1.94		7.82 ± 2.60	
Corneal involvement	15	11.04 ± 1.71	<0.001	8.94 ± 1.75	<0.001
No corneal involvement	9	7.01 ± 0.70		5.54 ± 1.00	

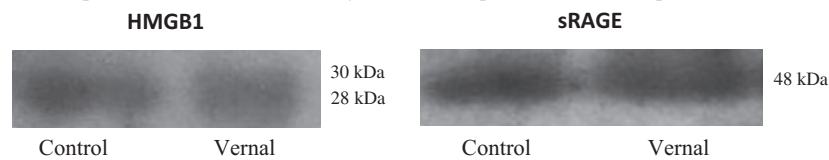
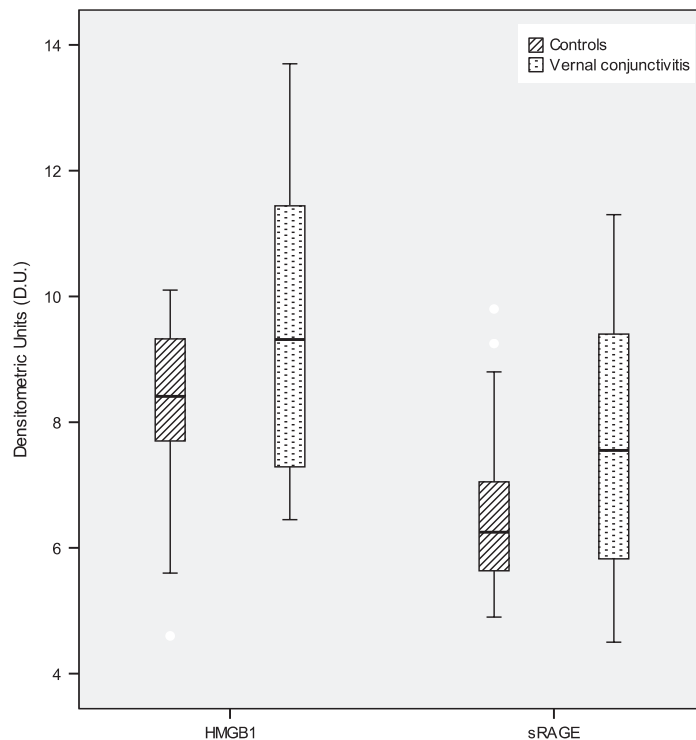
(a) a representative Western Blot of control and patient serum samples**(b)** Densitometric analysis of Western Blots. The horizontal bar within the box represents the median; the boxes represent a range of $\pm 25\%$ around the median. Vertical bars indicate 95% CI.

Figure 1 HMGB1 and sRAGE levels in children with vernal keratoconjunctivitis and in the controls. (a) a representative Western blot of control and patient serum samples. (b) Densitometric analysis of Western blots. The horizontal bar within the box represents the median; the boxes represent a range of $\pm 25\%$ around the median. Vertical bars indicate 95% CI.

characteristics of VKC. In this perspective, in our sample, only 50% of children with VKC show a clear allergic sensitization, in line with percentages reported in the literature (16–18).

Chronic conjunctiva changes in VKC are characterized by a prevalent Th2-type inflammatory response, and many authors (19) detected high levels of IL1,4,6,13 (IL1, IL4, IL6, and IL13) and transforming growth factor beta 1 (TGF β 1). Most of these studies were performed on the conjunctiva and underlined the chronic inflammation affecting the eyes of patients with VKC. Besides ocular lesions, most of the patients affected by VKC does not manifest systemic symptoms but many authors reported an increased presence of serum chemokines such as IL-1 and TNF α and other inflammatory mediators, eosinophil cationic protein, and soluble interleukin-2 receptor (20). Moreover, a predominance of IL-4-producing T-cells in peripheral blood was described as one of the immunological features of VKC (21). According to our knowledge, this is the first study that aims at assessing serum levels of HMGB1 and sRAGE in children affected by VKC before and after the treatment with CsA eye drops.

In 1999, Hingorani et al. (22) demonstrated that conjunctiva inflammatory infiltrates present a significant reduction after treatment with CsA eye drops. In 2010, Tesse et al. (16) confirmed that 1% CsA concentration, administered topically, is an effective treatment of VKC in children reporting a significant decrease in SS and OS mean scores already after 2 wks of treatment. Also our patients, after the treatment with topical CsA, showed a rapid decrease in scores for both severity scales. These findings might be explained by the effect of CsA that acts blocking helper T-lymphocyte proliferation and IL-2 production, inhibiting histamine release from human mast cells and basophils and reducing the IL-5 release (22).

HMGB1 is a proinflammatory mediator, and its release is stimulated by injury. This mediator activates cells to produce proinflammatory response after the binding to the receptor RAGE. When HMGB1 interacts with RAGE, mediates chemotaxis and stimulates cell growth, differentiation of immune cells and upregulation of cell surface receptors, including RAGE. The role of HMGB1 in systemic inflammatory reactions has been largely investigated both 'in vitro' and

(a) a representative Western Blot of one VKC patient before and after therapy

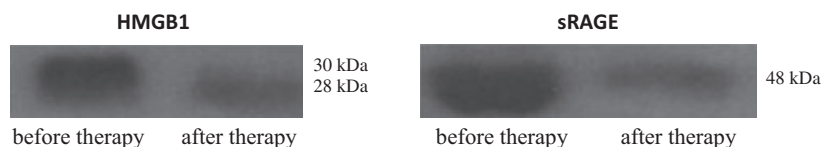
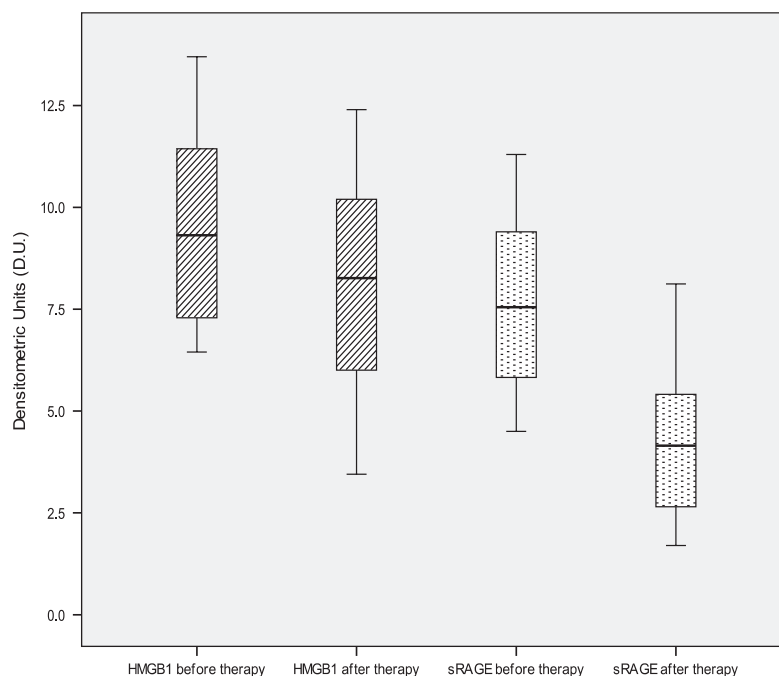
(b) Densitometric analysis of Western Blots. The horizontal bar within the box represents the median; the boxes represent a range of $\pm 25\%$ around the median. Vertical bars indicate 95% CI.

Figure 2 HMGB1 and sRAGE in a child affected by vernal keratoconjunctivitis before and after treatment with topical cyclosporine. (a) a representative Western blot of one vernal keratoconjunctivitis patient before and after therapy before therapy after therapy. (b) Densitometric analysis of Western blots. The horizontal bar within the box represents the median; the boxes represent a range of $\pm 25\%$ around the median. Vertical bars indicate 95% CI.

'*in vivo*', and serum level of HMGB1 was found increased in many diseases characterized by chronic inflammation as pancreatitis, gastrointestinal inflammation, arthritis, and other autoimmune diseases (5). On the other hand, the role played by sRAGE in inflammation is still not completely known. In fact, if many studies reported its decrease in chronic inflammatory diseases, others showed that the presence of detectable levels of sRAGE is associated with increased levels of HMGB1. Serum sRAGE levels have been reported not only to be higher in patients with lupus erythematosus compared with healthy subjects, but also to be increased during reactivation periods (23).

The involvement of HMGB1 in the pathogenesis of allergic diseases is the object of many studies. It has been recently demonstrated that nasal HMGB1 is significantly increased in children affected by allergic rhinitis when compared with non-allergic subjects, and HMGB1 level seems related to symptom severity. Moreover, HMGB1 level is related with the percentages of neutrophils and eosinophils in the induced sputum of asthmatic patients (11–13). Milutinovic et al. (24) demonstrated in a house-dust mite mouse model of asthma/allergic airway disease that the

absence of RAGE abolishes airway hypersensitivity, eosinophilic inflammation, and airway remodeling and that the treatment with an inhibitor of RAGE markedly reduces inflammation, suggesting that RAGE inhibition may represent a promising therapeutic strategy. We have found an increased serum level of both HMGB1 and circulating sRAGE in patients affected by VKC compared with the controls, and this difference was more evident in patients with corneal impairment. This result allows us to hypothesize that an immune-mediated dysregulation, leading to an increased production of the soluble form of sRAGE, is involved in the most severe phenotypes of VKC. sRAGE may capture and eliminate circulating HMGB1 and may be one of the determinants of circulating HMGB1 levels. In fact, sRAGE is able to act as a decoy to avoid interaction of RAGE with its proinflammatory ligands (AGEs, HMGB1, S100 proteins). In light of these findings, we speculated that sRAGE may represent an inflammatory marker of disease and it may provide useful information on the severity of the ocular disease. Furthermore, the reduction in circulating sRAGE and HMGB1 after CsA eye drops could be considered a parameter of the therapy efficacy.

The integrity of the conjunctiva extracellular matrix is impaired by the remodeling and the induction of matrix metalloproteinases (MMPs), and this injury may facilitate the outwards passage as well as the inward invasion of inflammatory cells (2). Furthermore, HMGB1 may stimulate the production and the activation of the MMPs (25, 26). In this perspective, VKC might be associated not only to a local inflammation, but also to a systemic inflammatory response (27).

In children with VKC, a corneal involvement is often reported and it is characterized by punctate keratitis or sterile corneal ulcers (19) that may induce serious visual changes.

The cornea and the conjunctiva are separated only by a thin film of tear fluid and immune cells (conjunctiva-associated lymphoid tissue) and vasculature with weak barrier properties are typical of the conjunctiva, while the cornea with its epithelium possesses strong barrier properties. The loss of this barrier functions might increase the exposure of stromal fibroblasts of the cornea to all the chemical mediators and cytokines released into tear fluid from the conjunctiva (27) determining all the previously described complications of VKC. In 2003, Pucci et al. (28) reported higher levels of ECP in patients affected by the tarsal and mixed forms of VKC supposing that the severity of the local disease may influence systemic immunological responses and inflammation. A few years later, Leonardi et al. (19) found higher mean values of interferon-gamma (IFN γ) in tears of patients who also presented the corneal involvement.

The evidence of higher serum levels of HMGB1 and sRAGE in children affected by VKC with corneal involvement seems to

confirm that systemic inflammation may also be influenced by the development of ocular complications and not only by the presence of ocular inflammation. The evaluation of HMGB1 and sRAGE in the conjunctiva tissue or tear fluid would be an interesting issue to investigate in future researches on VKC to better clarify their role in the complex framework of ocular inflammation.

We did not find any detectable levels of systemic CsA at the end of our study, but serum levels of HMGB1 and sRAGE showed a significant reduction after 4 wks of therapy. Our findings lead us to hypothesize that the topical administration of CsA may influence not only the ocular immunological responses but also the regulation of systemic inflammation.

Further studies on larger cohorts of patients are warranted to confirm our results and to clarify the role played by HMGB1 and sRAGE in the immunopathogenesis and the evolution of VKC.

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This study was approved by the Institutional Review Board of 'Sapienza' University of Rome.

Conflict of interest

In relation to this manuscript, we declare that there are no conflict of interest or financial support.

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