Evaluation of Inflammatory Mediators in the Tear Film Before and After Corneal Cross-Linking

Avaliação dos Mediadores Inflamatórios no Filme Lacrimal Antes e Depois do *Cross-Linking* Corneano

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ABSTRACT

INTRODUCTION: Keratoconus' pathogenesis is not completely understood. One suspected contributor is inflammation at the corneal surface, often signaled by elevated levels of pro-inflammatory cytokines in the tear film. Corneal cross-linking is a recognized treatment to slow or halt disease progression. While corneal cross-linking has demonstrated promising results, there is notable variability in outcomes among patients, and the reasons for this remain incompletely understood. Our hypothesis posits that favorable post-operative outcomes may be linked to alterations in the pro-inflammatory cytokine content in a patient's tear film. This study aims to explore the relationship between tear film pro-inflammatory cytokines and the effectiveness of corneal cross-linking treatment outcomes.

METHODS: Clinical evaluations captured corneal tomographic data, best-corrected distance visual acuity, and past medical history from participants. Two tear film samples were taken from individuals undergoing corneal cross-linking and from age-matched controls. These samples were collected both before the procedure and six months afterward using Schirmer strips. The cytokine analysis in these samples was performed using the commercially available Th1/Th2/Th9/Th17 Cytokine 18-Plex Human ProcartaPlex™ Panel.

RESULTS: We recruited 35 patients for the treatment group (57% females; mean age: 27.61±8.94 years) and 19 for the control group (47% females; mean age: 24.13±7.18 years). After corneal cross-linking, significant enhancements were observed in best-corrected distance visual acuity (p<0.0001) and tomographic indices such as Kmax (p=0.0417). Pre-operative mean total protein content stood at 1.54±0.46 µg/µL10 for the treatment cohort and 1.48±0.27 µg/µL10 for controls. Six months post-procedure, these figures were 1.40±0.428 µg/µL10 and 1.90±0.45 µg/µL10 for the treatment and control groups, respectively. Though there was a discernible trend of elevated pro-inflammatory cytokine levels in the pre-operative treatment group relative to both controls and post-corneal cross-linking results, the mean difference was not statistically significant.

CONCLUSION: Our research indicates that elevated pro-inflammatory cytokine levels are seen in patients with keratoconus. We were unable to confirm our hypothesis that treatment with CXL may reduce PIC levels. Future studies with expanded sample sizes are essential to under-

stand pro-inflammatory cytokines' influence on both keratoconus pathophysiology and corneal cross-linking outcomes.

KEYWORDS: Corneal Cross-Linking; Cytokines; Inflammation; Keratoconus/drug therapy.

RESUMO

INTRODUÇÃO: A patofisiologia do queratocone não está totalmente esclarecida. Um potencial fator de risco é o microambiente inflamatório na superfície corneana, traduzido por níveis elevados de citocinas pró-inflamatórias no filme lacrimal. O *cross-linking* corneano é um tratamento estabelecido para desacelerar ou interromper a progressão da doença. Apesar do *cross-linking* ter demonstrado resultados promissores, existe variabilidade nos *outcomes* entre os doentes. A nossa hipótese é que os resultados pós-operatórios favoráveis podem estar relacionados com as citocinas pró-inflamatórias no filme lacrimal. Este estudo visa explorar a relação entre as citocinas pró-inflamatórias no filme lacrimal e os outcomes do *cross-linking*.

MÉTODOS: Dados tomográficos corneanos, melhor acuidade visual corrigida e dados clínicos dos participantes foram colhidos. Foram colhidas duas amostras do filme lacrimal dos doentes submetidos a *cross-linking* e de controlos saudáveis. As amostras foram colhidas antes do procedimento e seis meses depois do *cross-linking*, usando tiras de Schirmer. A análise das citocinas nas amostras foi realizada com recurso ao painel Th1/Th2/Th9/Th17 Cytokine 18-Plex Human ProcartaPlex™.

RESULTADOS: Recrutamos 35 doentes para o grupo de tratamento (57% mulheres; idade média: 27,61±8,94 anos) e 19 para o grupo de controlo (47% mulheres; idade média: 24,13±7,18 anos). Após o *cross-linking*, foram observadas melhorias significativas na acuidade visual (p<0.0001) e em índices tomográficos como o Kmax (p=0,0417). O conteúdo médio total de proteínas no pré-operatório foi de 1,54±0,46 μg/μL10 para o grupo de tratamento e 1,48±0,27 μg/μL10 para o grupo de controlo. Seis meses após o procedimento, estes números foram de 1,40±0,428 μg/μL10 e 1,90±0,45 μg/μL10 para os grupos de tratamento e controlo, respetivamente. Observamos uma tendência para maiores níveis de citocinas pró-inflamatórias no grupo de tratamento pré-operatório comparativamente aos controlos e aos resultados após *cross-linking*. No entanto, a diferença média não foi estatisticamente significativa.

CONCLUSÃO: O estudo sugere que os doentes com queratocone poderão ter níveis mais elevados de citocinas pró-inflamatórias. No entanto, não foi possível confirmar a hipótese de que o tratamento com *cross-linking* reduz os níveis de citocinas pró-inflamatórias. Serão necessários estudos com amostras mais robustas para compreender a influência das citocinas pró-inflamatórias no queratocone e nos resultados cirúrgicos do *cross-linking*.

PALAVRAS-CHAVE: Citocinas; Cross-Linking da Córnea; Inflamação; Queratocone.

INTRODUCTION

1. STATE OF THE ART

1.1. Keratoconus

Keratoconus (KCN) is characterized by progressive corneal ectasia into a conical shape protrusion.[1] Most cases arise in adolescence and progress into the third and fourth decades of life,[1] although the disease onset, progression, or arrest can occur at any time.[2] Clinically, this condition can result in irregular astigmatism, myopia, and often irreversible loss of visual acuity.[2,3] Although epidemiologic data varies, a mean prevalence of 54 cases per 100 000 white European individuals is estimated1 and a recent meta-analysis suggests a global prevalence of 138 per 100 000.[4] Even though KCN was first described over a century ago in 1854,[4,5] its etiopathogenesis is yet to be completely understood.[4,6] Still, it is considered to occur under oxidative stress of environmental or endogenous origin on a systemic and corneal level in genetically susceptible individuals.[3,4] Environmental factors are frequently related to mechanical trauma, such as hard contact lens wear and eye rubbing.[1,2] Although it occurs more frequently as an isolated entity,[2] its association with comorbidities such as connective tissue disorders, atopy,[1,4] and obesity has been proposed as an etiological factor.[4] Re-

gardless of the trigger, the metabolic activity in the cornea is altered, resulting in biochemical instability and ultimate tissue loss[1] with the hallmark histopathological characteristics of corneal stroma thinning, tears in the Bowman's layer, and iron deposition in the basal layers of the corneal epithelium.[2] In addition to the observed downregulated expression of collagen[1,4] and the related decrease in the number of lamellae in the stroma,[1] it has been proposed that collagen is not only lost but redistributed by slippage between the lamellae.[1]

1.2. Keratoconus and Inflammation

Although the typical clinical presentation of KCN does not include macroscopic inflammatory signs or symptoms (corneal edema, redness, pain, or intraocular inflammation), current evidence suggests that several inflammatory pathways contribute to corneal damage.[3,7] Proinflammatory changes may therefore be responsible for the characteristic proteolytic environment of KCN corneas, with increased proteinase activity and decreased expression of proteinase inhibitors, resulting in loss of biochemical stability.[1,3,4] Furthermore, etiologic factors such as contact lens wear, frequent eye rubbing, and atopy are reflected in the immunological profile of patients' tears.[3] For example, chronic contact lens wear leading to corneal trauma and induction of proinflammatory cytokines (PIC) may contribute to the loss of keratocytes with abnormal susceptibility to apoptosis.[3,4]

The role of inflammatory pathways in the pathogenesis of KCN is supported by the discovery in several studies of altered levels of cytokines, chemokines, and immune mediators in the tear film of KCN patients, in comparison with unaffected individuals.[3]

Inflammatory markers associated with KCN include interleukin (IL)-1, IL-4, IL-5 IL-6, IL-8, IL-17, tumor necrosis factor (TNF)-α, transforming growth factor (TGF)-β, and chemokine ligand 5 (CCL5).[8] The interplay between these mediators is summarized is Fig. 1.

Previous efforts have been made to use PIC as biomarkers for KCN, albeit with inconsistent results. Lema *et al* found a significant positive correlation between the concentration of IL-6, TNF-α, and matrix metalloproteinase (MMP)-9 in KCN and the steepest keratometric reading K2.[8] Kolozsvári *et al* studied the tear fluid from 14 eyes of 11 KCN patients and found that CCL5 and MMP-13 positively correlated to the severity of the disease, while IL-6 and IL-13 were negatively associated with the severity of the disease.[9] Recently, Fodor *et al* attempted to predict KCN progression using the levels of inflammatory mediators in the tear samples of 42 KCN patients and found that the level of IL-13 in combination with that of nerve growth factor could predict the progression of KCN with 100% specificity and 80% sensitivity.[10] Although more research is required, these results highlight the potential of PIC as a useful biomarker in KCN.

2. CROSS-LINKING

KCN is one of the most common indications for keratoplasty.[1,11] However, the need for this procedure severely

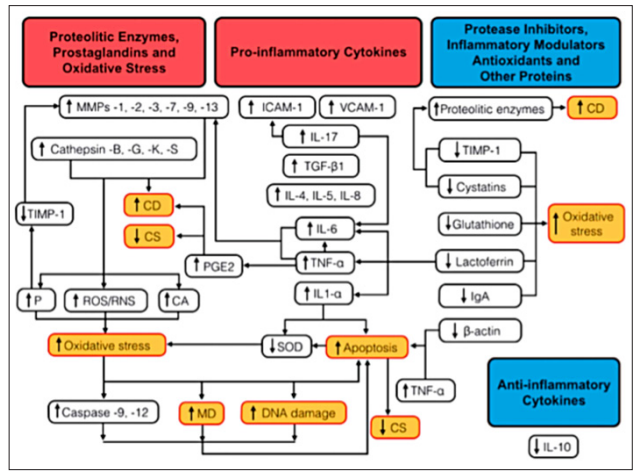

Figure 1. The abnormal balance between pro-inflammatory (red) and anti-inflammatory (blue) mediators in KCN. TNF-α induces the release of PGE2 that inhibits collagen synthesis (CS) and increases collagen degradation (CD). There is also an elevation of proteases such as lysosomal cathepsin-B, -G, -K, and -S, and metalloproteinases (MMPs) which cause higher production of ROS, RNS, cytotoxic aldehydes (CAs) and peroxynitrates (Ps) (which decreases the activity of TIMP-1 and increases MMP-2) and, with a low level of SOD related to IL-1α, an environment with high oxidative stress is created. This causes an activation of the caspases (caspase-9 and -12), mitochondrial dysfunction (MD), and DNA damage that leads to increased apoptosis of keratocytes. The pro-inflammatory environment is also due to an increased level of pro-inflammatory molecules: IL-1α, -4, -5, -6, -8, and -17, TNF-α, TGFβ-1, intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) and a decrease of antioxidant or anti-inflammatory molecules (SOD, glutathione, lactoferrin, IgA, and IL-10) and protease inhibitors such as cystatins (inhibitors of cysteine proteases) and TIMP-1 (inhibitor of MMPs).

Adapted from Galvis V, *et al*. Keratoconus: an inflammatory disorder?, Eye. 2015; 29:843–59.[8]

decreased with the advent of collagen cross-linking (CXL), which has become the gold-standard procedure to stop the progression of this disease.[12]

The artificial induction of cross-links in the corneal tissue using ultraviolet-A (UV-A) light and riboflavin was first proposed by Spoerl *et al* in 1998.[14] In 2003, Wollensak *et al* developed the Dresden Protocol,[15] which is currently the most widely accepted procedure.[13] This achieves biomechanical stiffening of the cornea and stops disease progression.[12,16] The effect is obtained by photopolymerization,[17] in which riboflavin reacts with UV light to create free radicals that induce new chemical bonds between carbonyl groups of collagen molecules at an intra- or interfibrillar level.[13,17] Riboflavin functions not only as a free radical generator but also as a radical scavenger at high concentrations, creating a balance between the formation and destruction of free radicals.[17]

Furthermore, UV radiation alone reaches a small penetration depth in the cornea, hence the need for a photosensitizer such as riboflavin.[14]

Regardless of the reported efficacy and safety of the corneal CXL procedure, there have been reports of continued disease progression and worsening of visual acuity, the reasons for which are not well understood.[18] Efforts to use corneal pachymetry, keratometry, and patient factors such as age, gender, and visual acuity as predictors of success

have yielded inconsistent results.[12] A possible determinant impacting on the efficacy of CXL, and its morphological outcomes, is the cornea's molecular microenvironment, especially concerning inflammatory mediators. As far as we are aware, this connection remains unexplored in the existing literature.

3. PURPOSE

The purpose of this study was to correlate PIC in tear film of patients who undergo CXL for the treatment of KCN and postoperative outcomes.

MATERIALS AND METHODS

1. ETHICS AND CONFIDENTIALITY

Institutional approval (Ethics Committee of Faculty of Medicine of the University of Coimbra, Coimbra, Portugal) was obtained for this study.

2. PARTICIPANTS

We included patients by applying a convenience sampling strategy. The inclusion criteria were:

- Study group: 1) patients with KCN, 2) scheduled CXL procedure, and 3) no history of other ocular disease, ocular surgery, or autoimmune/systemic inflammatory disease.
- Control group: healthy age and gender-matched volunteers.

3. WORKPLAN

This was a prospective, non-randomized study with at least 6 months of follow-up.

- **Step 1:** Participants selection (study and control groups) based on established criteria.
- **Step 2:** Interview and recording of study group corneal tomography indexes, thinnest corneal thickness (TCT) and maximal corneal curvature (Kmax), and Best Corrected Distance Visual Acuity (BCDVA) values.
- **Step 3:** Collection of the first tear fluid simple in study and control groups.
- **Step 4:** Corneal cross-linking in the study group.
- **Step 5:** Collection of the second tear fluid sample in the study and control groups 6 months after the first collection.
- **Step 6:** Recording of study group corneal tomography indexes (TCT and Kmax), and BCVA values 6 months after corneal cross-linking.
- **Step 7:** Quantification of total protein and cytokine in study and control groups.
- **Step 8:** Analysis and discussion of results.

4. CXL PROTOCOL AND POSTOPERATIVE MEDICATION

i. CXL procedure adhered to Athens or Cretan protocol, depending on elegibility criteria for each patient.
ii. Post-operative regimen included dexamethasone and ofloxacin 6 times daily for 1 week, then dexamethasone 3 times daily for 3 weeks.

5. TEAR COLLECTION

The method of tear fluid extraction has an impact on protein and cytokine quantification. Thus, the choice of a tear collection method should be careful, and the analysis of the results must take this into account to best correlate the outcomes of the CXL with the PIC concentrations.[26]

There are two different methods to collect tears, direct and indirect, that are both safe and well tolerated. The direct sampling methods, such as microcapillary tubes or micropipettes, are more aseptic and simpler to process, only requiring quick centrifugation. However, it needs specialized personnel for its correct fulfillment and requires previous stimulation or instillation of different volumes of saline (100–200 µL) into the *cul-de-sac*, which decreases the concentration of the cytokines.

The indirect method, with Schirmer strips, has the advantage of being simpler and readily available in most centers. It can be performed by any non-specialized personnel, and it is a non-invasive method. In addition, the tear fluid volume is larger than the volume extracted by direct methods which allows us to have a more representative sample. However, processing is much harder, as it requires additional dilutions for further analysis, and it is more uncomfortable for patients. It causes reflex tearing, which could provide less protein content because of possible dilution and protein binding to the matrix.[19-22]

For this study, tear fluid was collected with Schirmer strips which consists of an indirect collection by placing a 35 mm paper strip on the closed eye for 5 minutes without any anesthetic. After that, the Schirmer strips were cut at the level of the wetted portion, to decrease the loss of tear fluid sample, and reserved in 2 mL Eppendorf tubes which were housed in a plastic cup with ice and processed on the same day. All these procedures were performed under aseptic conditions with sterilized Schirmer strips, gloves, scissors, Eppendorf tubes, and plastic cups.

5.1. Tear Processing

The first step of tear fluid processing is the dilution with soaking buffer (100 µL of 0.9% NaCl) and 1-hour incubation on an orbital shaker at room temperature until it is well diluted. To waste as little volume as possible, the Schirmer strip is transferred to a perforated 0.5 mL Eppendorf tube, which is placed inside a 2 mL Eppendorf tube and centrifuged for 5 minutes at 4ºC and 10 000 relative centrifugal force. Both fluids (eluted and recovered) are mixed and then distributed in five aliquots (one aliquot of 10 µL and four aliquots of 50 µL) and frozen at -80ºC for posterior analysis.

6. TEAR ANALYSIS

6.1. Total protein quantification

The quantification of total protein content was done with a bicinchoninic acid total protein assay (Thermo Fisher Scientific, Pierce, Rockford, IL, USA) whose protocol was followed exactly as indicated.[26] This assay consists in the reduction of Cu2+ to Cu+ by protein in an alkaline fluid and the formation of a purple liquid. The intensities of the color obtained are measured colormetrically by a spectrophotometer and the absorbance at 570 nm is proportional to the concentration of total protein.[24]

6.2. Cytokine quantification

By utilizing Luminex xMAP technology, the Human High Sensitivity 9-Plex ProcartaPlex Panel™ was used to study immune function by analyzing 9 protein targets in a single well: IFN gamma, IL-1 beta, IL-2, IL-4, IL-6, IL-10, IL-12p70, IL-17A (CTLA-8), and TNF alpha. This panel has been specifically designed to measure samples with sensitivities for all analytes in the femtogram range (10-15 g).[25]

ProcartaPlex immunoassays are based on the same principles as sandwich ELISA, which involves the use of two highly specific antibodies that bind to different epitopes of a single protein to measure all protein targets simultaneously through a Luminex instrument. A multiplexed assay involves labeling each spectrally unique bead with antibodies specific to a single target protein. Bound proteins are then detected using biotinylated antibodies and streptavidin-R-phycoerythrin. By conjugating protein-specific antibodies to distinct beads, it becomes possible to analyze multiple targets in a single well.[25]

The detection and calculation of cytokine content is done with Luminex® 100/200™ System which is based on the principles of flow cytometry that analyses up to 100 analytes in a single microplate well (Fig. 2).

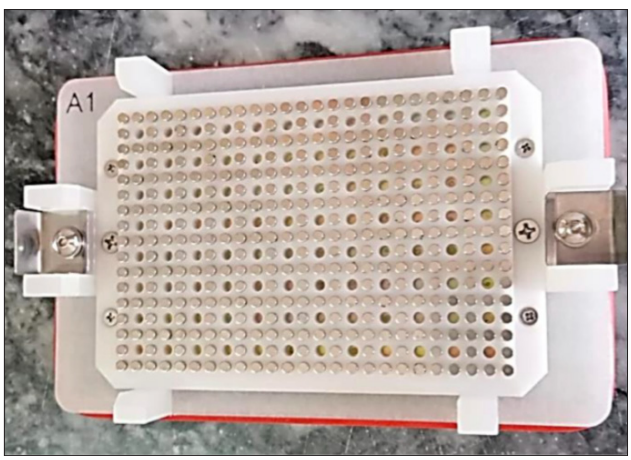

Figure 2. A photograph of the magnetic plate used to invert the 96-well flat bottom plate during washing steps.

6.3. Statistical Analysis

For statistical analysis, GraphPad (Software Inc., California, USA) and IBM® SPSS® version 26 (IBM, New York, USA) were used. Data distribution for normality was checked with the Shapiro-Wilk test. The Mann-Whitney test was used to compare the two pre- and post-operative tear sample collections in the study group in terms of best-corrected distance visual acuity (BCDVA) and corneal tomography measures (Kmax and TCT). To look for differences in cytokine content (already parameterized by the protein content) between the control and study groups, as well as between the pre- and 6-month post-CXL in the study group, the Kruskal-Wallis test was used. A *p*-value below 0.05 was considered statistically significant.

RESULTS

In this study, 35 eyes from 35 patients in the study group (15 males and 20 females, mean age: 27.61 years ± 8.94) and 19 eyes from 19 patients in the control group (10 males and 9 females, mean age: 24.13 years ± 7.18) were tested. The tear samples were collected at a 6-month interval in 11 volunteers of the control group and in 10 patients of the study group (pre- and 6 months post-CXL).

1. TOPOGRAPHIC CHANGES 6 MONTHS AFTER CXL

Fig. 3 shows the differences between topographic measures in the study group: Kmax and TCT. After CXL, the Kmax was significantly lower than pre-operative (from 58.42 ± 5.17 to 55.19 ± 5.94) with *p*-value = 0.0417 and TCT was also significantly lower (from 450.18 ± 29.74 to 419.42 ± 31.62) with *p*-value = 0.0003.

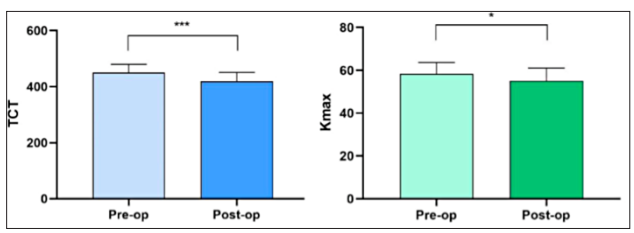

Figure 3. Comparison of pre-operative and 6 months after post-operative corneal topographic changes: TCT: thinnest corneal thickness and Kmax: maximal corneal curvature.

*: Statistically significant with *p*-value < 0.05
***: Statistically significant with *p*-value < 0.001

2. VISUAL ACUITY CHANGES 6 MONTHS AFTER CXL

Fig. 4 shows that 6 months after CXL, patients significantly improved their BCDVA, as they went from 0.492 ± 0.241 logMAR to 0.247 ± 0.172 logMAR, with a *p*-value of <0.0001.

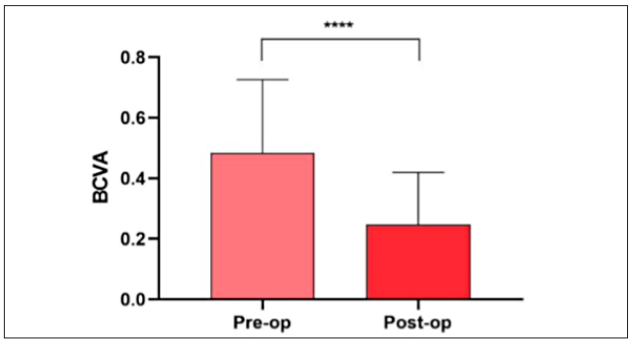

Figure 4. Comparison of pre-op and 6 months post-op BCDVA changes in logMAR values.

3. CYTOKINE CONTENT CHANGES 6 MONTHS AFTER CXL

The tear film PIC comparison between the control group and the pre-operative of study group, represented in Table 1, was not statistically significant. The difference between the second sample of controls and post-operative (Table 1) was also not statistically significant (Fig. 5).

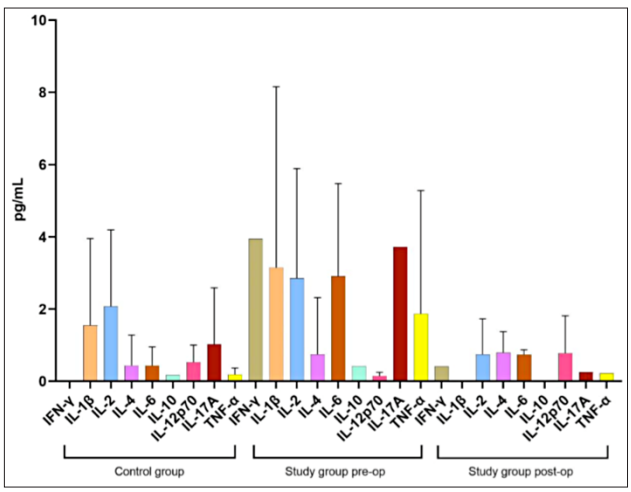

Figure 5. Comparison of PIC between the control group, pre-operative, and post-operative study group.

DISCUSSION

CXL has a positive impact on slowing the progression of KCN.[26-28] Our study corroborates these findings, showing an improvement in patients' visual and topographic outcomes after CXL. However, the significance of the results should be interpreted with caution because combined CXL and excimer laser partial photorefractive keratectomy were done in the majority of the study group population.

Initially, we planned to quantify each cytokine for each patient so that a paired comparison could be made between pre-operative versus post-operative and healthy controls versus KCN. However, due to processing limitations in the sample analysis phase, we were able to retrieve specific cytokine values for some patients. This happened because we were unable to achieve the minimum doseable concentration required for a valid result in 25 patients. Nevertheless, we were able to quantify total protein content, which serves as a proxy for total tear film inflammatory burden. In our study, a trend was seen towards higher levels of PIC in patients with KCN when compared to healthy individuals. There was also a reduced burden of PIC 6 months post-CXL. However, given the absence of statistical significance, these findings should be approached with prudence.

The present study has some limitations. The strength of our study is constrained by the reduced sample size. Also, only those with progressive disease were included in the study population. Therefore, our findings may not be entirely generalizable to all patients with KCN, including those at different stages of the disease.

The method chosen for tear fluid extraction has an impact on protein and cytokine quantification. Our study used the indirect method, with Schirmer strips, which is less aseptic, and the processing of the samples requires additional dilutions for further analysis, so the likelihood of errors occurring during the process is not insignificant.[19-22]

Also, we used different CXL protocols across participants, which introduces a potential confounding variable, as variations in protocol may have influenced the outcomes. As a result, it is difficult to isolate the specific effects of the treatment and draw definitive conclusions about its efficacy. Standardizing CXL protocols in future research will be crucial to minimize these confounding effects and improve the validity of the results.

CONCLUSION

Our investigation into the relationship between PIC and the effectiveness of CXL treatment outcomes in KCN patients has provided valuable insights. Even though keratoconus patients indeed exhibited elevated levels of PIC, we were unable to definitively confirm a reduction in these levels following CXL. As such, it is essential to interpret our findings with caution, considering the relatively small sample size and the refered confounders. To gain a comprehensive understanding of the influence of PIC on both keratoconus pathophysiology and CXL surgical outcomes, larger-scale studies are warranted. Additionally, the

Table 1. First sample – control group versus pre-operative treatment group. Second sample – control group versus 6 months post-CXL.

	1st sample			2nd sample		
	Control group	Treatment group		Control group	Treatment group	
Mean protein content (μg/μL[10])	1.48±0.27	1.54±0.46	$p > 0.05$	1.90±0.45	1.40±0.43	$p > 0.05$

clinical improvements in BCDVA and tomographic indices, such as Kmax, emphasize the potential efficacy of CXL in halting disease progression and improving visual acuity.

Our findings contribute to the growing body of knowledge surrounding KCN and CXL treatment. It is crucial for future research to delve deeper into the complex interplay between PIC, corneal biomechanics, and CXL to optimize patient selection, treatment protocols, and post-operative care. Ultimately, we hope that our study serves as a stepping stone for further investigations, aiming to enhance the effectiveness of CXL in managing keratoconus and improving the quality of life for affected individuals.

PRESENTATIONS

Part of this study will be presented at the 66th Portuguese Congress of Ophthalmology in 2023.

CONTRIBUTORSHIP STATEMENT / DECLARAÇÃO DE CONTRIBUIÇÃO

BT, CC, PS, and AR: Responsible for gathering data, presenting results, and writing the manuscript.

RF, LC, PM, AR: Responsible for performing complementary exams and helping in laboratory tasks.

AR, JG, EC, MJQ, JM: Supervised the project and contributed with their expertise to its conclusion.

All authors read and approved the final manuscript.

BT, CC, PS, e AR: Responsável pela recolha de dados, apresentação de resultados e redação do manuscrito.

RF, LC, PM, AR: Responsável pela realização de exames complementares e auxílio nas tarefas laboratoriais.

AR, JG, EC, MJQ, JM: Supervisionaram o projeto e contribuíram com os seus conhecimentos para a sua conclusão.

Todos os autores leram e aprovaram o manuscrito final.

RESPONSABILIDADES ÉTICAS

Conflitos de Interesse: Os autores declaram a inexistência de conflitos de interesse na realização do presente trabalho.

Fontes de Financiamento: Não existiram fontes externas de financiamento para a realização deste artigo.

Confidencialidade dos Dados: Os autores declaram ter seguido os protocolos da sua instituição acerca da publicação dos dados de doentes.

Proteção de Pessoas e Animais: Os autores declaram que os procedimentos seguidos estavam de acordo com os regulamentos estabelecidos pela Comissão de Ética responsável e de acordo com a Declaração de Helsínquia revista em 2013 e da Associação Médica Mundial.

Proveniência e Revisão por Pares: Não comissionado; revisão externa por pares.

ETHICAL DISCLOSURES

Conflicts of Interest: The authors have no conflicts of interest to declare.

Financing Support: This work has not received any contribution, grant or scholarship

Confidentiality of Data: The authors declare that they have followed the protocols of their work center on the publication of data from patients.

Protection of Human and Animal Subjects: The authors declare that the procedures followed were in accordance with the regulations of the relevant clinical research ethics committee and with those of the Code of Ethics of the World Medical Association (Declaration of Helsinki as revised in 2013).

Provenance and Peer Review: Not commissioned; externally peer reviewed.

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