Tear Film Mediators After Corneal Cross-Linking: Methodology Validation

Mediadores do Filme Lacrimal Após Cross-Linking Corneano: Validação de Metodologia

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Recebido/Received: 2021-12-05 | **Aceite/Accepted:** 2023-01-04 | **Published online/Publicado online:** 2023-03-16 | **Publicado/Published:** 2023-03-28

DOI: https://doi.org/10.48560/rspo.25967

ABSTRACT

INTRODUCTION: Currently, the gold-standard treatment of progressive keratoconus (KC) is corneal collagen cross-linking (CXL), in which riboflavin and ultraviolet-A radiation increase the corneal biomechanical rigidity, arresting progression. However, post-operatory results vary significantly among patients. Although it has been suggested that corneal thickness, cone location and patient's age are important factors, their contribution is controversial. The reason for this variability remains obscure.

This project aims to find biomarkers that could explain the variability within surgical results after corneal CXL by investigating the correlation between the local corneal inflammatory environment and these results.

We hypothesize that better post-operatory results are related to a reduction in the inflammatory cytokine levels in the patients' tear film.

Ultimately, we aim to optimize the patients' surgical outcomes according to their corneal inflammatory environment.

METHODOLOGY: The research protocol was established after testing different sample collection methods. Patients are interviewed to assess their medical history and relevant habits. Their corneal tomographic and best corrected visual acuity data are registered before and 6 months after surgery.

The patient´s tear film is sampled twice 6 months apart using Schirmer strips, as the volume yield is larger compared to direct methods (microcapillary tubes or micropipettes), which are more difficult to perform and require stimulation or the instillation of saline into the cul-de-sac, originating reflex tearing.

The samples are analyzed for their total protein content (BCA Protein Assay Kit) and cytokine concentration using Multiplex technology (Th1/Th2/Th9/Th17 Cytokine 18-Plex Human Procarta-Plex™ Panel) and not ELISA, as we tried before.

Statistical analysis to assess sample differences and the correlation between cytokine concentration and tomographic indexes and visual acuity values for each group and subgroup is performed at 6 months.

RESULTS: Twenty patients and eight control subjects have been enrolled in the study and the first collection of tears has been carried out. CXL surgeries for the twenty patients were per-

formed from October 2020 to September 2021. Two samples were lost due to excessively length of dry Schirmer strip, which we improved by cutting the dry portion.

Additionally, the tear fluid from the first eight samples from patients and from control participants was successfully processed for their total protein content.

CONCLUSION: Tear film analysis poses several challenges concerning the small amount of material available without reflex production. This fact precludes the use of conventional ELISA, requiring multiplex modified technology. When dealing with tears it is also important to avoid contamination by using sterilized material, to avoid dilution and to establish an efficient flow of samples from the clinic to the laboratory. This pilot study enabled to identify several methodological frailties and to establish a solid research protocol that can be replicated by other researchers.

Understanding the correlation between inflammatory cytokines and surgical outcomes of CXL may allow for the optimization and personalization of CXL. In addition, the results of this research protocol are expected to establish prognostic factors for CXL-related surgical outcomes, ultimately improving the quality of life of KC patients.

KEYWORDS: Corneal Cross-Linking; Cytokines; Keratoconus Inflammation Mediators; Tears.

RESUMO

INTRODUÇÃO: Atualmente, o tratamento *gold-standard* do queratocone (QC) progressivo é o *cross-linking* do colagénio corneano (CXL), no qual a riboflavina e a radiação ultravioleta-A aumentam a rigidez biomecânica da córnea, parando a progressão. No entanto, os resultados pós-operatórios variam significativamente entre os doentes. Embora se tenha sugerido na literatura que a espessura da córnea, a localização do cone e a idade do doente são fatores importantes, a contribuição destes fatores é controversa. A razão para esta variabilidade permanece incógnita.

Este projeto visa descobrir biomarcadores que possam explicar a variabilidade dos resultados cirúrgicos após CXL corneano, investigando a correlação entre o ambiente inflamatório local da córnea e estes resultados.

A nossa hipótese é que melhores resultados pós-operatórios estão relacionados com uma redução nos níveis de citocinas inflamatórias no filme lacrimal dos doentes.

Em última instância, pretendemos otimizar os resultados cirúrgicos dos pacientes de acordo com o seu ambiente inflamatório corneano.

MÉTODOS: O nosso protocolo de pesquisa foi estabelecido após testagem de diferentes métodos de colheita de amostras. Os doentes são entrevistados para avaliar os seus antecedentes pessoais e hábitos relevantes. Os dados tomográficos da córnea e a melhor acuidade visual corrigida são registados antes e 6 meses após a cirurgia.

Amostras de filme lacrimal dos doentes são colhidas duas vezes, com 6 meses de intervalo, usando tiras de Schirmer, pois o volume conseguido é maior em comparação com os métodos diretos (tubos microcapilares ou micropipetas), que são mais difíceis de realizar e requerem estimulação ou instilação de solução salina no fundo de saco conjuntival, originando lacrimejo reflexo.

As amostras são analisadas quanto ao seu conteúdo de proteína total (BCA *Protein Assay Kit*) e concentração de citocinas através de tecnologia Multiplex (Th1/Th2/Th9/Th17 Cytokine 18-Plex Human ProcartaPlex™ Panel) e não ELISA, como previamente tentado.

A análise estatística para avaliar as diferenças entre as amostras e a correlação entre a concentração de citocinas e índices tomográficos e valores de acuidade visual para cada grupo e subgrupo é realizada aos 6 meses.

RESULTADOS: Vinte doentes e oito controlos foram incluídos no estudo e a primeira colheita de lágrimas foi realizada. As cirurgias de CXL dos vinte doentes foram realizadas de outubro de 2020 a setembro de 2021. Duas amostras foram perdidas devido ao comprimento excessivo da tira de Schirmer, facto que melhorámos cortando a parte seca da tira.

Além disso, as amostras de filme lacrimal dos primeiros oito doentes e dos 8 controlos foram processadas com sucesso para obtenção do conteúdo de proteína total.

CONCLUSÃO: A análise do filme lacrimal apresenta vários desafios, tendo em conta a pe-

quena quantidade de material disponível sem estimulação da produção de lágrimas reflexa. Este facto impossibilita o uso de ELISA convencional, exigindo assim tecnologia multiplex modificada. Ao lidar com lágrimas, também é crucial evitar a contaminação, através do uso de material esterilizado, evitar a diluição e estabelecer um eficiente fluxo de amostras do hospital para o laboratório. Este estudo piloto permitiu identificar várias fragilidades metodológicas e estabelecer um protocolo de investigação sólido que pode ser replicado por outros investigadores.

Compreender a correlação entre citocinas inflamatórias e resultados cirúrgicos do CXL pode permitir a otimização e personalização do CXL. Além disso, é expectável que os resultados deste protocolo estabeleçam fatores prognósticos para os resultados cirúrgicos do CXL, melhorando, em última análise, a qualidade de vida dos doentes com QC.

PALAVRAS-CHAVE: Cross-linking; Citocinas; Lágrimas; Mediadores da Inflamação; Queratocone.

INTRODUCTION

A) STATE OF THE ART

a) Keratoconus

Keratoconus (KCN) is an ophthalmic disorder characterized by corneal ectasia with 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 estimated[1] 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]

Regardless of the trigger, the metabolic activity in the cornea is altered, resulting in biochemical instability and tissue loss[1] with the histopathological characteristics of corneal stroma thinning, tears in the Bowman's layer, and iron deposition in the epithelial basal layers.[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] Moreover, a reduction in the expression of decorin, lumin, biglycan and keratocan accompanied by an increase in abnormally configured proteoglycans has been observed,[1,4] which may contribute to the slippage of the lamellae.[1]

b) Keratoconus and Inflammation

Although the typical clinical presentation of KCN does not include macroscopic inflammatory signs or symptoms (corneal oedema, redness, pain, or intraocular inflammation), current evidence suggests that several inflammatory pathways contribute to corneal damage.[3,7] Pro-inflammatory 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] Contact lens wear leading to corneal trauma and induction of proinflammatory cytokines 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 keratoconic 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, tumour necrosis factor (TNF)-α, transforming growth factor (TGF)-β, and chemokine ligand 5 (CCL5). The interplay between these mediators is summarised in Fig. 1.

Previous efforts have been made to use pro-inflammatory cytokines 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

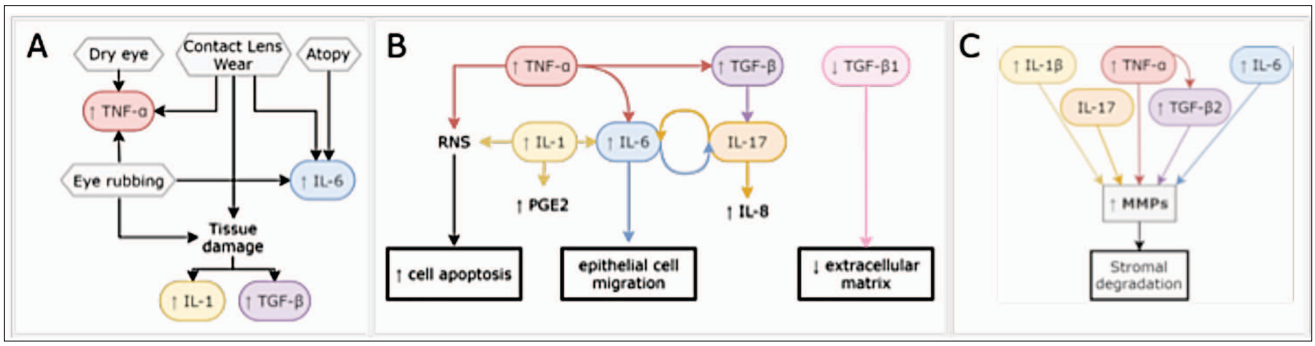


Figure 1. **Inflammatory mediators in Keratoconus. 1A.** Etiological factors such as dry eye disease[3], contact lens wear6 and eye-rubbing[3,7] are associated with increased production of TNF-α. Similarly, eye-rubbing[3,7], contact lens wear[3,6] and atopy[3] have been shown to elevate the expression of IL-6 and corneal injury in general leads to an increase in IL-1 and TGF-β levels[3]. **1B.** TNF-α could have a role in the pathogenesis of KCN by synergistically acting with IL-1 to increase cell apoptosis through the formation of reactive nitrogen species (RNS) and therefore contributing to corneal thinning. Together with IL-1 and IL-17, TNF-α is also responsible for upregulating the expression of IL-6, which promotes epithelial cell migration and IL-17 expression. IL-17 expression is also stimulated by TGF-β and results in a rise in IL-8[3]. There is evidence of reduced expression of TGF-β1 in KCN. In normal conditions, TGF- β1 promotes myofibroblast differentiation and extracellular matrix secretion. In KCN this pathway could be hindered and thus be responsible for corneal fibrosis and scar formation in severe forms of the disease[3]. **1C.** MMP expression is stimulated by IL-1β, IL-6, IL-17, TNF-α, and TGF-β2, resulting in degradation of the corneal stroma and alterations in collagen distribution, with corneal thinning.

IL-, interleukin. MMP – matrix metalloproteinases. PGE2, prostaglandin E2. RNS, reactive nitrogen species. TGF-, tumour growth factor. TNF, tumour necrosis factor.

patients and found that the level of IL-13 in combination with that of nerve growth factor (NGF) could predict the progression of KCN with 100% specificity and 80% sensitivity.[10] Although more research is required, these results highlight the potential of inflammatory mediators as useful biomarkers in the management of KCN.

c) Cross-linking

Keratoconus is one of the most common indications for keratoplasty.[1,11] However, the need for this procedure severely decreased with the advent of collagen cross-linking, 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.[13] In 2003, Wollensak *et al* developed the Dresden protocol,[14] which is currently the most widely accepted procedure.[12] It consists of applying a 0.1% riboflavin solution to the affected cornea after epithelium removal for 30 minutes. Then, an 8 mm area of the central cornea is exposed to UV-A at a wavelength of 370 nm and an irradiance of 3 mW/cm^2 (total dose of 5.4 J/cm2) for 30 minutes, while riboflavin is reapplied at a 5-minute interval.[12,14] This achieves biomechanical stiffening of the cornea and stops disease progression.[11,15]

This effect is obtained by photopolymerization,[16] 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.[12,16] 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.[16] Furthermore, UV radiation reaches a small penetration depth in the cornea, hence the need for a photosensitizer such as riboflavin.[13]

Based on the Bunsen-Roscoe law of reciprocity, which states that the same photochemical effect can be achieved by increasing the irradiation intensity while decreasing the illumination time, accelerated cross-linking (A-CXL) protocols (Fig. 2) have been developed.[12,16-18] These vary in irradiation time and intensity but keep a cumulative dose of 5.4 J/

Figure 2. Epithelium-off accelerated corneal collagen cross-linking **2A.** Phototherapeutic keratectomy followed by photorefractive keratectomy. **2B.** Riboflavin instillation. **2C.** UV-A irradiation.

cm^2 or, more recently, 7.2 J/cm^2.[12] Hammer *et al* have found, however, that the stiffening effect is reduced with higher radiation intensity / lower irradiation times.[12,19] Nonetheless, a recent meta-analysis has found the Dresden protocol and the accelerated protocols to be comparable in terms of results and safety.[18]

To minimize the complications of CXL procedures like corneal haze and infectious keratitis, mostly related to epithelial removal, transepithelial cross-linking protocols were developed ("epithelium-on" CXL in contrast with the standard "epithelium-off" CXL).[17] Because of the hydrophilic nature of riboflavin, it is difficult for it to penetrate through the lipophilic epithelium, and its diffusion is limited by the tight junctions.[16,17] For this reason, techniques to increase the diffusion of riboflavin across the epithelium were developed, such as changing its physicochemical properties, mechanically disrupting the corneal epithelium, or greatly increasing the duration of application.[17] However, the available evidence is insufficient to conclude that transepithelial CXL is as effective as "epithelium-off" CXL.[16]

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.[20] Efforts to use corneal pachymetry, keratometry and patient factors (age, gender and visual acuity) as predictors of success have yielded inconsistent results.[20]

B) OBJECTIVES

We hypothesize that the heterogeneity in therapeutic outcomes may derive from the variability in the inflammatory microenvironment of the patients' corneas.

The goal of this study is therefore to evaluate the differences in the tear film profile of patients who undergo corneal cross-linking and to relate them to the outcomes of the procedures.

On a larger scale, this project aims to broaden the knowledge of KCN and of CXL as the gold-standard treatment for KCN. Furthermore, it has the objective of contributing to the personalization and optimization of CXL procedures.

METHODOLOGY

A) ETHICS AND PRIVACY

This monocentric study will adhere to the tenets of the Declaration of Helsinki and has been approved by the Ethics Committee of the Centro Hospitalar e Universitário de Coimbra (CHUC). The principles established by the Clinical Trials Regulation (EU) 536/2014 and the General Data Protection Regulation (EU) 2016/679 will be followed.

Informed unambiguous consent for participation and for the processing of personal data must be given if an individual is to enrol in this study. No minors or other individuals who are incapable of giving consent will be recruited.

Subjects' data will be registered in case report forms and data of personal and medical nature will be registered separately. Only relevant information will be collected, namely:

- Test and Control Group Participants– obtained through patient inquiry:
  - Personal Data (Identification) – Full Name, Sex, Date of Birth, Preferred Contact Method;
- Test Group Participants Only
  - Special Personal Data (Health Data)
    - Obtained through patient inquiry:
      - Personal and family history of ophthalmic disease;
      - History of present illness (KCN);
      - Personal history of atopy and autoimmune or systemic inflammatory disease;
      - Eye rubbing frequency;
    - History of contact lens use;
      - Obtained through consultation of medical records:
      - Best corrected visual acuity prior to and 6 months after CXL;
      - Corneal tomography data (from prior to and from 6 months after CXL) – thinnest corneal thickness (TCT), steepest corneal curvature (Kmax).

All collected data will be coded (pseudonymised) by the investigators and the key to the coded data will be eliminated after a maximum period of 5 years after the end of this project.

It should be noted that the created code will not make use of the first letters of the participants' names, their birthdates or any other data that could allow easy decryption of the subjects' identities.

The processed data will be documented in a database which will only be accessible to the investigators.

B) RESEARCH DESIGN

This will be a prospective, non-randomized, comparative clinical study with a minimum follow-up period of 6 months, which will follow the plan below:

1. Participant selection according to the established criteria (study and control groups).
2. Patient interview and consultation of medical records to register the most recent Corneal Tomography indexes (Kmax and TCT) and BCVA value (study group only).
3. First tear fluid collection (study and control groups).
4. CXL (study group only).
5. Second tear fluid collection, 6 months after the first collection (study and control groups).
6. Consultation of medical records to register the Corneal Tomography indexes (Kmax and TCT) and BCVA value 6 months after CXL (study group only).
7. Total protein and cytokine quantification (study and control groups).
8. Statistical Analysis.

C) PARTICIPANTS

The test group will consist of 30 patients who have been diagnosed with KCN and who have scheduled CXL procedures in the department of Ophthalmology of CHUC. Ex-

clusion criteria will be as follows:

- Past individual history of ocular surgery;
- Individual history of autoimmune or systemic inflammatory disease;
- Individual history of dry eye disease.

The control group will be comprised of subjects who will be matched for sex and, tentatively, age with the study group participants. 30 healthy volunteers will be recruited with the following exclusion criteria:

- Age under 18 or over 45 years;
- Past individual history of ophthalmological, autoimmune, or systemic inflammatory disease.

D) SPECIFIC TASKS

a) Interview

A short interview will be conducted in order to obtain information from the test group participants regarding the following aspects:

- Eye rubbing frequency: Never, every day, or every week;
- Past individual history of atopy, specifically history of atopic dermatitis, atopic rhinitis, allergic asthma, or other known atopic disorders;
- Past individual history of allergies, specifically history of food, drug, or other known allergies;
- History of contact lens use – currently or in the past.

Data from the patients' last corneal tomography prior to corneal cross-linking and from the one performed 6 months after surgery will also be recorded. This includes the thinnest corneal thickness (TCT), maximum anterior sagittal curvature (K max) and best corrected visual acuity (BCVA).

b) Tear Collection

Disease-specific alterations have been documented in the tear fluid of KCN patients.[3] This may either be a consequence of the corneal alterations that are characteristic of KCN or could represent an etiological role in the disease.[7]

Although the composition of the tear fluid can also reflect alterations of the lacrimal gland or the conjunctiva, and although its levels can vary according to the collection method,[3] the ease of access to patients' tears and their role in the corneal microenvironment make them a feasible and practical biomarker source.

Alternatives to tear fluid collection include the more invasive and rarely feasible anterior chamber tap or corneal tissue harvest.[3] It should also be noted that the differences in the levels of inflammatory mediators found in the ocular environment of KCN patients are not reflected in these patients' sera, as demonstrated by Jun *et al*.[21]

Tear fluid will be collected twice, with an interval of 6 months in between, using Schirmer strips.

Diagnostic (Schirmer) strips are an indirect method of tear fluid collection in which the elution of proteins from the filter matrix is incomplete and non-uniform.[22,23] Furthermore, care should be had to avoid irritation of the ocular surface and subsequent reflexive tearing, as stimulated tears contain a higher proportion of the lacrimal gland secretion[22,24] and are not equivalent to non-stimulated tears.[22,25,26]

Still, the use of Schirmer strips is advantageous as these are readily available in any Ophthalmology department and their application is simple and non-invasive. Besides, the volume yield using this method is larger compared to direct methods such as microcapillary tubes or micropipettes, which are more difficult to perform and often require stimulation or the instillation of saline into the cul-de-sac.[22] The diluting effect of saline suppresses the concentration of the most abundant cytokines in tears and may result in concentrations of other cytokines below the detection limit. Moreover, these methods may cause discomfort for the subjects and are interrupted by blinking.[22]

Thus, tear samples will be collected by placing a Schirmer strip in the individuals' inferior cul-de-sac without any anaesthetic (Fig. 3A) and removing it after 5 minutes, during which the subjects will have their eyes closed (Fig. 3B). The strips will then be placed in sterile 2 mL Eppendorf microcentrifuge tubes (Fig. 3C) and stored on ice for a maximum of 3 hours for processing within the same day.[27]

c) Tear Processing and Analysis

In order to elute the tears from the strips, these will be placed in soaking buffer[28] (100 µL of 0.9% NaCl) (Fig. 3D) and incubated on an orbital shaker at room temperature for 1 hour (Fig. 3E). For recovery of residual liquid, the strip will then be transferred with clean tweezers into a perforated 0.5 mL microcentrifuge tube, which will be placed inside a 2 mL microcentrifuge tube and centrifuged at 4ºC and 10.000 relative centrifugal force (RCF) for 5 minutes[23,29] (Fig. 3F).

The eluted and recovered tears will then be split into one aliquot of 10 µL and four aliquots of 50 µL and frozen at -80ºC for posterior processing.

d) Total Protein Quantification

To determine the total protein content of the sample, the bicinchoninic acid (BCA) total protein assay (Thermo Fisher Scientific, Pierce, Rockford, IL, USA) will be used.

This assay is based on the reduction of Cu^{2+} to Cu^{1+} by protein in an alkaline environment; Cu^{1+}, in turn, forms an intense purple complex with the bicinchoninic acid. The intensity color is proportional to the protein concentration and can be measured colourimetrically.[30]

The manufacturer's protocol[31] will be followed, with minor modifications (Fig. 3G-I):

1. An albumin standard - bovine serum albumin (BSA) will be sequentially diluted in Milli- Q® ultrapure water to create a standard concentration curve (Subtable 1A).
2. NaCl 0.9% will be diluted in MilliQ® ultrapure water in a 1:9 proportion (50 µL of NaCl 0.9% in 450 µL of H2O). Similarly, tear samples ("unknowns") will be diluted in MilliQ® ultrapure water in a 1:9 proportion (6 µL of sample in 54 µL of H2O).

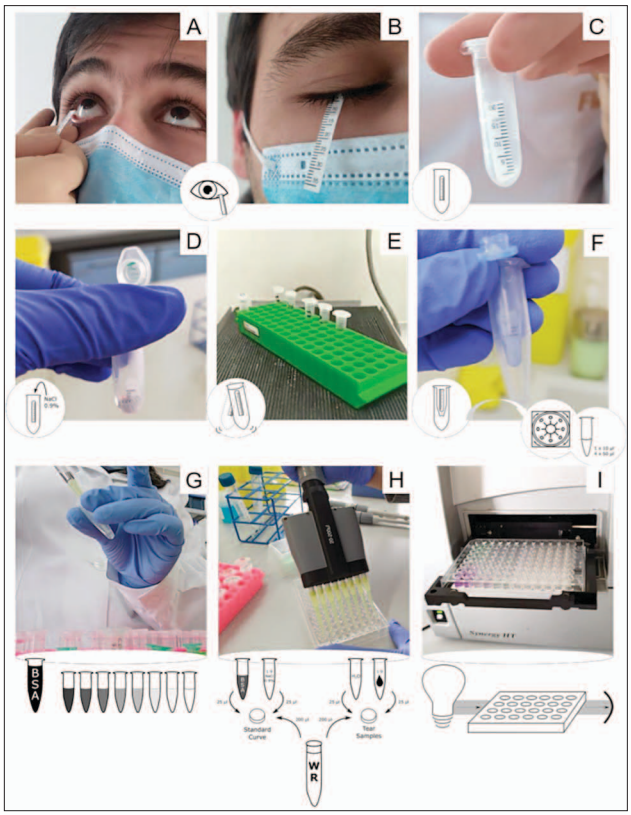


Figure 3. **Tear fluid collection, processing and analysis. A-C:** tear fluid collection using Schirmer strips. **D-F:** tear fluid elution. **G-I:** total protein quantification.

3. Working Reagent (WR) will be prepared by mixing 50 parts of reagent "A" and 1 part of reagent "B" from the PierceTM BCA Protein Assay Kit. The amount of Working Reagent necessary is calculated according to the following formula:
(# standards + # unknowns) × (# replicates) × (volume of WR per sample) = total volume WR required ⇔ (8+16) × 2 × 200 µL= 900 µL
4. A flat-bottom 96-well microplate will be filled with the preparations from the dilution scheme in Subtable 1B.

e) Cytokine Quantification

The levels of several cytokines will be measured using the commercially available Th1/Th2/Th9/Th17 Cytokine 18-Plex Human ProcartaPlexTM Panel (Thermo Fisher Scientific, Pierce, Rockford, IL, USA). The protocol provided by the manufacturer will be followed.

This method is similar to a sandwich ELISA, in which two antibodies bind to the target protein in order to measure it. However, the ProcartaPlexTM assays use Luminex® beads attached to the protein-specific capture antibodies, which would be adsorbed to the microplate in a sandwich ELISA. The beads are read using Luminex® xMAP® detection systems, which are composed of two lasers. One of the lasers identifies the signature of each bead (based on its unique proportions of red and infrared fluorophores). The other quantifies the amount of fluorescence provided by the biotinylated detection antibody that is bound to streptavidin–R- phycoerythrin, which is proportional to the amount of analyte in the sample.[23,32]

f) Corneal Collagen Cross-Linking

Accelerated epithelium-off collagen cross-linking will be performed according to the Athens protocol[33,34]:

1. Epithelium removal – Phototherapeutic keratectomy at a 7.0 mm zone and at a depth of 50 µm (WaveLight® ALLEGRETTO WAVE® Excimer Laser System, Alcon Inc.) – Fig. 2A;
2. Partial topography-guided photorefractive keratectomy with an effective optical zone diameter of 5.5 mm and 70% treatment of cylinder and sphere (WaveLight® ALLEGRETTO WAVE® Excimer Laser System, Alcon Inc.);
3. Application of 0.02% mitomycin C solution onto the de-epithelialized surface for 20 seconds;
4. CXL
   i. Instillation of 0.1% riboflavin (VibeX Rapid, Avedro Inc.) onto the corneal surface every 2 minutes with a total soaking time of 10 minutes – Fig. 2B.
   ii. UVA irradiation for 10 minutes (KXL I System,

Table 1. BCA Total Protein Assay. Subtable A – Dilution Scheme for Standard Curve. Subtable B – Dilution Scheme for Microplate Procedure.

Subtable 1A – Dilution Scheme for Standard Curve			
Vial	**Final [BSA] (µg/mL)**	**Volume and Source of BSA (µL)**	**Volume of Diluent - H2O (µL)**
A	800	80 from Stock (BSA 2mg/mL)	120
B	400	100 from vial A	100
C	200	100 from vial B	100
D	100	100 from vial C	100
E	50	100 from vial D	100
F	25	100 from vial E	100
G	12,5	100 from vial F	100

Subtable 1B – Dilution Scheme for Microplate Procedure					
	Volume of diluted BSA (µL)	**Volume of diluted NaCl (µL)**	**Volume of sample (µL)**	**Volume of H2O (µL)**	**Volume of Working Reagent (µL)**
Blank	25 (Vial "Blank" = H2O)	25	0	0	200
Standard Curve (Vials A-G)	25	25	0	0	200
Samples	0	0	25	25	200

BSA – bovine serum

Avedro Inc.): exposure of continuous UVA 365-nm light at an irradiance of 10 mW/cm² (total energy 6 J/cm²) – Fig. 2C.

g) Statistical Analysis

The studied variables can be described as follows:

- Numerical
  - discrete: age
  - continuous: total protein content, cytokine concentration, thinnest corneal thickness (TCT), steepest corneal curvature (Kmax), best corrected visual acuity (BCVA, in logMAR)
- Categorical
  - Nominal
  - Sex (Female, Male, Other / Would Rather Not Say)
  - Binary:
    - o Atopy (Yes or No: 'Yes' is considered if one or more of the following is present: atopic dermatitis, atopic rhinitis, allergic asthma, or other known atopic disorder)
    - o Allergy (Yes or No: 'Yes' is considered if there is a past individual history of allergies, specifically a history of food, drug, or other known allergies)
    - o Contact Lens Use (Yes or No: 'Yes' is considered if the patient has a history of contact lens use at the time of the interview or in the past)
- Ordinal: eye rubbing habits (Never; Daily – every day of every week; Weekly – every week, but not every day)

The following groups and subgroups will be compared:

- Control Group (subdivided into Control Group at t0 and Control Group at t1 - 6 months after);
- Study Group - subdivided according to:
  - Timeline: t0 and t1 (6 months after);
  - Atopy status, allergy status, eye-rubbing habits, contact lens use history.

The data will be summarized using descriptive statistics and analyzed with inferential statistic tests.

The Mann-Whitney nonparametric test will be used to test the statistical difference between groups for the following variables:

- Total protein content
- Total protein content variation (t1-t0)
- Cytokine concentration
- Cytokine concentration variation (t1-t0) – for each cytokine
- For each corneal tomography index and for BCVA (appliable only to the Study group and subgroups)

Subgroup analysis will be performed using the Kruskal-Wallis nonparametric test for the same variables.

The Spearman's rank test will be used to assess correlation:

- Between pairs of cytokines;
- Between each cytokine and each corneal tomography and BCVA.

E) BUDGET

We present our budget (Table 2). Some tasks are already part of the patients' diagnostic and therapeutic routine. The costs pertaining to the outpatient ophthalmology appointments (diagnosis, visual acuity determination and follow-up), the corneal tomographies and cross-linking procedures are not contemplated here.

PRELIMINARY RESULTS AND DISCUSSION

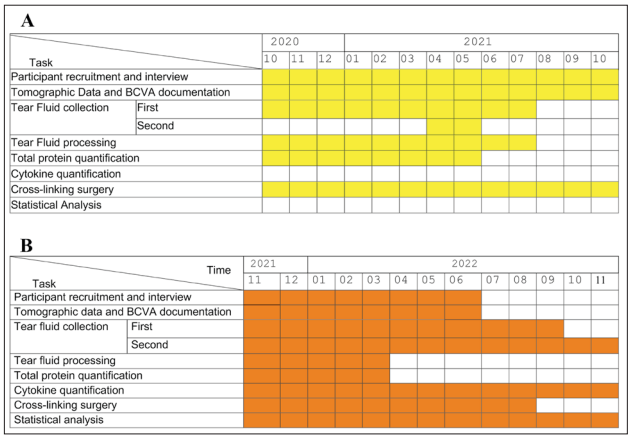
A) TIMELINE

We present our timeline (Table 3) - our previous work, work in progress and planned work.

Table 2. Budget.

Study cost*			Total (€)
Database support and electronic case report forms elaboration			1590 €
Data analysis and biostatistical support			1050
Manuscript preparation and submission fees			850 €
Insurance for participants			970 €
Congress abstract and travel costs			1385 €
Devices / equipment cost (A)			4010 €
Total site budget			**9855 €**
Devices / Equipment Cost (A)			
Tear fluid collection	Schirmer strips		30 €
	surgical gloves		30 €
	Laboratory material		200 €
Tear processing and analysis	Equipment rental		200 €
	Total protein quantification	Pierce™ BCA protein assay kit (Thermo Fisher Scientific)	155 €
	cytokine concentration quantification	Th1/Th2/Th9/Th17 Cytokine 18-Plex Human ProcartaPlex™ Panel (Thermo Fisher Scientific)	3 395 €
			Total 4010 €

Table 3. Timeline. A, in yellow, represents our previous work and work in progress until November 2021. B, in orange, represents the planned work.



BCVA – Best Corrected Visual Acuity.

B) PRELIMINARY WORK

A significant portion of this project has been executed from March of 2020 to March of 2021. This investigation was affected by the SARS-CoV-2 pandemic, with an impact on the number of surgeries, outpatient appointments, medical tests performed and access to laboratories. This resulted in fewer chances to enrol patients and to obtain data.

So far twenty patients and eight control subjects have been enrolled in the study and the first collection of tears has been carried out. Cross-linking surgeries for the twenty patients were performed from October 2020 to October 2021. The tear fluid from these samples has been eluted from the Schirmer strips and the total protein content has been calculated for 8 patients and for the controls. The data regarding corneal tomography indexes and best corrected visual acuity prior to CXL has also been recorded.

The mean total protein concentration was 90.95 μg/μL for the eight patients group and 62.26 μg/μL for the control group, not a statistically significant difference (p>0.05); we concluded that the groups are comparable and the cytokine quantification feasible.

We keep doing all the possible tasks weekly and earning fundus to perform the cytokine quantification.

CONCLUSION

Keratoconus is a progressive disorder affecting patients from an early age. Current data suggests that its global prevalence is larger than 1:2000 and that therefore it is not a rare disease anymore.[4] KCN impairs quality of life early on due to its clinical consequences (myopia, irregular astigmatism and corneal scarring). As it affects young patients, it impairs quality of life throughout the entire life. It can be debilitatingly severe, with up to 20% of patients requiring a corneal transplant.

Cross-linking has been a major development in the management of KCN, which was previously one of the main indications for keratoplasty.[1,11] Although this approach has been shown to be successful in halting the progression of the disease, it is not equally effective in all cases, with a recent study reporting a procedure failure rate as high as 19.87%.[20]

CXL affects the cornea not only mechanically, but also molecularly. It leads to a decrease in the concentration of proinflammatory biomarkers in the corneal microenvironment,[35-39] therefore restoring corneal homeostasis.[37] Our results are expected to reinforce these findings.

Efforts have been made to identify the factors involved in outcome variability, but no definite conclusion has been reached. This project is innovative and ambitious, since it aims to answer this question by assessing whether the altered inflammatory environment of KCN corneas is responsible for the inconsistent results of CXL, a question still unanswered, by establishing the correlation between laboratory and clinical-topographic parameters, finding the missing link between surgical outcomes and the microenvironmental milieu of the ocular surface.

This will allow for the optimization of the CXL procedure, with fewer expected complications and KCN progression. Specifically, briefly, we may use the information about each patient tear profile to tailor the topical anti-inflammatory treatment in the post-operative period. This project will lead us to perform the most appropriate treatment for each patient, personalizing not only the optical component of the treatment, but also the treatment of the local inflammatory environment. This will increase patients' quality of life and reduce disease-associated costs.

This work is essential to make great strides towards the treatment of the patient and not only of the disease. We have technologically capable resources and a translational team, willing to work towards increasing our patients' quality of life, reducing disease-associated costs and the burden caused by more invasive strategies.

PRESENTATIONS / APRESENTAÇÕES

Part of this study will be presented at the 64th Portuguese Congress of Ophthalmology in 2021.

Parte deste estudo será apresentado no 64º Congresso Português de Oftalmologia em 2021.

Parte deste estudo foi apresentado no Prémio MSD de Investigação em Saúde 2021.

CONTRIBUTORSHIP STATEMENT / DECLARAÇÃO DE CONTRIBUIÇÃO:

MA, CC and AR: Responsible for gathering data, presenting results and creating the manuscript.

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

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

All authors read and approved the final manuscript.

RESPONSABILIDADES ÉTICAS

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

Fontes de Financiamento: Parte deste estudo foi apresentado no Prémio MSD de Investigação em Saúde 2021, sendo selecionado para a sessão de apresentação final e tendo arrecadado, por agora, 1500 euros.

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 pelos responsáveis da Comissão de Investigação Clínica e Ética 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: Part of this study was presented at the Prémio MSD de Investigação em Saúde 2021, being selected to the final presentation session and earned, till now, 1500 euros.

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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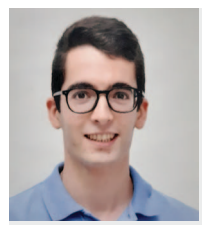

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