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Increased inflammatory mediators in the ocular surface tissue in keratoconus

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Purpose: This study aimed to characterize the inflammatory mediators present in the tear film of patients with keratoconus (KC). It also aimed to investigate the gene expression of these mediators in corneal epithelial cells and their immune activity in conjunctival epithelial cells in patients with KC compared to a control group.

Methods: This transversal study included 30 patients with KC and 23 control group participants. Tear samples were collected by washing the ocular surface with 60 µL of sterile buffered saline solution. The levels of interleukin IL-5, IL-13, IL-2, IL-6, IL-10, interferon-gamma, tumor necrosis factor-alpha, and IL-4 were measured using a LEGEND plex HU Th1/Th2 panel kit and analyzed using flow cytometry. Corneal epithelial samples were obtained via manual keratectomy from KC patients scheduled for corneal crosslinking and from individuals scheduled for photorefractive keratectomy (control group). These samples were immediately stored at −70 °C for mRNA extraction and subsequent reverse transcription polymerase chain reaction analysis to measure *IL-5* and *IL-6* gene expression. Conjunctival epithelium samples were collected using impression cytology and analyzed using immunohistochemistry and confocal microscopy to detect IL-5 and IL-6 immunoreactions.

Results: Our study found no statistically significant differences in the tear film cytokine concentrations between the two groups. In addition, the gene expression of *IL-5* and *IL-6* in the corneal epithelium was higher in the KC group than in the control group, with *IL-5* showing a 50% increase and IL-6 showing a 20% increase. Immunohistochemical analysis revealed a greater immunostaining of IL-5 and IL-6 in the conjunctival epithelium of patients with KC compared to the control group.

Conclusions: In this study, despite higher levels of IL-5 and IL-6 in the tear film of patients with KC, there was no statistically significant difference compared to the control group. However, there was heightened immune activity in the corneal and conjunctival epithelial cells of patients with KC based on IL-5 and IL-6 gene expression and their immunodetection, respectively.

Although keratoconus (KC) was originally described as a noninflammatory corneal disorder, growing evidence suggests that inflammatory activity plays a role in its pathogenesis [1]. Several factors have been associated with the etiology of KC, including various candidate genes [2-13], altered structural morphology of the extracellular matrix [14-19], and a proinflammatory biochemical environment [1,20-34]. The prevalence of KC varies worldwide, likely due to variations in ethnicity, geographic location, and the diagnostic criteria used in epidemiological investigations [12,35-46]. KC can significantly impair visual acuity and quality of life, particularly affecting young working-age individuals and potentially causing severe low vision [47-51]. A better understanding of the etiology of KC and the identification of risk factors for disease progression can help in reducing visual impairment and increasing workforce participation.

Several studies have investigated the role of inflammatory activity in KC using distinct methodologies. It is believed that ocular allergy plays a crucial role in the development of KC, as there is a higher prevalence of allergic diseases in patients with KC than in healthy individuals [17,52-58]. Cytokines and other inflammatory tear molecules have been detected in various ocular surface conditions, including dry eye disease, ocular allergy, and ocular graft-versus-host disease [59-62]. In patients with KC, increased levels of tear film molecules, such as interleukin IL-6, epidermal growth factor, matrix metalloproteinase (MMP)-1, 3, 7, 9, and 13, and tissue inhibitor of metalloproteinase-1, have been reported. Conversely, decreased levels of interferon-gamma (IFN-γ), IL-4, IL-5, IL-6, IL-8 (C-X-C motif chemokine ligand 8), IL-12, IL-13, CCL5, and vascular endothelial growth factor have been observed in patients with KC [22-24,28,34,63,64].

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In a previous study, Moura et al. from our group reported a significant correlation between KC severity and IL-6 levels, with more severe cases exhibiting higher IL-6 concentrations in the tear film [1]. Balasubramanian et al. discussed the association between corneal tissue damage in patients with KC and elevated tear levels of MMPs, IL-1, IL-6, and tumor necrosis factor-alpha (TNF-α), as well as an imbalance between proteolytic and lysosomal enzymes and decreased concentrations of protease inhibitors [14-16].

However, it is important to note that these studies do not rule out the involvement of other inflammatory mediators in the development of KC, and nor do they explore potential interactions or signaling between distinct molecules. Furthermore, these studies do not investigate the cellular sources, targets, or receptor activity of the measured mediators in tears.

This study aimed to characterize inflammatory mediators present in the tear film of patients with KC. Additionally, it aimed to investigate *IL-5* and *IL-6* gene expression in corneal epithelial cells and examine their immune activity in conjunctival epithelial cells in patients with KC compared to a control group.

METHODS

This transversal study was conducted at the Department of Ophthalmology and Visual Sciences at the Federal University of São Paulo (UNIFESP) in collaboration with the Department of Immunology at the University of São Paulo. The study was approved by the UNIFESP Ethics Committee (0884/2018) and adhered to the tenets of the Declaration of Helsinki. Written informed consent was obtained from all participants. The study included 31 eyes of 31 patients with KC who underwent corneal crosslinking and 30 eyes of 23 patients who underwent photorefractive keratectomy (control group). The inclusion criteria for the KC group were regular follow-up at the Cornea Clinic at UNIFESP and being candidates for corneal crosslinking due to disease progression. The control group consisted of individuals who met the normal criteria for photorefractive keratectomy (eligible for refractive surgery with no suspicion of keratoconus). Exclusion criteria for both groups included having hydrops, a previous history of herpetic keratitis, collagen diseases, autoimmune diseases, other systemic diseases such as diabetes mellitus; the topical and systemic use of hormonal or nonhormonal anti-inflammatory drugs; the use of rigid or soft contact lenses within one month before tear collection; and pregnancy or lactation.

The severity of KC was determined based on keratometric measurements and qualitative patterns in the topographic map, the pachymetry map, and the posterior corneal elevation map obtained from corneal tomography using the Pentacam® system. KC was classified into four stages: mild (less than 45 diopters in both meridians), moderate (45–52 diopters in both meridians), advanced (52–62 diopters in both meridians), and severe (more than 62 diopters in both meridians) [65]. KC progression was defined as an increase of at least 0.75 diopters in the apical keratometry (K) value of the corneal topography within 6–12 months.

Tear collection and processing: Tears were collected via ocular surface washing. Sixty microliters of sterile buffered saline solution were gently instilled into the inferior conjunctival fornix using a sterile micropipette without touching the ocular surface or eyelids. This procedure was performed without anesthesia. Patients were then asked to move their eyes in different directions (up, down, right, and left), and the fluid was collected with a micropipette at the inferior conjunctival fornix. The collected fluid (60 µl) was transferred into 1.5-mL Eppendorf tubes (Eppendorf, Fremont, CA) and immediately stored on ice for transport. Subsequently, the samples were stored at −70 °C until further analysis by immunoassay. The tear sample processing and analysis of all patient samples was conducted once all the samples were collected, with all samples being stored at −70 °C.

The levels of IL-5, IL-13, IL-2, IL-6, IL-10, IFN-γ, TNF-α, and IL-4 were measured using a LEGEND plex HU Th1/Th2 Panel Kit (Biolegend, San Diego, CA), following the manufacturer's instructions. Briefly, 25 µl of the lacrimal sample were incubated with 25 µl of beads in a 96-well plate for 2 h at room temperature under agitation at 800 rpm. After centrifugation, the plate was washed twice and incubated with 25 µl of the detection antibody for 1 h under agitation at room temperature. Without washing, 25 µl of streptavidin–phycoerythrin were added, and the plate was then incubated for 30 min under agitation. The plate was washed, and the samples were resuspended in 200 µl of Wash Buffer and transferred to 5-ml tubes for flow cytometric analysis (FACS Calibur, BD Biosciences, San Jose, CA). A total of 4,000 events were saved per sample, and quantitative data were obtained using LEGENDplex™ Data Analysis software (Biolegend).

Conjunctival epithelium collection and processing: Conjunctival epithelium samples were collected using impression cytology (IC) under topical anesthesia with 0.5% proxymetacaine hydrochloride (Anestalcon® 0.5%, Alcon, São Paulo, Brazil). For IC sampling, an acetate cellulose filter strip paper with a 0.45-micron pore size (Millipore HAWP304, Bedford, MA) was placed onto the superior-temporal bulbar conjunctiva quadrant. The strip was gently pressed for 5 s and then peeled off. The filter strips were immediately fixed

for approximately 1 h in a solution of 4% paraformaldehyde and phosphate-buffered saline (PBS), washed with PBS, and stored in a solution of 0.1% sodium azide in PBS until analysis. The samples were analyzed by immunohistochemistry, and confocal microscopy was used to observe IL-5 and IL-6 immunoreactions.

Primary antibodies used were streptavidin–phycoerythrin anti-mouse/antihuman IL-5 antibody (1:20 dilution in blocking buffer solution; catalog no. 504,303; Biolegend) and fluorescein isothiocyanate antihuman IL-6 antibody (1:10 dilution in blocking buffer solution; catalog no. 501,105; Biolegend). The filter papers were incubated with the antibodies overnight at 4 °C. Next, the filter papers were washed three times for 10 min each with tris-buffered saline (TBS)–Tween-20 and incubated for 15 min in TBS–Hoechst solution to demarcate the cell nucleus. They were subsequently washed with TBS–Tween-20 to remove excess Hoechst solution. The samples were then mounted on slides using 5 µl–10 µl of ProLong™ Diamond Antifade Mountant (catalog no. P36965, ThermoFisher, Waltham, MA, USA) and covered with a square coverslip. The samples were stored at 2 °C–6 °C until analysis. Confocal microscopy was used to evaluate IL-5 and IL-6 immunofluorescence, allowing for a comparative analysis between the two groups. Confocal images were captured using a Zeiss LSM-780 NLO microscope (CEFAP, ICB–USP, FAPESP 2009/53994–8) equipped with an EC Plan-Neofluar 20×/0.5 1.4 NA lens and 1× digital zoom. For each slide, three images were acquired in a single optical plane with a format of 1024 × 1024 pixels and a pixel draw time of 3.15 µs using a four-line averaging method. The pinhole was set to 1 Airy unit for each wavelength to optimize image quality. To avoid interference between the fluorophores, excitation and detection of each channel were performed sequentially. Images were analyzed using a custom Fiji protocol [66]. The average fluorescence intensity of the entire image for IL-5 and IL-6 channels was divided by the number of nuclei in the image under analysis to determine the average fluorescence intensity per cell for each channel. The obtained results were analyzed using a nonparametric *t* test and plotted on a bar graph for better visualization.

Corneal epithelia collection and processing: Corneal epithelia were collected by manual keratectomy from patients with KC and control group participants under topical anesthesia with 0.5% proxymetacaine hydrochloride (Anestalcon® 0.5%, Alcon, São Paulo, Brazil). The samples were immediately stored at −70 °C until mRNA extraction and reverse transcription polymerase chain reaction (RT–PCR) analysis to measure *IL-5* and *IL-6* gene expression.

Briefly, total RNA was isolated from corneal epithelial samples using Trizol® (Invitrogen, Carlsbad, CA). cDNA was synthesized from 2 µg of total RNA after treatment with DNase (RQ1 DNase free RNase; Promega, Madison, WI) to avoid DNA contamination. Oligo(dT)$_{12–18}$ (0.2 µg; GE Healthcare, Aylesbury, Buckinghamshire, UK), BSA (20 ng), deoxynucleotides triphosphates (0.1 µmol; Promega), and M-MLV reverse transcriptase Moloney murine leukemia virus (400 U; Promega) were added to the reaction. The mixture was incubated to allow the transcription reaction. Real-time PCR was performed using Power Sybr® Green PCR Mastermix (Life Technologies LTD, Woolston Warrington, UK) for amplification and quantification on a QuantStudio 3 thermocycler (Applied Biosystem–ThermoFisher, Waltham, MA). The thermocycling conditions for the Power Sybr® Green PCR Mastermix Mix are standardized and universal for any primer. The gene expression of *IL-6* (QIAGEN QuantiTect®; catalog no. QT00083720, QIAGEN, Germantown, MD) and *IL-5* (QIAGEN QuantiTect®; catalog no. QT00001435, QIAGEN) was determined relative to the reference gene *PPIA* (primer sense: CAA ATG CTG GAC CCA ACA CA and antisense: TTG CCA AAC ACC ACA TGC TT) using a comparative cycle threshold (CT) method. For each experiment, the CT values of the target genes were normalized to their respective reference genes, and the relative expression of the target genes was calculated using the $2^{-\Delta\Delta CT}$ method. In this way, relative mRNA levels were expressed as a difference of "n" times in relation to a control sample. This method allowed for the comparison of *IL-6* and *IL-5* mRNA expression in the corneal epithelium between patients with KC and the control group.

Data were collected and presented in contingency tables as mean (standard deviation) or frequency (proportion). Individuals or eyes were compared using the *t* test or chi-square test for means and proportions. The generalized estimating equation was used to account for the correlation between eyes when both eyes from the same individual were included in the analysis. Some analyses were adjusted for age and allergy status as confounders. Individuals with KC were categorized as having moderate, advanced, or severe KC to analyze the association between levels of IL-5 and IL-6 in tears and conjunctival epithelium. All statistical analyses were performed using Stata v.17 (College Station, TX), and p values less than 0.05 were considered statistically significant.

RESULTS

Demographics: This study included 54 participants, with 31 (57.41%) in the KC group and 23 (42.59%) in the control group. The two groups were comparable in terms of gender

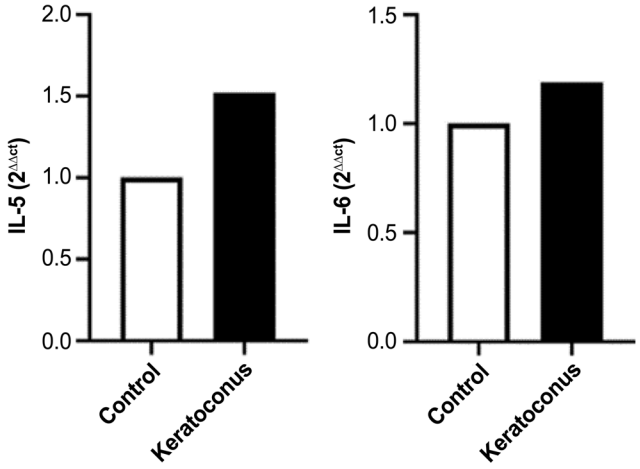

Figure 1. Bar graph demonstrating *IL-5* and *IL-6* gene expression in corneal epithelial cells from the keratoconus and control groups.

(p=0.846), but the KC group were younger (p=0.001) and had a higher prevalence of ocular allergy (p=0.013).

Tear film cytokines: The concentrations of seven cytokines and the INF-γ:IL-10 ratio, which represents the Th1 cell:Th2 cell ratio, were measured in the tear film of the KC and control groups, and no statistically significant differences were found between the two groups (Table 1). This analysis was repeated after excluding outliers and revealed no significant differences between the two groups.

Gene expression of IL-6 and IL-5 in the corneal epithelium: Quantitative RT–PCR analysis revealed higher *IL-5* and *IL-6* gene expression in corneal epithelial cells from patients with KC compared to the control group. The CT values of the target genes (*IL-5* and *IL-6*) were normalized using their respective reference genes, and the relative expression was determined using the $2^{-\Delta\Delta CT}$ method. *IL-5* gene expression was increased by 52% in patients with KC compared to the control group, whereas *IL-6* expression increased by 19% in patients with KC compared to the control group (Table 2, Figure 1).

Immune activity of IL-6 and IL-5 in the conjunctival epithelium: The mean cytoplasmic fluorescence intensity in conjunctival epithelial cells was found to be higher in the KC group compared to the control group for IL-6 (72.51±25.87

Table 1. Cytokine concentrations and Th1/Th2 ratios in tear film of keratoconus and control groups.

Variable	IL-5	IL-13	IL-6	IL-10	INF-γ	TNF-α	IL-4	Th1/Th2
Keratoconus (mean ± SD); n=31	1.24±1.29	2.72±5.22	7.95±15.06	2.91±3.21	205.94±119.22	13.77±14.74	3.52±3.41	112.95±70.89
Control (mean ± SD); n=30	1.36±4.36	2.05±4.19	4.33±5.96	1.76±3.43	129.91±106.96	7.23±15.50	1.82±2.20	104.65±78.36
P value	0.218	0.415	0.721	0.183	0.269	0.865	0.419	0.595

Table 2. *Interleukin IL-5* and *IL-6* gene expression in corneal epithelial cells from the keratoconus and control groups.

Sample	*PPIA*	*IL-5*	Δ*ct (C-B)*	ΔΔ*ct(Δ-14.49)*	*2*− ΔΔct
Control	17.447	31.941	14.49	0.00	**1.00**
Keratoconus	17.507	31.391	13.88	−0.61	**1.52**
Sample	***PPIA***	***IL-6***	**Δ*ct (C-B)***	**ΔΔ*ct(Δ-16.50)***	***2*− ΔΔct**
Control	17.586	34.091	16.50	0.00	**1.00**
Keratoconus	17.648	33.892	16.24	−0.26	**1.19**

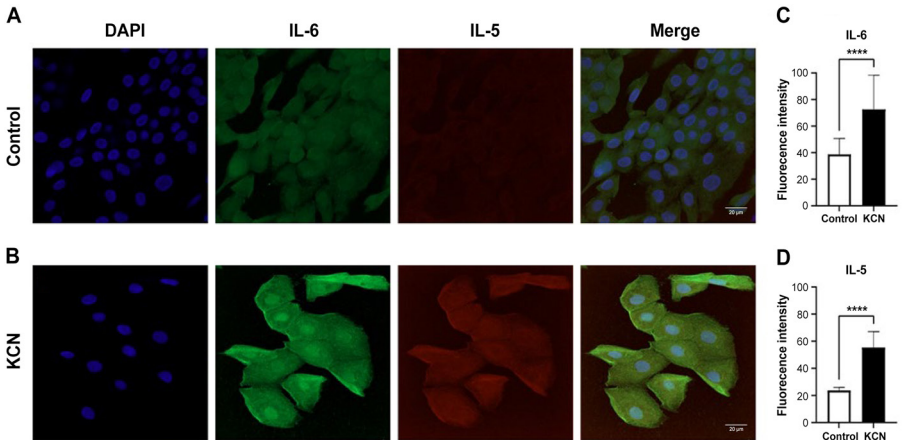

Figure 2. Immunofluorescence of conjunctival epithelial cells showing higher levels of IL-5 and IL-6 in patients with keratoconus compared to the control group. A. Immunofluorescence of control conjunctival epithelial cells labeled for 4′,6-diamidino-2-phenylindole (DAPI, blue), IL-6 (green), IL-5 (red), and merge. B. Immunofluorescence of keratoconus conjunctival epithelial cells labeled for DAPI (blue), IL-6 (green), IL-5 (red), and merge (bars=20 um). C. Intracellular fluorescence intensity of IL-6. D. Intracellular fluorescence intensity of IL 5. ****p<0.0001.

and 38.72±11.89, respectively; p<0.001) and IL-5 (55.41±11.61 and 23.65±2.25, respectively; p<0.001; Figure 2).

Correlation between tear film cytokines, immune activity in the conjunctival epithelium, and disease severity: The correlation analysis between the levels of IL-5 and IL-6 in the tear film and the immunofluorescence intensity in the conjunctival epithelium of patients with KC in relation to disease severity did not show statistically significant differences (Table 3). The correlation analysis between IL-5 levels in the tear film and its fluorescence in the conjunctival epithelium of patients with KC showed a moderate and significant correlation (r=0.4340, p=0.0033). However, there was no significant correlation observed between IL-6 levels in the tear film and its fluorescence in the conjunctival epithelium (r=−0.0859, p=0.5792).

DISCUSSION

In this study, we observed that there was no significant difference in the levels of IL-5 and IL-6 in the tear film of patients with KC compared to the control group. However, we observed heightened immune activity in the corneal and conjunctival epithelial cells of patients with KC based on IL-5 and IL-6 gene expression and their immunodetection, respectively.

The presence and concentration of cytokines and other inflammatory molecules in tears have been investigated in various conditions that affect the ocular surface, particularly dry eye, ocular allergy, and graft-versus-host disease [59-61]. Regarding the concentrations of cytokines in the tear film, no statistical difference was found between the two groups, despite higher levels being observed in six out of the seven samples in the KC group. In our previous study [1], we analyzed 21 cytokines and found that IL-6 and IL-5 were the most relevant, with a correlation between the level of IL-6 and the degree of disease severity. Tears were collected using microcapillary tubes, and cytokines were analyzed using Luminex 200 (Luminex Corporation, Austin, TX). In the present study, cytokine readings were performed using a flow cytometer, and quantitative analyses were conducted using LEGENDplexTM Data Analysis software (Biolegend). With this methodology, we were unable to reproduce the results obtained by Moura et al. This discrepancy might be due to: (1) differences in collection methods; (2) different methodologies for analyzing and quantifying different cytokines; and/

Table 3. Correlation between tear film levels of IL-5 and IL-6 and their immunodetection in the conjunctival epithelium of patients with keratoconus and disease severity.

Keratoconus classification	Sample	IL-5 (tear film)	IL-6 (tear film)	IL-5 (conjunctiva)	IL-6 (conjunctiva)
Moderate to advanced*	23	1.31±1.47	8.76±16.93	55.78±13.26	70.95±28.62
Severe**	8	1.03±0.52	5.64±7.99	54.61±7.92	75.89±20.58
P value		0.54	0.52	0.93	0.89

or (3) large intragroup quantitative variability and lack of standardization in the normal population as a reference for tear analysis. It is reasonable to infer that collection using microcapillary tubes may cause some discomfort and serve as an inflammatory trigger, potentially interfering with the secretion of proinflammatory cytokines. In contrast, the wash method used in the present study may act as a diluting factor, although it was applied to both groups. Regarding the methods of analysis and quantification of tear cytokines, Khan et al. [67] compared different kits from different manufacturers using aliquots of the same serum samples and compared the results with the results of enzyme-linked immunosorbent assays for specific cytokines. They found that cytokine levels followed similar patterns but reported quantitative differences between kits. When comparing the enzyme-linked immunosorbent assay and Luminex from the same manufacturer, the results were similar. This could explain the high intragroup variability observed, regardless of the methodology and kit supplier, as well as the lack of standardization or reference to normal values in measuring tear cytokines in the normal population. To adjust the large intragroup variability and the variability between the two groups, we performed a new analysis excluding outliers without compromising the sample size, but it did not alter the original results. It is possible that larger sample sizes would provide more representative results. However, the limitations of conducting large experiments with these kits must be considered, particularly due to their high cost.

Several authors have investigated tear film cytokines in patients with KC using different sampling and analysis techniques. Shetty et al. [29] evaluated the levels of inflammatory molecules in the tears of Indian patients with KC using a flow cytometer and reported a significant difference in cytokines associated with inflammation, which indicates an altered inflammatory environment on the ocular surface of patients with KC. Sorkhabi et al. [31] investigated the level of inflammatory molecules in the tear film of patients with KC compared to a control group. They found increased levels of IL-1β, IL-6, and IFN-γ in patients with KC, as well as a lower level of IL-10. Ionescu et al. [22] reported significant expressions of IL-1β, IL-4, IL-6, IL-10, IFN-γ, and TNF-α in the tear film of patients with KC compared to a control group. Jun et al. [23] observed elevated levels of IL-6 and reduced levels of IL-4, CCL5, IFN-γ, and TNF-α in the tear film of patients with KC compared to a control group. Lema et al. [24] reported higher levels of IL-6, TNF-α, and MMP-9 in the tear film of patients with KC compared to a control group. Balasubramanian et al. [14] reported increased tear concentrations of MMP-1, −3, −7, and −13; IL-4, −5, −6, and −8; and TNF-α and β in patients with KC. In contrast to the aforementioned authors, who used microcapillary tubes for sampling, Peyman et al. [28] used Schirmer strips and reported elevated levels of IL-6 and TNF-α in the tear film of patients with KC. Regarding the studies mentioned above, it is noteworthy that many replicated the finding of higher tear concentrations of certain cytokines, such as IL-6, in patients with KC.

It is worth highlighting the correlation between KC and ocular allergy, as well as the potential interference in cytokine concentration. In the Collaborative Longitudinal Evaluation of Keratoconus study, Wagner et al. [68] reported that 53% of patients had a history of atopy and approximately 50% had the habit of eye rubbing. Agrawal et al. [69] reported symptomatic ocular allergy in 24.45% of patients with KC. In our study, the KC group had a significantly higher prevalence of allergy than the control group, which could potentially confound the main outcomes. Therefore, the analyses involving the measurement of tear film cytokine concentration, gene expression of *IL-6* and *IL-5* in the corneal epithelium, and protein detection of IL-6 and IL-5 in the conjunctival epithelium were controlled for age and allergy to avoid potential confounding factors. Ideally, a study with a better distribution of the allergy factor between the two groups or excluding patients with KC and ocular allergy could better elucidate the potential interference of allergy. Another interesting analysis relating to ocular allergy pertains to the Th1:Th2 ratio represented by the IFN-γ:IL-10 ratio between the two groups. We speculated and intuitively expected that there might be a Th2 deviation in the KC group due to the higher prevalence of ocular allergy in this group. However, there was no statistical difference observed between the two groups in terms of this ratio.

Regarding the gene expression of *IL-6* and *IL-5* in the corneal epithelium, we found that it was higher in the KC group. Pahuja et al. [70] evaluated the gene expression of inflammatory mediators, including *IL-6*, in the corneal epithelium of KC patients and a control group. They compared the epithelium of the KC apex with that of the corneal periphery. The cone apex epithelium of patients with KC showed increased levels of inflammatory mediators. A similar analysis was performed using stromal tissue and epithelium from five patients with advanced KC and Bowman's layer rupture who underwent corneal transplantation. Epithelium collected from the cone apex of patients with KC and Bowman's layer rupture demonstrated significantly elevated levels of MMP-9, TNF-α, and IL-6 and reduced levels of IL-10, tissue inhibitor of metalloproteinase-1, and collagen IVA1. Shetty et al. [30] demonstrated that the gene expression of *MMP-9*, *TNF-α*, and *IL-6* in the corneal epithelium was higher in patients with KC

compared to a control group. In another study, Shetty et al. reported that the transcription levels of *MMP-9* and *IL-6* were upregulated in the corneal epithelium of patients with KC compared to normal participants [71]. These findings support our findings regarding the gene expression of inflammatory mediators, specifically *IL-6*, in the corneal epithelium of patients with KC.

Regarding the immunodetection of IL-6 and IL-5 in the cytoplasm of conjunctival epithelium, we detected heightened fluorescence intensity in patients with KC compared to the control group. Despite an extensive literature review, we did not find any studies investigating the presence of inflammatory mediators in the conjunctival epithelium of patients with KC using techniques such as IC, immunofluorescence, and confocal microscopy analysis. Studies exploring inflammatory mediators in the conjunctiva have mainly focused on conditions such as dry eye and allergic conjunctivitis. For instance, Narayanan et al. evaluated the expression of cytokines, including IL-6, on the ocular surface of individuals with moderate dry eye and healthy controls [72].

Another important finding in this study was the correlation between the immunodetection of IL-5 in the conjunctival epithelium of patients with KC and its tear film level, whereas no such correlation was observed for IL-6. Further investigations using cultured and stimulated conjunctival epithelial cells from patients with KC could provide insights into their secretory capacity for IL-5 and/or IL-6. However, the levels of IL-5 and IL-6 in the tear film and their immunodetection in the conjunctival epithelium did not show any correlation with the degree of KC severity. Peyman et al. evaluated the tear levels of IL-6 and TNF-α in patients with KC compared to normal individuals and reported a correlation between these tear cytokines and the severity of KC [28]. Contradictory to the present study findings, Moura et al. [1] reported a positive correlation between tear levels of IL-6 and the degree of KC severity. Nonetheless, the results presented in this study support the notion that inflammatory activity plays a role in the etiopathogenesis of KC. The characterization of inflammatory mediators in the tear film and their relationship with the immunological microenvironment of the ocular surface in patients with KC adds to the body of knowledge. Although cytokine levels in the tear film can be easily measured, it remains unclear whether corneal and/or conjunctival cells interact with these interleukins or serve as a source of these dysregulated proteins.

The expression of *IL-5* and *IL-6* mRNA in the corneal epithelium, along with the detection of IL-5 and IL-6 in the conjunctival epithelium at the posttranscriptional level and the observed correlations between cytokines in the tear film and their conjunctival immunodetection suggest a potential deviation or the predominance of the Th2 response in the immunological microenvironment of the ocular surface in KC. However, it is important to note that these findings do not establish a causal or temporal relationship between intercellular interactions in the cornea, conjunctiva, and tear film.

Indeed, further studies involving the culture of corneal epithelial cells, keratocytes, and conjunctival epithelial cells, as well as coculture experiments under specific stimuli and blockages of inflammatory pathways are necessary to better understand the individual roles of these cell groups and their interaction in the inflammatory pathway that leads to the degradation of the corneal extracellular matrix in patients with KC. The identification of biomarkers and/or the characterization of immuno-inflammatory pathways can facilitate the early diagnosis of KC, predict its progression, and aid in the development of therapeutic strategies to prevent functional visual loss in KC patients.

Conclusion: In this study, despite observing higher levels of IL-5 and IL-6 in the tear film of patients with KC, there was no statistically significant difference compared to the control group. However, when examining the corneal epithelium, it was found that patients with KC exhibited higher gene expression of *IL-5* and *IL-6* than the control group. Likewise, the conjunctival epithelial cells of patients with KC showed increased immunodetection of IL-5 and IL-6 compared to the control group.

Interestingly, there was no correlation between the levels of IL-5 and IL-6 in the tear film and their immunodetection in the conjunctival epithelium and disease severity. However, when analyzing the correlation between IL-5 and IL-6 levels in the tear film and their immunodetection in the conjunctival epithelium, a moderate and significant correlation was found for IL-5, while no correlation was found for IL-6.

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