Changes in tear biomarker levels in keratoconus after corneal collagen crosslinking

Jose Ignacio Recalde,1 Juan Antonio Duran,1,2 Iñaki Rodriguez-Agirretxe,1,3 Javier Soria,4 Miguel Angel Sanchez-Tena,5 Xandra Pereiro,5 Tatiana Suarez,4 Arantxa Acera6

1Instituto Clínico-Quirúrgico de Oftalmología, Bilbao, Bizkaia, Spain; 2Department of Ophthalmology, University of the Basque Country, Experimental Ophtalmo-Biology Group UPV/EHU, Leioa, Bizkaia, Spain; 3Hospital Universitario Donostia, San Sebastian, Gipuzkoa, Spain; 4Bioftalmik Applied Research, Bizkaia Science and Technology Park, Derio, Bizkaia, Spain; 5Universidad Europea de Madrid, Madrid, Spain; 6Department of Cell Biology and Histology, University of the Basque Country, Experimental Ophtalmo-Biology Group UPV/EHU, Leioa, Bizkaia, Spain

Purpose: The purpose of this work was to analyze the expressions of matrix metalloproteinase 9 (MMP-9), calcyclin (S100A6), and cystatin S (CST4) in the tears of keratoconus (KC) patients. The correlations between the expressions of these proteins and the values of various ocular surface parameters were examined after accelerated corneal crosslinking (A-CXL) with pulsed ultraviolet light.

Methods: This prospective, observational study enrolled patients with different grades of KC, scheduled to undergo the A-CXL procedure, as well as healthy subjects. Tear samples were analyzed by employing customized antibody microarray assays for MMP-9, S100A6, and CST4 proteins. The keratometry readings at the maximum keratometry (Kmax) and the simulated keratometry (SimK) values were obtained for examining the postoperative evolution of corneal topography. The state of the ocular surface was evaluated using the results of the Ocular Surface Disease Index (OSDI) questionnaire, tear osmolarity (OSM) test, Schirmer test (SCH), Tear Break Up Time (TBUT), tear clearance (CLR), and fluorescein (FLUO) and lissamine green (LG) corneal staining.

Results: A total of 18 patients (22 eyes) and 10 healthy subjects were studied. The concentrations of MMP-9 and S100A6 decreased in tears, from 104.5 ± 78.98 ng/ml and 350.20 ± 478.08 ng/ml before the surgery to 48.7 ± 24.20 ng/ml and 55.70 ± 103.62 ng/ml, respectively, after 12 months of follow up. There were no changes in the CST4 concentration after 12 months of follow up (2202.75 ± 2863.70 versus 2139.68 ±2719.89 ng/ml). When the patients were divided into three groups according to the evolutive stage of KC, the trends for the three biomarkers in each group were the same as in the general group. Basal concentrations of MMP-9 and S100A6 from healthy subjects and KC patients were compared. The levels of MMP-9 and S100A6 in tears were (9.8 ± 5.11 and 104.55 ± 78.98 ng/ml, p<0.01; and 11.35 ± 3.18 and 350.26 ± 478.06 ng/ml, respectively, p<0.01). This was not the case for CST4, which did not exhibit statistically significant differences between the two groups (2261.94 ± 510.65 and 2176.73 ± 2916.27 ng/ml respectively, p=0.07).

Conclusions: A-CXL promoted a decrease in the concentrations of MMP-9 and S100A6 in the tear film. This effect may be related to the restoration of corneal homeostasis and the consequent repair of the tissue damage caused by KC. Moreover, the A-CXL treatment did not produce lasting alterations in the ocular surface, and the values of the evaluated clinical parameters did not change significantly.

The tear film covers and protects the ocular surface, and it is essential in maintaining ocular homeostasis [1]. It contains several molecules that include a wide variety of proteases and protease inhibitors [2,3], where the concentrations can change in various local and systemic diseases, such as dry eye syndrome (DES) [4-8], keratoconus (KC) [9-15], primary open-angle glaucoma, and proliferative diabetic retinopathy, among others [16]. This specific molecular signature can help in understanding the etiology of the disease and to help in the diagnosis or prognosis of some ocular surface conditions.

KC is a progressive ectasia of the cornea of unknown origin. It is characterized by the thinning and protrusion of the cornea, leading to irregular astigmatism and myopia, thereby affecting the visual performance [17]. The thinning of the cornea in KC may be due to tissue degradation that involves the remodeling of the extracellular matrix as a result of collagen deficiency [18] and increases in the levels of proinflammatory cytokines, cell adhesion molecules, and matrix metalloproteinases (MMPs) [9,19-21].

MMPs, a group of zinc-dependent endopeptidases that includes gelatinases (MMP-2, 9), collagenases (MMP-1, 8, 13), stromelysins (MMP-3, 10), and matrilysins (MMP-7, 26)
synthesized by corneal epithelial cells and stromal cells, have long been suspected of having a significant role in KC [11]. MMP-9 is a gelatinase produced in the corneal epithelium and activated in tear film. Its concentration is significantly higher in patients with KC disease, as shown by Lema et al. [9], who reported that the tear film of those patients showed increased levels of the proinflammatory cytokines interleukin 6 (IL-6) and tumor necrosis factor α (TNF-α), as well as higher levels of MMP-9; thus, this enzyme could be implicated in the remodeling process of the cornea in KC. It is known that the levels of epithelial and stromal structural proteins of KC corneas are altered, suggesting that they are affected due to structural remodeling during the development and progression of KC.

There are some structural proteins implicated in corneal epithelium integrity that appear upregulated in the tears from some ocular surface diseases [8,22]. One of these is the calcyclin (S100A6) protein. This is an S100 calcium-binding protein that exhibits upregulated expression in proliferating and differentiating cells. S100A6 was found to be expressed at high levels in fibroblasts and epithelial cells with high proliferating activity, as well as those undergoing differentiation [23]. Moreover, it has been shown to interact in vitro in a calcium dependent manner with Annexin A2 (ANXA2) and Annexin A5 (ANXA5) [24,25]. In contrast, ANXA2 has been found to be downregulated in stroma of KC corneas, suggesting a possible role in progression of KC disease. In addition, S100A6 has been used for monitoring patients’ response to changing glaucoma treatment, suggesting the potential application of this protein as a prognostic biomarker [26].

Cystatins are natural inhibitors of cysteine proteinases. These proteinases are one of the most abundant protein-degrading enzymes in mammalian cells [27]. They are involved in the initial phases of degradation of intracellular proteins and can provoke tissue degradation after being released into the extracellular medium. The activity of cysteine proteinases is controlled by their physiologic inhibitors, the cystatins, which are known to be generally present in tears [28]. Extracellular cystatins have a protective role against the damaging effects of lysosomal proteinases, which can be secreted under physiological conditions for the degradation and regeneration of tissues, as well as under pathological conditions associated with infection by bacteria or viruses [29]. Our group has previously described a decrease in cystatins levels in KC patients that is potentially related to the degradation of tear proteins [10].

Corneal crosslinking (CXL) with ultraviolet radiation (UVA) and riboflavin is a technique used to strengthen the softened and deformed cornea [30,31]. This procedure results in tightening of the interlocking mesh of the corneal collagen fibers and formation of a dense weblike structure. In most cases, the procedure stops the progression of diseases like KC, pellucid marginal degeneration of the cornea, and iatrogenic ectasias. The aim of this research was to determine the concentration of biomarkers related to the remodeling process of the ocular surface in the tears of KC patients before A-CXL treatment and examine the potential changes in the concentration of these markers 12 months after the surgery.

**METHODS**

This prospective observational study was performed at the Cornea Unit of the Instituto Clínico-Quirúrgico de Oftalmología (ICQO) of Bilbao, Spain. The research was conducted by medically qualified personnel after receiving the approval of the Cruces Hospital Ethics Committee. The study was conducted in strict accordance with the tenets of the Declaration of Helsinki on Biomedical Research Involving Human Subjects. Before tear collection, a signed informed consent was obtained from all patients once the nature and possible consequences of the study had been explained.

**Subjects:** Twenty-eight subjects (32 eyes) over 18 years of age were enrolled in the study. Ten healthy subjects and 18 patients with clinically evident and progressive KC, with an increase of at least one diopter in maximum keratometry (Kmax) during the previous year and scheduled for the A-CXL surgery were considered for the study. The exclusion criteria were ocular surgery performed in the preceding 3 months, a systemic condition (active allergy), or medication (anti-inflammatories) that could interfere with the interpretation of the results, as well as the concomitant administration of topical medications (except artificial tears).

The diagnosis of KC was performed by topographic evaluation using a Pentacam HR (Oculus Optikgeräte GmbH, Wetzlar, Germany), following the classical criteria established by Rabinowitz [32]. The criteria were an inferior/superior index greater than 1.5, maximum keratometry at the corneal apex (Kmax) greater than 47 D, and a difference between the Kmax of the two eyes of more than 1 D.

As there is no consensus on the most appropriate classification of the various progression degrees of KC, it was decided to modify the classification proposed in 2013 slightly, which is based mainly on the Kmax [33]. The grades 1 and 2 in this classification (suspected and subclinical KC, respectively) are not suitable for the CXL treatment, which is reserved for grades 3, 4, and 5 (initial, moderate, and advanced KC). For our study, the division between the
moderate and advanced grades was assumed at a maximum keratometry of 55 D (not 57 D as suggested by Rabinowitz).

**Surgical intervention:** The surgical technique employed in the A-CXL treatment was the method with corneal de-epithelialization (Epi-Off technique). After de-epithelializing 6.5 mm of the central cornea using an Amoils epithelial scrubber (Innovative Excimer Solutions Inc., Toronto, Canada), an iso-osmotic riboflavin solution (Vibex Rapid, Avedro Inc., Waltham, MA) was instilled every 2 min for 10 min.

The XKL system (Avedro Inc.) was used to produce the UVA radiation. We employed pulsed light (UVA exposure, 1 s on, 1 s off) at 30 mW/cm² for 8 min. This corresponds to a yield of 7.2 J/cm² of total administered energy.

Finally, a hydrophilic contact lens was placed on the cornea as a bandage. It was removed within 5–7 days, when re-epithelization of the cornea was completed. Antibiotic and steroidal eye drops (TobraDex®, Alcon-Cusi, El Masnou, Spain) were instilled, in a descending pattern, for a month. During this period, the eye was moistened with artificial tears on demand.

**Calendar of visits:** Before the surgery, all the patients underwent a complete ophthalmic examination, including refraction, topography, corneal biomicroscopy, and the examination of the fundus of the eye. Control examinations were scheduled at 3, 6, and 12 months after the operation. All visits took place between 10 and 12 AM.

**Clinical examinations during each visit:** For examining the clinical evolution of KC and the changes in the ocular surface after A-CXL, the following clinical and biochemical parameters were studied: keratometry (Kmax and simulated keratometry [SimK]), lacrimal osmolarity (OSM) measurements [34], the Ocular Surface Disease Index (OSDI) questionnaire [35], the Tear Break Up Time (TBUT) test, lacrimal clearance (CLR) [36], fluorescein staining (FLUO) using the Oxford scheme [37], Schirmer test with anesthesia (SCH), and lissamine green (LG) staining following the van Bijsterveld scheme [38].

The concentrations of the MMP-9, S100A6, and CST4 biochemical biomarkers in the tear film were also analyzed in the study. The manner of sample collection and order of tests were always the same. First, the OSDI questionnaire was used to assess the symptoms of ocular irritation and their effect on the vision-related functions. Second, the OSM was tested, after which a drop of topical anesthetic was applied to the ocular surface and SCH, TBUT, FLUO, CRL, and LG tests were performed. The tear samples were collected one day later to avoid any interference between the clinical tests and biochemical studies.

All tear samples were collected without anesthesia, using calibrated 10-μl glass microcapillary tubes (BLAUBRAND intraMark, Wertheim, Germany), from the inferior temporal tear meniscus, taking care to minimize the irritation of the ocular surface. The samples were placed in Eppendorf tubes and stored at −80 °C until analysis. Each sample was labeled with a code identifying the patient and visit number.

**Immunobassay protocol:** Customized arrays for quantitative determination of the three selected biomarkers S100A6, MMP-9, and CST4 were developed (Figure 1). The process of customized microarray preparation for quantitative tear biomarker analysis included several steps, as previously described [39]. The final integration of the antibodies into the microarray system used in the study was performed as follows: A set of customized microarrays was generated. Briefly, antibodies and markers were diluted in the printing solution. A format of 24 arrays, consisting of eight replicas of each antibody surrounded by three replicas of the marker, was spotted onto functionalized glass slides (IMG Pharma, Bizkaia, Spain) using a Nano-Plotter NP 2.1 (GeSiM, Grosserkmannsdorf, Germany). The slides were printed at room temperature, and the microarrays were stored at −20 °C until required. The reaction volume was 70 μl/well for all the steps in the immunoassay. Tear samples were diluted (1/30) in 10 mM phosphate-buffered saline (PBS, Sigma Aldrich, St. Louis, MO) for microarray analysis. Subsequently, the samples from healthy subjects and KC patients were incubated for 1 h with rabbit detection antibodies. Finally, after washing the slides with 1X Tris -buffered saline supplemented with 0.05% of Tween-20 (TBS-T), the secondary Alexa Fluor 647-labeled anti-rabbit antibodies were added and incubated for 1 h. The fluorescence of the spots was measured using an Agilent High-Resolution Microarray Scanner (Agilent Technologies, Santa Clara, CA) at 633 nm, and protein concentration was determined based on the standard curve intensity values.

**Statistics:** A mixed-model design (split-plot analysis of variance [ANOVA]) was used in this study, in which two factors were studied simultaneously: One factor (a fixed-effect factor) was the between-subjects variable, and the other (a random-effect factor) was the within-subjects variable (repeated measurements). Therefore, significant differences between the groups were determined using the Games-Howell post hoc test nonparametric approach. Means between healthy subjects and patients were compared via the Mann–Whitney U test. Finally, Spearman correlation analysis was performed to assess the correlations between clinical parameters and protein levels. The level of statistical significance was set at
RESULTS

Twenty-two eyes of 18 patients were followed up successfully for 12 months. Six patients were women (33.3%) and 12 were men (66.6%). The mean age was 28.2 ± 10.7 years. Seven eyes (31.8%) presented grade 1 KC (incipient), 8 eyes (36.3%) presented grade 2 (moderate), and 7 eyes (31.8%) presented grade 3 (advanced).

Fourteen patients underwent operation on one eye. Four patients underwent operation on both eyes and were included for analysis; since each eye had a different KC grade, they were analyzed independently. In the latter group, two eyes were grade 1 (incipient), two eyes were grade 2 (moderate), and four eyes were grade 3 (advanced).

A control group was included to know the control values of the biomarkers in healthy subjects. The control group included 10 eyes of 10 subjects. Five were women (50%) and five were men (50%). The mean age was 25.7 ± 6.5 years. The variables related to the ocular surface condition were measured and served as inclusion criteria to include a subject in the control group. The means of the control group values were as follows: 299.5 ± 11.24 mOsm/l (OSM), OSDI 8.65 ± 12.47, TBUT 13.5 ± 2.83 s, and Schirmer test 18.3 ± 6.66 mm. They did not show corneal or conjunctival staining, and the variables related to KC were not evaluated. Twelve months after the treatment, only a slight increase in Kmax and a slight decrease in SimK were observed, although none of the variables showed statistically significant differences in comparison with the baseline. None of the studied parameters of lacrimal function exhibited statistically significant changes after the 12-month follow up (Table 1).

Twelve months after the surgery, the concentration of MMP-9 in the tears of patients with KC decreased significantly, from 104.5 ± 78.98 ng/ml to 48.7 ± 24.20 ng/ml. The concentration of S100A6 was also reduced significantly, from 350.26 ± 478.08 ng/ml to 55.79 ± 103.62 ng/ml. However, there was no significant change in the CST4 levels (2202.75 ± 2863.70 ng/ml versus 2139.6 ± 2719.89 ng/ml; Table 2, Figure 2). A similar trend was observed when the samples were grouped according to the severity of KC (Table 3). However, they did not show statistically significant differences when comparing the basal levels of the biomarkers analyzed according to the degree of KC, indicating that there was no relationship between the severity and concentration of the biomarkers present in the tears.

When the basal concentrations of the healthy subjects and KC patients were compared, the levels of MMP-9 and S100A6 in tears showed statistically significant differences (9.8 ± 5.11 and 104.55 ± 78.98 ng/ml, p<0.01, and 11.35 ± 3.18 and 350.26 ± 478.08 ng/ml, respectively, p<0.01). This was not the case for CST4, which did not show statistically significant differences between the two groups (2261.94 ± 510.65 and 2202.75 ± 2863.70 ng/ml, respectively, p = 0.07).
At 12 months after surgery, the levels of MMP-9 and S100A6 tended to show decreased concentrations approaching control values.

At 6 and 12 months after the procedure, a statistically significant positive correlation was observed between S100A6 levels and the damage to the ocular surface, reflected by the FLUO and LG variables. As S100A6 is directly related to cellular apoptosis, a decrease in its tear concentration reduces the corneal staining, indicating diminished tissue damage. Furthermore, a positive correlation was observed between the OSDI questionnaire results and the MMP-9 concentration.

### Table 1. Summary of clinical outcomes of KC patients.

| Variables | Basal          | 12 month        | P value |
|-----------|----------------|-----------------|---------|
| Kmax      | 52.07 ± 6.12   | 52.54 ± 11.02   | 0.203   |
| SimK      | 45.85 ± 3.34   | 45.87 ± 3.04    | 0.078   |
| OSM       | 302.46 ± 9.40  | 300.9 ± 13.5    | 0.676   |
| OSDI      | 28.29 ± 22.77  | 17.07 ± 21.35   | 0.513   |
| BUT       | 12.50 ± 4.16   | 14.45 ± 3.85    | 0.139   |
| SCH       | 16.58 ± 8.51   | 17.05 ± 7.42    | 0.863   |
| CLR       | 4.46 ± 1.72    | 3.63 ± 1.21     | 0.13    |
| FLUO      | 0.33 ± 0.56    | 0.52 ± 0.81     | 0.166   |
| VL        | 1.22 ± 1.45    | 1.5 ± 1.6       | 0.131   |

K max: maximum keratometry (diopters); SimK: simulated keratometry (diopters); OSM: Osmolarity (mOsm/L); OSDI: Ocular Surface Disease Index; TBT: Tear Break Up Time (seconds); SCH: Schirmer’s test (mm); CRL: Tear clearance; FLUO: Fluorescein staining (Oxford staining score); LG: Lissamine green (van Bijsterveld schema); Values are expressed as mean ± SD *p<0.05.

### Table 2. Biomarkers concentration in tears along de study.

| Variables    | Basal          | 3 month       | 6 month       | 12 month      |
|--------------|----------------|---------------|---------------|---------------|
| MMP9 (ng/ml) | 104.55 ± 78.98 | 65.25 ± 24.20* | 56.57 ± 15.76* | 48.76 ± 24.20* |
| S100A6 (ng/ml)| 350.26 ± 478.08| 112.39 ± 103.62* | 64.07 ± 45.52* | 55.79 ± 103.62* |
| CST4 (ng/ml) | 2202.75 ± 2863.70| 2714.94 ± 2719.89 | 1792.47 ± 2719.89 | 2139.68 ± 2719.89 |

Values are expressed as mean ± SD *p<0.05 show statistically significant differences.
Table 3. Biomarker’s concentrations in tears attending severity of keratoconus.

| KC grade     | Incipient |               |       | Moderate |               |       | Advanced |               |       |
|--------------|-----------|---------------|-------|----------|---------------|-------|----------|---------------|-------|
| Variables    | Basal     | 12 month      | P     | Basal    | 12 month      | P     | Basal    | 12 month      | P     |
| MMP-9 (ng/ml)| 115.03 ± 78.98 | 46.08 ± 8.52 | 0.018* | 91.59 ± 51.93 | 46.47 ± 5.39 | 0.012* | 108.87 ± 100.72 | 54.04 ± 18.67 | 0.028* |
| S100A6 (ng/ml)| 296.36 ± 478.08 | 64.07 ± 45.52 | 0.028* | 395.39 ± 584.73 | 61.04 ± 65.84 | 0.036* | 352.63 ± 501.97 | 44.06 ± 29.74 | 0.028* |
| CST4 (ng/ml) | 1407.37 ± 2469.11 | 1147.18 ± 2489.34 | 0.612 | 2749.12 ± 3203.54 | 2866.15 ± 3259.62 | 0.484 | 2373.72 ± 3076.16 | 2302.09 ± 3144.63 | 0.463 |

Values are expressed as mean ± SD *p<0.05 show statistically significant differences.
values. Otherwise, a statistically significant negative correlation was found between S100A6 levels and CLR values after 12 months. Finally, CST4 levels showed a negative correlation with CLR at 3 months and FLUO staining at 3 and 6 months (Table 4).

DISCUSSION

KC is a multifactorial disease involving complex interactions between genetic and environmental factors. Traditionally, it has been defined as a noninflammatory disease of the cornea, but increasing studies show overexpression of several cytokines in KC [9,11].

In our study, during the first year after the A-CXL treatment, the behaviors of three biomarkers (S100A6, CST4, and MMP-9) in the tears of patients with KC were studied. The high preoperative concentration of MMP-9 observed in our study is consistent with the positive regulation of MMP-9 gene expression reported previously in KC [9,11,19]. This finding agrees with the results obtained by Kolozsvári et al. [15], who showed reduced levels of cytokines, chemokines, enzymes, and growth factors in the tears of CXL-treated KC patients in response to the corneal tissue redistribution. Our results clearly show a downward trend in the concentration of MMP-9 protein in tears after the treatment. The diminished levels of MMP-9 and increased corneal stiffness induced by A-CXL (making the extracellular matrix more resistant to degradation by MMP-9) reduced the tendency of the cornea to deform. Although it could be suggested that the regularization of MMP-9 expression may be linked to postoperative changes in the ocular surface, this does not seem to be the case. None of the lacrimal function parameters was significantly altered after the A-CXL treatment in our study.

Shetty et al. [40] reported that the topical application of 0.05% cyclosporin A (CsA) inhibits the appearance of MMP-9 in tears; their paper described a combined in vivo and in vitro study. The researchers demonstrated in vitro that CsA inhibits the expression of MMP-9 and cytokines in cultured epithelial cells from patients with KC. They also found that the disease progression was halted for 6 months in a group of KC patients treated with a topical preparation of CsA (Restasis, Allergan, Inc., Irvine, CA). The anti-inflammatory effect of the application of CsA or A-CXL treatment (decrease in the concentration of MMP-9) seems to play a key role in the stabilization of KC. This may open a way for new methods of KC management. Even if A-CXL cannot be performed, immunomodulatory drugs can be used to stabilize the KC, at least temporarily.

Our study also showed a reduction in S100A6 levels in tears from the patients after A-CXL. The S100A proteins are involved in inflammatory processes of the ocular surface [41] and neovascularization of the cornea [42]. Moreover, their levels in tears increase under various pathological conditions, such as pterygium [43], ocular surface tumors [44], and dry eye [8]. Apart from their role in inflammation, these proteins are involved in apoptosis induced by reactive oxygen species (ROS), which are abundant in oxidative stress processes. The oxidative damage by cytotoxic products ROS and reactive nitrogen species (RNS) generated by lipid peroxidation and the nitric oxide pathway has been reported in KC [45]. We postulate that the increased concentration of S100A6 in KC tears may be related to apoptosis and oxidation stress in the cornea. The reduction in the level of this protein after A-CXL may be associated with a positive response to this treatment.

In contrast with MMP-9 and S100A6, the CST4 concentration did not change significantly during the study in any of the studied groups. This may indicate that the levels of CST4 were not modified by A-CXL treatment. In previous studies conducted by our group [10], we saw that the tear concentration of this protein was lower in KC patients than it was in healthy subjects; however, the difference was low, and it was not statistically significant. In addition, the analysis was performed with a semiquantitative technique, in contrast with this study. Due to the high variability in the concentration of this protein in tears, we cannot say that there is a lower concentration in patients with KC compared with control subjects. This hypothesis should be supported in future investigations with a greater number of KC patients.

The positive correlation between the OSDI results and MMP-9 levels deserves some attention, although it was not significant throughout the study. The correlation observed between the preoperative levels of MMP-9 and the OSDI results at different times after the operation indicates that the baseline MMP-9 may be a predictive factor for the postoperative symptoms. That is, the greater the preoperative concentration of MMP-9, the more subjective symptoms will be reported by the patients in the postoperative period. There was a positive correlation between the S100A6 levels and ocular surface staining and a negative correlation between S100A6 levels and Schirmer test values. These results showed a relationship between corneal damage, tear volume, and biomarker concentration. As the CST4 protein is produced in the lacrimal gland, its concentration depends on tear clearance; this explains the negative correlation of CST4 levels with CLR and FLUO results (after 3 months). Finally, our data showed no important differences between the correlations of clinical and biochemical variables in the different KC evolutive stage groups.
| Variables | OSM 0 | OSM 3 | OSM 6 | OSM 12 | CSTH 0 | CSTH 3 | CSTH 6 | CSTH 12 |
|-----------|-------|-------|-------|--------|--------|--------|--------|---------|
| OSM 0     | 0.39  | -0.21 | 0.16  | 0.12   | -0.45  | -0.24  | -0.45  | -0.24   |
| OSM 3     | 0.26  | 0.20  | 0.40  | -0.44  | -0.11  | -0.16  | -0.16  | -0.35   |
| OSM 6     | -0.23 | 0.03  | -0.23 | -0.29  | 0.16   | -0.28  | -0.32  | -0.29   |
| OSM 12    | 0.46  | 0.30  | 0.39  | 0.43   | 0.19   | 0.31   | 0.56   | 0.48    |
| OSGD 0    | 0.23  | 0.34  | 0.93  | 0.50   | 0.56   | 0.45   | 0.31   | 0.61    |
| OSGD 3    | 0.24  | 0.28  | 0.28  | 0.31   | 0.58   | 0.32   | 0.51   | 0.40    |
| OSGD 6    | 0.17  | 0.62* | 0.62* | 0.62*  | 0.88*  | 0.62*  | 0.88*  | 0.62*   |
| OSGD 12   | 0.06  | 0.42  | 0.42  | 0.42   | 0.47   | 0.87*  | 0.87*  | 0.47    |
| OSME 0    | 0.38  | 0.54  | 0.54  | 0.54   | 0.39   | 0.79*  | 0.79*  | 0.39    |
| OSME 3    | 0.06  | 0.20  | 0.20  | 0.20   | 0.28   | 0.71*  | 0.71*  | 0.28    |
| OSME 6    | 0.28  | -0.47 | -0.47 | -0.47  | 0.16   | 0.23   | 0.23   | 0.16    |
| OSME 12   | 0.16  | 0.28  | 0.28  | 0.28   | 0.31   | 0.58   | 0.58   | 0.31    |
| TBUT 0    | 0.12  | -0.21 | -0.21 | -0.21  | 0.16   | 0.23   | 0.23   | 0.16    |
| TBUT 3    | -0.028| -0.167| -0.167| -0.167 | -0.38  | -0.036| -0.036| -0.38   |
| TBUT 6    | -0.028| -0.167| -0.167| -0.167 | -0.38  | -0.036| -0.036| -0.38   |
| TBUT 12   | -0.028| -0.167| -0.167| -0.167 | -0.38  | -0.036| -0.036| -0.38   |
| SCH 0     | -0.18 | -0.356| -0.356| -0.356| -0.036| -0.036| -0.036| -0.036  |
| SCH 3     | -0.362| -0.408| -0.408| -0.408| 0.16   | 0.346| 0.346 | 0.16    |
| SCH 6     | -0.362| -0.408| -0.408| -0.408| 0.16   | 0.346| 0.346 | 0.16    |
| SCH 12    | -0.362| -0.408| -0.408| -0.408| 0.16   | 0.346| 0.346 | 0.16    |
| CLR 0     | 0.28  | 0.25  | 0.25  | 0.25   | 0.15   | 0.15   | 0.15   | 0.15    |
| CLR 3     | 0.032 | -0.036| -0.036| -0.036| 0.013  | 0.013  | 0.013  | 0.013   |
| CLR 6     | -0.080| -0.207| -0.207| -0.207| 0.013  | 0.013  | 0.013  | 0.013   |
| CLR 12    | -0.080| -0.207| -0.207| -0.207| 0.013  | 0.013  | 0.013  | 0.013   |
| FLUO 0    | 0.131 | 0.147 | 0.147 | 0.147 | 0.213  | 0.213  | 0.213  | 0.213   |
| FLUO 3    | -0.131| -0.147| -0.147| -0.147| 0.213  | 0.213  | 0.213  | 0.213   |
| FLUO 6    | 0.096 | 0.096 | 0.096 | 0.096 | 0.213  | 0.213  | 0.213  | 0.213   |
| FLUO 12   | 0.096 | 0.096 | 0.096 | 0.096 | 0.213  | 0.213  | 0.213  | 0.213   |
| LG 0      | 0.239 | 0.239 | 0.239 | 0.239 | 0.239  | 0.239  | 0.239  | 0.239   |
| LG 3      | 0.356 | 0.356 | 0.356 | 0.356 | 0.356  | 0.356  | 0.356  | 0.356   |
| LG 6      | 0.356 | 0.356 | 0.356 | 0.356 | 0.356  | 0.356  | 0.356  | 0.356   |
| LG 12     | 0.356 | 0.356 | 0.356 | 0.356 | 0.356  | 0.356  | 0.356  | 0.356   |

*Rho significant differences: p<0.05; 0=pre-surgery; 3,6 and 12= months after surgery.
In conclusion, our study shows that A-CXL could produce a certain anti-inflammatory effect favoring corneal homeostasis. This anti-inflammatory effect may be an additional benefit, apart from the increase in corneal rigidity, produced by A-CXL. Thus, the crosslinking of corneal collagen may not be the only factor responsible for the stabilization of KC after the A-CXL, as it is currently assumed.

REFERENCES

1. Dart DA, Willcox MD. Complexity of the tear film: importance in homeostasis and dysfunction during disease. Exp Eye Res 2013; 117:1-3. [PMID: 24280033].

2. De Souza GA, Godoy LM, Mann M. Identification of 491 proteins in the tear fluid proteome reveals a large number of proteases and protease inhibitors. Genome Biol 2006; 7:R72.[PMID: 16901338].

3. Zhou L, Zhao SZ, Koh SK, Chen L, Vaz C, Tanavde V, Li XR, Beuerman RW. In-depth analysis of the human tear proteome. J Proteomics 2012; 75:3877-85. [PMID: 22634083].

4. Stern ME, Beuerman RW, Fox RI, Gao J, Mircheff AK, Pfugfelder SC. The pathology of dry eye: the interaction between the ocular surface and lacrimal glands. Cornea 1998; 17:584-9. [PMID: 9820935].

5. Perumal N, Funke S, Pfeiffer N, Grus FH. Proteomics analysis of human tears from aqueous-deficient and evaporative dry eye patients. Sci Rep 2016; 6:29629. [PMID: 27436115].

6. Li B, Sheng M, Li J, Yan G, Lin A, Li M, Wang W, Chen Y. Tear proteomic analysis of Sjögren syndrome patients with dry eye syndrome by two-dimensional-nano-liquid chromatography coupled with tandem mass spectrometry. Sci Rep 2014; 4:5772-[PMID: 25159733].

7. Von Thun Und Hohenstein-Blaul N, Funke S, Grus FH. Tears as a source of biomarkers for ocular and systemic diseases. Exp Eye Res 2013; 117:126-37. [PMID: 23880526].

8. Soria J, Durán JA, Etxebarría J, Merayo J, González N, Reigada R, García I, Acera A, Suárez T. Tear proteome and protein network analyses reveal a novel pentamarker panel for tear film characterization in dry eye and meibomian gland dysfunction. J Proteomics 2013; 78:94-112. [PMID: 23201116].

9. Lema I, Durán JA. Inflammatory molecules in the tears of patients with keratoconus. Ophthalmology 2005; 112:654-9. [PMID: 15808258].

10. Acera A, Vecino E, Rodriguez-Agirretxe I, Aloria K, Arizmendi JM, Morales C, Durán JA. Changes in tear protein profile in keratoconus disease. Eye (Lond) 2011; 25:1225-33. [PMID: 21701529].

11. Galvis V, Sherwin T, Tello A, Merayo J, Barrera R, Acera A. Keratoconus: an inflammatory disorder? Eye (Lond) 2015; 29:843-59. [PMID: 25931166].

12. Garcia B, García-Suárez O, Merayo-Llones J, Ferrara G, Alcalde I, González J, Lisa C, Alfonso JF, Vazquez F, Quirós LM. Heparanase Overexpresses in Keratoconic Cornea and Tears Depending on the Pathologic Grade. Dis Markers 2017; 2017:3502386.-[PMID: 29379222].

13. Nishitaka K, Pahuja N, Shetty R, Nuijts RM, Ghosh A. Tear biomarkers for keratoconus. Eye Vis (Lond) 2016; 3:19-[PMID: 27493978].

14. Karamichos D, Zieske JD, Sejersen H, Sarker-Nag A, Asara JM, Hjortdal J. Tear metabolic changes in keratoconus. Exp Eye Res 2015; 132:1-8. [PMID: 25579606].

15. Kolozsvári BL, Berta A, Petrovski G, Miháltz K, Gogolák P, Rajnavölgyi E, Hassan Z, Széles P, Fodor M. Alterations of tear mediators in patients with keratoconus after corneal crosslinking associate with corneal changes. PLoS One 2013; 8:e76333.-[PMID: 24124547].

16. Pavlenko TA, Chesnokova NB, Davydova HG, Okhotsimskaia TD, Beznos OV, Grigor’ev AV. Level of tear endothelin-1 and plasminogen in patients with glaucoma and proliferative diabetic retinopathy. Vestn Oftalmol 2013; 129:20-3. [PMID: 24137977].

17. Davidson AE, Hayes S, Hardcastle AJ, Tuft SJ. The pathogenesis of keratoconus. Eye (Lond) 2014; 28:189-95. [PMID: 24357835].

18. Shetty R, Sathyarayaranyamoorthy A, Ramachandra RA, Arora V, Ghosh A, Srivatsa PR, Pahuja N, Nuijts RM, Sinha-Roy A, Mohan RR, Ghosh A. Attenuation of lysyl oxidase and collagen gene expression in keratoconus patient corneal epithelium corresponds to disease severity. Mol Vis 2015; 21-12-25. .

19. Lema I, Sobrino T, Durán JA, Brea D, Diez-Feijoo E. Subclinical keratoconus and inflammatory molecules from tears. Br J Ophthalmol 2009; 93:820-4. [PMID: 19304583].

20. Lema I, Durán JA, Ruiz C, Diez-Feijoo E, Acera A, Merayo J. Inflammatory response to contact lenses in patients with keratoconus compared with myopic subjects. Cornea 2008; 27:758-63. [PMID: 18650659].

21. Balasubramanian SA, Mohan S, Pye DC, Willcox MD. Proteases, proteolysis and inflammatory molecules in the tears of people with keratoconus. Acta Ophthalmol 2012; 90:e303-9. [PMID: 22413749].

22. Zhou L, Beuerman RW, Chan CM, Zhao SZ, Li XR, Yang H, Tong L, Liu S, Stern ME, Tan D. Identification of tear fluid biomarkers in dry eye syndrome using iTRAQ quantitative proteomics. J Proteome Res 2009; 8:4889-905. [PMID: 19705875].

23. Tonini GP, Casalaro A, Cara A, Di Martino D. Inducible Expression of Calcyclin, a Gene with Strong Homology to S-100 Protein, during Neuroblastoma Cell Differentiation and Its Prevalent Expression in Schwann-like Cell Lines. Cancer Res 1991; 51:1733-7. [PMID: 1998863].

24. Filipek A, Gerke V, Weber K, Kuźnicki J. Characterization of the cell-cycle-regulated protein calcyclin from Ehrlich ascites tumor cells. Identification of two binding proteins obtained by Ca2(+) dependent affinity chromatography. Eur J Biochem 1991; 14:795-800. [PMID: 1999197].
25. Zeng FY. Gerke V, Gabius HJ. Identification of annexin II, annexin VI and glyceraldehyde-3-phosphate dehydrogenase as calcyclin-binding proteins in bovine heart. Int J Biochem 1993; 25:1019-27. [PMID: 8365543].

26. Uusitalo H, Aapola U, Jylhä A, Näättinen J, Beuerman R. Improved tear fluid proteome and dynamics after switch from preserved latanoprost to preservative free tafluprost. A 1-year follow-up study. Invest Ophthalmol Vis Sci 2015; 56.

27. Bond JS, Butler PE. Intracellular proteases. Annu Rev Biochem 1987; 56:333-64. [PMID: 3304137].

28. Barka T, Asbell PA, Van der Noen H, Prasad A. Cystatins in human tear fluids. Curr Eye Res 1991; 10:25-34. [PMID: 2029847].

29. Takahashi M, Honda Y, Ogawa K, Barka T. Immunofluorescence localization of cystatins in human lacrimal gland and in the exorbital lacrimal gland of the rat. Acta ophthalmologica 1992; 70:625-31. [PMID: 1471486].

30. Wollensak G, Spoerl E, Seiler T. Riboflavin/ultraviolet-a-induced collagen crosslinking for the treatment of keratoconus. Am J Ophthalmol 2003; 135:620-7. [PMID: 12791068].

31. Raiskup F, Spoerl E. Corneal crosslinking with riboflavin and ultraviolet A. I. Principles. Ocul Surf 2013; 11:65-74. [PMID: 23583042].

32. Rabinowitz YS. Videokeratographic indices to aid in screening for keratoconus. J Refract Surg 1995; 11:371-9. [PMID: 8528916].

33. Hafezi F, Randleman B. Corneal Collagen Cross-Linking. Thorofare: Slack Incorporated. 2013.

34. Lemp MA, Bron AJ, Baudouin C, Benitez Del Castillo JM, Geffen D, Tauber J, Foulks GN, Pepose JS, Sullivan BD. Tear osmolarity in the diagnosis and management of dry eye disease. Am J Ophthalmol 2011; 151:792-8. [PMID: 21310379].

35. Schiffman RM, Christianson MD, Jacobsen G, Hirsch JD, Reis BL. Reliability and validity of the ocular surface disease index. Arch Ophthalmol 2000; 118:615-21. [PMID: 10815152].

36. Vico E, Benitez del Castillo JM, Gimenez RA, Fernandez C, Garcia Sanchez J. Arch Soc Esp Oftalmol 2004; 79:265-71. Tear function index validation for dry eye diagnosis [PMID: 15221672].

37. Bron AJ, Evans VE, Smith JA. Grading of corneal and conjunctival staining in the context of other dry eye tests. Cornea 2003; 22:640-50. [PMID: 14508260].

38. Doughty MJ. Rose Bengal staining as an assessment of ocular surface damage and recovery in dry eye disease – a review. Cont Lens Anterior Eye 2013; 36:272-80. [PMID: 23928365].

39. Soria J, Acera A, Merayo-Lloves J, Durán JA, González N, Rodriguez S, Bistolas N, Schumacher S, Bier FF, Peter H, Stöcklein W, Suárez T. Tear proteome analysis in ocular surface diseases using label-free LC-MS/MS and multiplexed-microarray biomarker validation. Sci Rep 2017; 7:17478. [PMID: 29234088].

40. Shetty R, Ghosh A, Lim RR, Subramani M, Mihir K, Reshma AR, Ranganath A, Nagaraj S, Nuijts RM, Beuerman R, Shetty R, Das D, Chaurasia SS, Sinha-Roy A, Ghosh A. Elevated expression of matrix metalloproteinase 9 and inflammatory cytokines in keratoconus patients is inhibited by cyclosporine A. Invest Ophthalmol Vis Sci 2015; 56:738-50. [PMID: 25648341].

41. Quintyn JC, Pereira F, Hellot MF, Brasseur G, Coquerel A. Concentration of neuron-specific enolase and S100 protein in the subretinal fluid of rhegmatogenous retinal detachment. Graefes Arch Clin Exp Ophthalmol 2005; 243:1167-74. [PMID: 15906069].

42. Li C, Zhang F, Wang Y. S100A proteins in the pathogenesis of experimental corneal neovascularisation. Mol Vis 2010; 16:2225-35. [PMID: 21139687].

43. Riau AK, Wong TT, Beuerman RW, Tong L. Calcium-binding S100 protein expression in pterygium. Mol Vis 2009; 15:335-42. [PMID: 19223989].

44. Kanoff JM, Colby J, Jakobiec FA. Incipient corneoscleral xanthogranuloma with S-100 positivity in a teenager. Cornea 2010; 29:688-90. [PMID: 20458239].

45. Wojcik KA, Kaminska A, Blasiak J, Szaflik J, Szaflik JP. Oxidative stress in the pathogenesis of keratoconus and Fuchs endothelial corneal dystrophy. Int J Mol Sci 2013; 14:19294-308. [PMID: 24065107].