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Changes in tear biomarker levels in keratoconus after corneal collagen crosslinking

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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.

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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 KXL 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/cm^2 for 8 min. This corresponds to a yield of 7.2 J/cm^2 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.

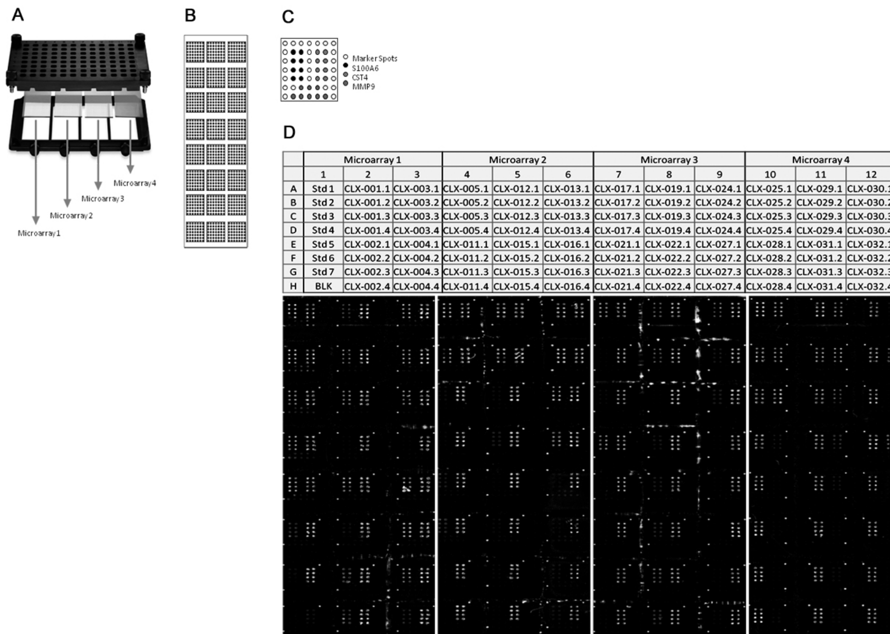
Clinical examinations during each visit: For examining the clinical evolution of KC and the changes in the ocular surface after A-CXL, the following clinical and biochemical parameters were studied: keratometry (Kmax and simulated keratometry [SimK]), lacrimal osmolarity (OSM) measurements [34], the 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.

Immunoassay 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



	Microarray 1			Microarray 2			Microarray 3			Microarray 4		
	1	2	3	4	5	6	7	8	9	10	11	12
A	Std 1	CLX-001.1	CLX-003.1	CLX-005.1	CLX-012.1	CLX-013.1	CLX-017.1	CLX-019.1	CLX-024.1	CLX-025.1	CLX-029.1	CLX-030.1
B	Std 2	CLX-001.2	CLX-003.2	CLX-005.2	CLX-012.2	CLX-013.2	CLX-017.2	CLX-019.2	CLX-024.2	CLX-025.2	CLX-029.2	CLX-030.2
C	Std 3	CLX-001.3	CLX-003.3	CLX-005.3	CLX-012.3	CLX-013.3	CLX-017.3	CLX-019.3	CLX-024.3	CLX-025.3	CLX-029.3	CLX-030.3
D	Std 4	CLX-001.4	CLX-003.4	CLX-005.4	CLX-012.4	CLX-013.4	CLX-017.4	CLX-019.4	CLX-024.4	CLX-025.4	CLX-029.4	CLX-030.4
E	Std 5	CLX-002.1	CLX-004.1	CLX-011.1	CLX-015.1	CLX-016.1	CLX-021.1	CLX-022.1	CLX-027.1	CLX-028.1	CLX-031.1	CLX-032.1
F	Std 6	CLX-002.2	CLX-004.2	CLX-011.2	CLX-015.2	CLX-016.2	CLX-021.2	CLX-022.2	CLX-027.2	CLX-028.2	CLX-031.2	CLX-032.2
G	Std 7	CLX-002.3	CLX-004.3	CLX-011.3	CLX-015.3	CLX-016.3	CLX-021.3	CLX-022.3	CLX-027.3	CLX-028.3	CLX-031.3	CLX-032.3
H	BLK	CLX-002.4	CLX-004.4	CLX-011.4	CLX-015.4	CLX-016.4	CLX-021.4	CLX-022.4	CLX-027.4	CLX-028.4	CLX-031.4	CLX-032.4

Figure 1. Antibody microarrays customized. **A**: Specific device for analyzing four microarrays simultaneously. **B,C**: Spotting pattern for the 24-subarray format. **D**: Representative image of the arrays showing the distribution of the standard calibration curve and samples. Only one microarray slide contains a standard calibration curve (left column) and the fluorescence acquisition for 16 tear samples (Microarray 1). Other slices (Microarrays 2–4) show the fluorescence for 24 tear samples. Fluorescence scans of the microarray multiplex assays were acquired at 633 nm.

$p<0.05$. Statistical analysis was performed using the SPSS 24.0 program (SPSS Inc., Chicago, IL).

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).

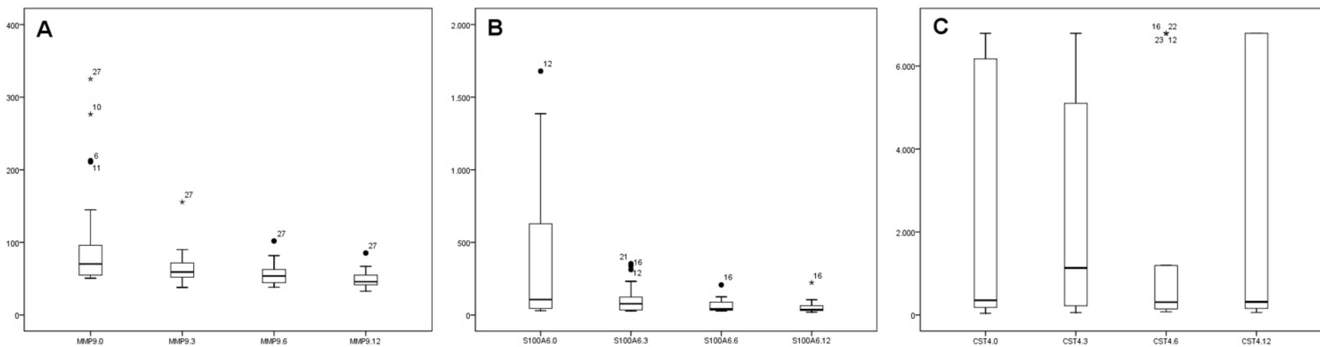


Figure 2. Concentrations in ng/ml of the matrix metalloproteinase (MMP-9), calcyclin (S100A6), and cystatin S (CST4) during the study (presurgery and 3, 6, and 12 months).

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; TBUT: 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 expresed 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* *P*=0.014	56.57 ± 15.76* *P*=0.006	48.76 ± 24.20* *P*=0.001
S100A6 (ng/ml)	350.26 ± 478.08	112.39 ± 103.62* *P*=0.012	64.07 ± 45.52* *P*=0.008	55.79 ± 103.62* *P*=0.007
CST4 (ng/ml)	2202.75 ± 2863.70	2714.94 ± 2719.89 *P*=0.533	1792.47 ± 2719.89 *P*=0.635	2139.68 ± 2719.89 *P*=0.941

Values are expressed as mean ± SD *p<0.05 show statistically significant differences.

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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 lachrymal 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.

Table 4. Correlations between clinical parameters and biomarkers concentrations in KC patients.

Variables	S100A6 0	S100A6 3	S100A6 6	S100A6 12	MMP9 0	MMP9 3	MMP9 6	MMP9 12	CST4 0	CST4 3	CST4 6	CST4 12
OSM 0	0.39	0.08	0.45	0.58*	-0.21	0.16	0.12	-0.45	-0.24	-0.06	-0.08	-0.14
OSM 3	0.26	-0.30	0.20	0.40	-0.44	-0.11	-0.16	-0.71*	-0.19	-0.35	-0.23	-0.42
OSM 6	0.24	-0.03	-0.05	0.03	-0.20	0.07	-0.33	-0.37	0.01	0.02	-0.30	-0.32
OSM 12	0.46	0.09	0.31	0.34	0.03	0.39	0.43	0.19	0.16	-0.29	-0.28	-0.03
OSDI 0	0.24	0.23	0.34	0.2	0.59*	0.00	0.31	0.56	0.43	0.48	-0.15	0.61
OSDI 3	0.17	0.62*	0.28	-0.47	0.87*	0.58	0.62*	0.86*	0.72*	0.50	0.19	0.45
OSDI 6	0.06	0.42	0.20	-0.31	0.71*	0.16	0.23	0.80*	0.62*	0.51	-0.09	0.07
OSDI 12	0.38	0.54	0.42	0.02	0.79*	0.55	0.47	0.80*	0.43	0.40	0.07	0.49
TBUT 0	0.12	0.074	-0.169	-0.16	-0.167	-0.38	-0.167	-0.004	0.226	0.238	0.454	0.19
TBUT 3	-0.028	0.25	-0.07	-0.57*	0.448	0.258	-0.036	0.316	0.143	0.392	0.197	0.251
TBUT 6	-0.190	-0.15	-0.254	-0.238	-0.175	-0.134	-0.392	-0.267	0.082	-0.140	-0.51	-0.064
TBUT 12	0.23	-0.04	0.098	-0.12	-0.145	-0.395	-0.194	-0.107	0.159	0.01	0.184	0.432
SCH 0	-0.158	-0.356	-0.101	-0.208	0.16	0.364	0.211	0.071	-0.196	-0.469	0.054	0.212
SCH 3	-0.301	-0.420	-0.314	-0.212	0.013	-0.230	-0.133	0.191	-0.078	-0.316	-0.105	-0.181
SCH 6	-0.410	-0.471	-0.296	-0.302	0.096	0.182	0.013	0.233	-0.148	-0.352	-0.288	0.057
SCH 12	-0.612*	-0.451	-0.408	-0.672*	0	0.085	-0.306	-0.068	-0.238	-0.316	-0.239	0.017
CLR 0	-0.033	-0.060	0.124	-0.032	0.394	0.226	0.325	0.550*	0.195	-0.070	-0.011	0.392
CLR 3	-0.157	-0.352	-0.080	-0.049	-0.207	0.117	-0.256	-0.226	-0.415	-0.508*	-0.133	0.014
CLR 6	0.105	0.106	0.154	-0.310	0.147	0.193	0.427	0.158	0.337	-0.248	0.416	0.627*
CLR 12	-0.870*	-0.485	-0.641*	-0.593*	0.1	0.003	-0.590*	0.202	-0.6*	-0.224	-0.444	-0.550
FLUO 0	-0.327	-0.246	-0.410	-0.205	0.123	-0.123	0.041	0.246	0.164	0.082	-0.041	0.207
FLUO 3	-0.309	-0.247	-0.062	-0.247	0.06	-0.062	-0.124	0.124	-0.186	-0.531*	-0.372	-0.124
FLUO 6	-0.317	-0.272	0.502*	-0.182	0.091	-0.136	0.045	0.227	0.136	0.046	-0.046	0.229
FLUO 12	-0.462	-0.463	-0.309	0.01	-0.231	-0.386	-0.386	-0.154	-0.077	0	-0.572*	-0.155
LG 0	0.293	-0.107	0.473	0.583*	-0.155	-0.110	-0.239	-0.187	-0.325	-0.203	-0.328	-0.315
LG 3	0.356	-0.092	0.377	0.381	-0.177	0.136	-0.061	-0.344	-0.216	-0.118	-0.166	-0.022
LG 6	0.279	0.21	0.682*	0.456	0.363	0.271	0.465	0.312	0.037	0.002	-0.183	0.151
LG 12	0.148	-0.279	0.224	0.753*	-0.093	0.236	0.279	0.093	-0.152	-0.342	-0.392	-0.205

*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.

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