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MARIANA JACINTO ARAÚJO

Mediadores no Filme Lacrimal Após Cross-Linking da Córnea

PROJETO DE INVESTIGAÇÃO

ÁREA CIENTÍFICA DE OFTALMOLOGIA

Trabalho realizado sob a orientação de:

PROFESSORA DOUTORA ANDREIA DE FARIA MARTINS ROSA

DR. JOÃO MANUEL BEIRÃO CARDOSO RIBEIRO QUADRADO GIL

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Tear Film Mediators After Cross-Linking

Mariana Jacinto Araújo △,

Dr. João Manuel Beirão Cardoso Ribeiro Quadrado Gil, MD, MSc △■○,

Prof. Andreia de Faria Martins Rosa, MD, PhD△■○

△ Faculty of Medicine, University of Coimbra, Portugal

■ Clinical Academic Center of Coimbra, Portugal

○ Department of Ophthalmology, Centro Hospitalar e Universitário de Coimbra, Portugal

Correspondence:

Andreia de Faria Martins Rosa

Centro Hospitalar e Universitário de Coimbra - Praceta Prof. Mota Pinto, 3000-075 Coimbra, Portugal

andreia.rosa@gmail.com

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Resumo

Contexto

O queratocone é uma patologia oftalmológica caracterizada por ectasia da córnea com protusão em formato cónico que clinicamente leva a astigmatismo irregular, miopia e perda de acuidade visual dificilmente reversível.

Atualmente o seu tratamento passa pela realização de cirurgia de *cross-linking*, que consiste na aplicação de riboflavina e uso de radiação ultravioleta A com o intuito de aumentar a rigidez biomecânica da córnea e parar a progressão da doença. No entanto, os resultados pós-operatórios variam significativamente entre pacientes. Embora fatores como a idade do doente e a curvatura ou localização do cone tenham sido propostos como importantes, o seu verdadeiro impacto é controverso, e a explicação para esta variabilidade permanece, em grande parte, por descobrir.

Objetivos

Pretende-se com este estudo procurar marcadores que expliquem a variabilidade nos resultados da cirurgia de *cross-linking,* investigando a correlação entre o microambiente inflamatório da córnea dos pacientes e estes resultados.

Colocamos a hipótese de que resultados pós-operatórios mais positivos estarão associados a uma redução nos níveis de citocinas inflamatórias no fluido lacrimal.

O propósito final é a otimização dos resultados pós-operatórios, de acordo com o microambiente inflamatório da córnea.

Métodos

Através de entrevista clínica, recolher-se-ão dados sobre os antecedentes patológicos e fisiológicos dos pacientes. Serão também registados dados tomográficos das córneas dos mesmos e relativos à sua acuidade visual.

Serão feitas duas colheitas de fluido lacrimal dos pacientes e dos controlos através de tiras de Schirmer, separadas por um período de 6 meses. Será depois quantificado o conteúdo proteico total das amostras, bem como a concentração de citocinas usando tecnologia Multiplex.

A análise estatística será feita para avaliar as diferenças entre as amostras e a correlação entre a concentração de citocinas e os parâmetros tomográficos e de acuidade visual dentro de cada grupo e subgrupo.

Aplicações

A identificação de fatores correlacionados com os resultados terapêuticos permitirá o ajustamento e personalização da terapêutica do queratocone, minimizando os casos de insucesso terapêutico e a necessidade de abordagens mais invasivas, e melhorando a qualidade de vida destes doentes.

Palavras-chave

Queratocone; Cross-linking; Citocinas; Mediadores da Inflamação; Lágrimas

Abstract

Background

Keratoconus is an ophthalmologic disease, which is characterized by corneal ectasia with a conic-shaped protrusion. Clinically this leads to irregular astigmatism, myopia, and almost irreversible loss of visual acuity.

Currently, the gold-standard treatment of progressive keratoconus consists in corneal collagen cross-linking, a surgical procedure in which riboflavin and ultraviolet-A radiation are used to increase the corneal biomechanical rigidity and arrest disease progression. However, post-operatory results vary significantly among patients. Although it has been suggested that corneal thickness, cone location and the patient's age are important factors, their true contribution is controversial. The reason for this variability thus remains largely obscure.

Objectives

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

Thus, 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.

Methods

The patients will be interviewed to assess their past medical history and relevant habits. Their corneal tomographic and best corrected visual acuity data will also be registered.

The patients' tear film will be sampled twice 6 months apart using Schirmer strips. The samples will then be analysed for their total protein content and cytokine concentration using Multiplex technology.

Statistical analysis will be carried out to assess sample differences and the correlation between cytokine concentration and tomographic indexes and visual acuity values for each group and subgroup.

Applications

By identifying biomarkers that correlate to therapeutic outcomes, this project may enable the personalization and adaptation of KCN treatment. This, in turn, could result in fewer cases of treatment failure, reducing the need for more invasive techniques, and improving the quality of life of KCN patients.

Keywords

Keratoconus; Corneal cross-linking; Cytokines; Inflammation Mediators; Tears

Introduction

State of the Art

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 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). 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).

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). For example, chronic 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 Figure 1.

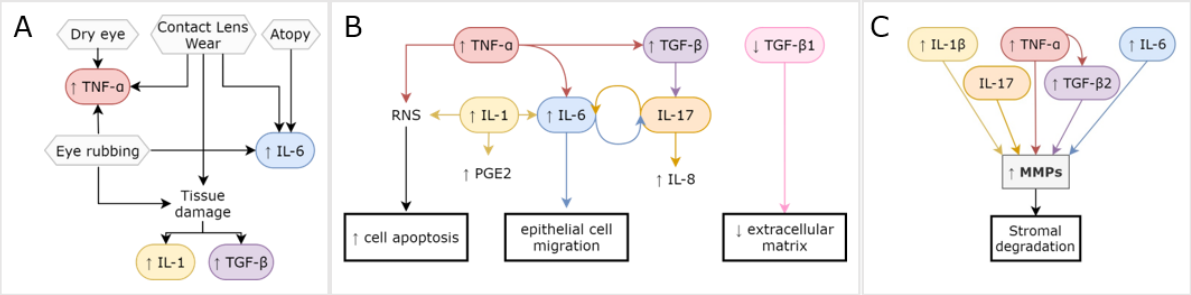

Figure 1 – Inflammatory mediators in Keratoconus.

IL-, Interleukin. MMP – Matrix Metalloproteinases. PGE2, Prostaglandin E2. RNS, Reactive Nitrogen Species. TGF-, Tumour Growth Factor. TNF, Tumour Necrosis Factor.

1A. Etiological factors such as dry eye disease (3), contact lens wear (6) 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.

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 K_2 (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 (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.

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).

This protocol 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/cm^2) for another 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 alone 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 (Figure 2) have been

developed (12,16–18). These vary in irradiation time and intensity but keep a cumulative dose of 5.4 J/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 such as corneal haze and infectious keratitis, which are mostly related to epithelial removal, efforts have been made to develop transepithelial cross-linking protocols ("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 of the cornea, and its diffusion is limited by the epithelial tight junctions (16,17). For this reason, several techniques were developed to increase the diffusion of vitamin B2 across the epithelium, such as changing its physicochemical properties, mechanically disrupting the corneal epithelium, or greatly increasing the duration of application of this substance (17). However, the available evidence is presently 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). In fact, efforts to use corneal pachymetry, keratometry and patient factors such as age, gender, and visual acuity as predictors of success have yielded inconsistent results (20).

Figure 2 – Epithelium-off Accelerated Corneal Collagen Cross-linking

2A. Phototherapeutic keratectomy followed by Photorefractive keratectomy.

2B. Riboflavin instillation.

2C. UV-A irradiation.

Preliminary Work

A significant portion of this project has been executed from March of 2020 to March of 2021. The completion of this investigation was affected by the onset of the SARS-CoV-2 pandemic, which had an impact on the number of crosslinking surgeries, outpatient appointments, and medical tests performed as well as on the access to laboratories. This resulted in fewer chances to enrol patients and to obtain data regarding them. This study is also dependent on the completion of other projects, which were likewise affected and delayed by the pandemic.

So far eight 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 eight patients were performed from October 2020 to January 2021. The tear fluid from these samples has been eluted from the Schirmer strips and their total protein content has been calculated. The data regarding corneal tomography indexes and best corrected visual acuity prior to CXL has also been recorded.

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 collagen cross-linking as the *gold-standard* treatment for KCN. Furthermore, it has the objective of contributing to the personalization and optimization of CXL procedures.

Methodology

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 in the study and for the processing of personal data must be freely 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.

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.

Participants

The study group will consist of 10 patients who have been diagnosed with KCN and who have scheduled CXL procedures in the department of Ophthalmology of CHUC. Exclusion 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. 10 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.

Specific Tasks

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).

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 (Figure 3A) and removing it after 5 minutes, during which the subjects will have their eyes closed (Figure 3B). The strips will then be placed in sterile 2 mL Eppendorf microcentrifuge tubes (Figure 3C) and stored on ice for a maximum of 3 hours for processing within the same day (27).

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) (Figure 3D) and incubated on an orbital shaker at room temperature for 1 hour (Figure 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 4º C and 10.000 Relative Centrifugal Force (RCF) for 5 minutes (23,29) (Figure 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 -80º C for posterior processing.

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 colour is proportional to the protein concentration and can be measured colourimetrically (30).

The manufacturer's protocol (31) will be followed, with minor modifications (Figure 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 H_2O). Similarly, tear samples ("unknowns") will be diluted in MilliQ® ultrapure water in a 1:9 proportion (6 µL of sample in 54 µL of H_2O).
3. Working Reagent (WR) will be prepared by mixing 50 parts of reagent "A" and 1 part of reagent "B" from the Pierce™ BCA Protein Assay Kit. The amount of Working Reagent necessary is calculated according to the following formula:

$$(\#\text{ standards} + \#\text{ unknowns}) \times (\#\text{ replicates}) \times (\text{volume of WR per sample}) = \text{total volume WR required} \Leftrightarrow (8+20) \times 2 \times 200\ \mu L = 11200\ \mu L$$

4. A flat-bottom 96-well microplate will be filled with the preparations from the dilution scheme in Subtable 1B.

Cytokine Quantification

The levels of several cytokines will be measured using the commercially available Th1/Th2/Th9/Th17 Cytokine 18-Plex Human ProcartaPlex™ 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 ProcartaPlex™ 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).

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.) – Figure 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 – Figure 2B.
    ii. UVA irradiation for 10 minutes (KXL I System, Avedro Inc.): exposure of continuous UVA 365-nm light at an irradiance of 10 mW/cm^2 (total energy 6 J/cm^2) – Figure 2C.

Table 1 – BCA Total Protein Assay

Subtable 1A – Dilution Scheme for Standard Curve

Vial	Final [BSA] (µg/mL)	Volume and Source of BSA (µL)	Volume of Diluent - H_2O (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
Blank	0	0	100

Subtable 1B – Dilution Scheme for Microplate Procedure

	Volume of diluted BSA (µL)	Volume of diluted NaCl (µL)	Volume of sample (µL)	Volume of H_2O (µL)	Volume of Working Reagent (µL)
Blank	25 (Vial "Blank" = H_2O)	25	0	0	200
Standard Curve (Vials A-G)	25	25	0	0	200
Samples	0	0	25	25	200

Table 1 – BCA Total Protein Assay. BSA – Bovine Serum Albumin

Subtable A – Dilution Scheme for Standard Curve.

Subtable B – Dilution Scheme for Microplate Procedure.

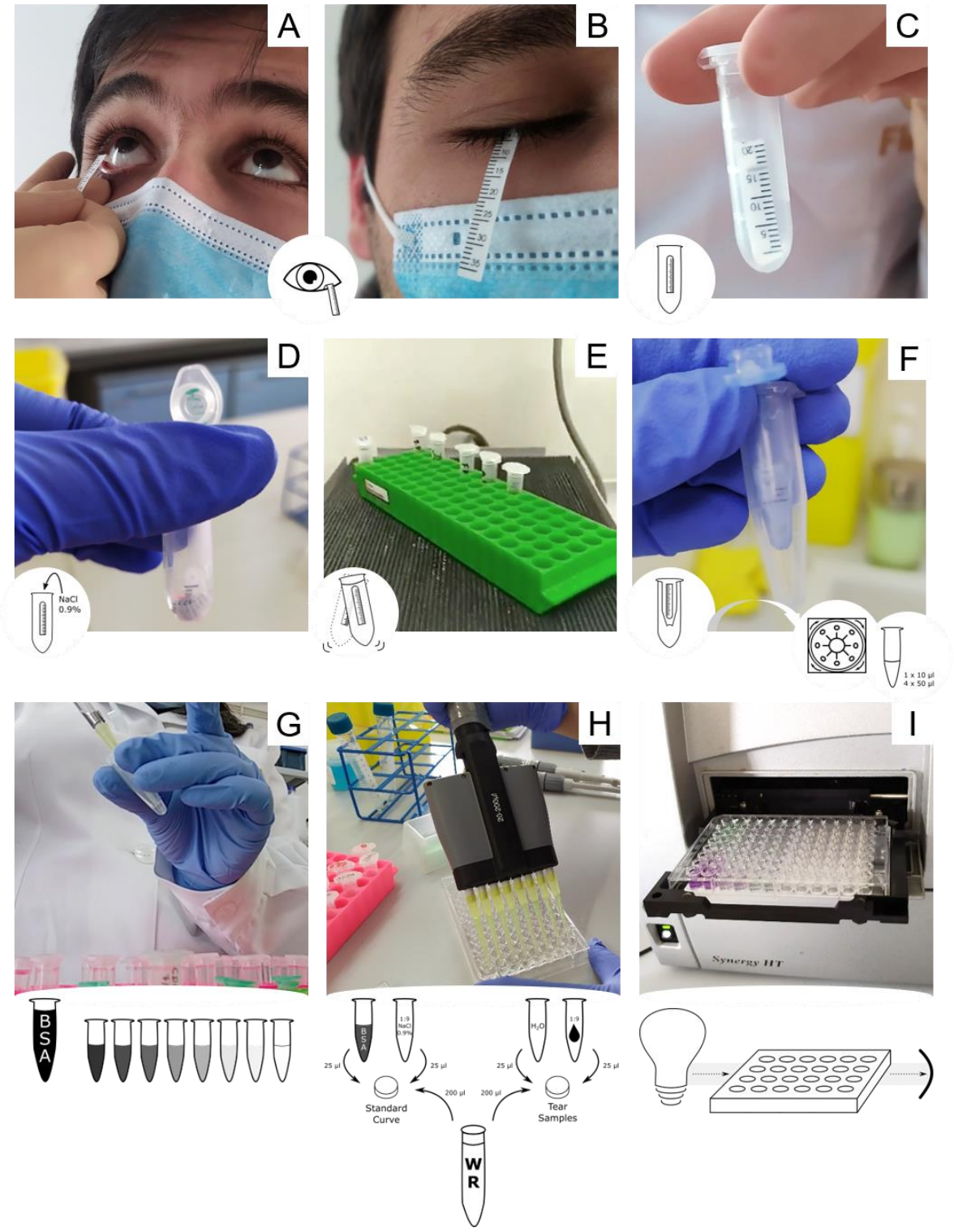

Figure 3 – Tear Fluid Collection, Processing, and Analysis.

A-C: Tear fluid collection using Schirmer Strips.

D-F: Tear fluid elution.

G-I: Total protein quantification.

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:
      - 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)
      - 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)
      - 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, which can be subdivided into Control Group at t0 and Control Group at t1 (6 months after);
- Study Group, which can be 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 posteriorly analysed 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.

Expected Results and Applications

Keratoconus is a progressive disorder affecting patients from an early age. Current data suggests that its global prevalence is larger than 1:2,000 and that therefore it is not a rare disease, as it was considered before (4). KCN impairs patients' quality of life early on due to its clinical consequences, including myopia, irregular astigmatism, and corneal scarring.

Corneal collagen cross-linking has been a major development in the management of KCN, which was previously one of the main indications for corneal transplant (1,11). Although this approach has been shown to be successful in halting the progression of the disease, and in reducing the costs associated with it, it is not equally effective in all cases, with a recent study reporting a procedure failure rate as high as 19.87% (20).

Efforts have been made to identify the factors involved in outcome variability, but no definite conclusion has been reached. This project aims to answer this question by assessing whether the altered inflammatory environment of KCN corneas is responsible for the inconsistent results of CXL.

Studies have shown that CXL affects the cornea not only mechanically, but also molecularly. It has been observed that CXL 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.

The demonstration of the correlation between biomarkers (namely inflammatory cytokines) and the therapeutic outcomes of CXL will allow for the optimization of the CXL

procedure, with fewer expected complications and cases of KCN progression. This, in turn, will increase patients' quality of life and reduce disease-associated costs.

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Timeline

Table 2 - Timeline													
Task \ Time		2020			2021								
		10	11	12	01	02	03	04	05	06	07	08	09	10
Participant recruitment and interview														
Tomographic Data and BCVA value documentation														
Tear Fluid collection	First													
	Second													
Tear Fluid processing														
Tear Fluid analysis														
Cross-linking surgery														
Statistical Analysis														

Table 2 – Timeline. BCVA – Best Corrected Visual Acuity.

Research Team

Principal Investigator

Name: Mariana Jacinto Araújo

Affiliation: Faculty of Medicine of the University of Coimbra

Email: mariana.araujo8@gmail.com

Mobile Phone: +351 93 938 922

Address: Rua da Liberdade, 77, 3º esq. 3020-112 Coimbra

Supervisors

Andreia Rosa, MD, PhD

- Department of Ophthalmology, Centro Hospitalar e Universitário de Coimbra (CHUC);
- Faculty of Medicine, University of Coimbra (FMUC);
- Clinical Academic Center of Coimbra (CACC);
- andreia.rosa@gmail.com

João Quadrado Gil, MD, MSc

- Department of Ophthalmology, Centro Hospitalar e Universitário de Coimbra (CHUC);
- Faculty of Medicine, University of Coimbra (FMUC);
- Clinical Academic Center of Coimbra (CACC);
- joaomggil@gmail.com

Budget and Funding

Budget

Please refer to Annex I.

The budget for this project includes costs associated with a research grant for an investigator with a master's degree, as established by the Portuguese Fundação para a Ciência e a Tecnologia. This covers 12 months of research, as described in the timeline.

Additionally, it is expected that the results derived from this study will be published in a peer-reviewed, Q1, journal. Expenses related to submission fees and travelling for presentation in congresses have thus also been considered.

The price of the material required for tear fluid collection, processing, and analysis is registered under the "Devices / Equipment Cost" section.

Finally, some tasks essential to this project are already of part of the patients' diagnostic and therapeutic routine. Therefore, the costs pertaining to the outpatient ophthalmology appointments (necessary for diagnosis, visual acuity determination and follow-up), the corneal tomographies and cross-linking procedures are not contemplated in this budget.

Funding

To cover the costs detailed in this project's budget, funding will be sought by applying for grants and awards. Some of these include:

- PhD Grant, Sociedade Portuguesa de Oftalmologia: up to 10.000€
- ESCRS Pioneer Award: 50.000 €
- ESCRS Clinical Research Award: 75.0000 €
- Prémio MSD Investigação em Saúde: 10.000 €
- Bolsa D. Manuel de Melo: 10.000 €
- Pfizer Investigator Sponsored Research: no specified limit.

Annex I - Budget

Study cost*	Total (€)
Human Resources (A)	15165,36 €
Data management and Biostatistical support	0 €
Manuscript preparation and Submission fees	1000 €
Congress Abstract and Travel costs	2500 €
Devices / Equipment cost (B)	4 010 €
Total site budget	**22325,36**

Human Resources (A)			
Research Grant (12 months)	Average **Monthly** Costs	Monthly Grant	1104,64
		Social and Work Accident Insurance	159,14
		Monthly Total	1263,78
		Total	**15165,36**

Devices / Equipment Cost (B)			
Tear Fluid Collection	Schirmer Strips		30 €
	Surgical Gloves		30 €
Tear Processing and Analysis	Laboratory Material		200 €
	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	**4 010 €**

Annex II - "Regulamento das Bolsas de Doutoramento
Sociedade Portuguesa de Oftalmologia"

Regulamento das Bolsa de Doutoramento

Preâmbulo

A investigação clínica é uma necessidade da Oftalmologia Portuguesa e, simultaneamente, dos sócios da Sociedade Portuguesa de Oftalmologia (SPO).

A investigação clínica exige meios materiais e financeiros que ultrapassam as disponibilidades habituais das instituições, especialmente no âmbito de uma tese de Doutoramento.

Com a criação desta bolsa, a SPO pretende associar-se ao financiamento parcial destes projetos, tentando contribuir para o desenvolvimento da Oftalmologia Portuguesa criando um incentivo ao doutoramento de sócios da SPO.

I. Objectivo

Apoio à investigação científica nas áreas afins à Oftalmologia, englobada numa tese de Doutoramento.

II. Modo

Financiamento total ou parcial de um projecto original de investigação científica, a decorrer total ou parcialmente no território nacional, no decurso de uma tese de Doutoramento.

III. Candidatos

1. O Investigador principal seja membro da SPO, com quotas actualizadas;

2. A instituição responsável (Hospital, Universidade ou outra) terá de estar sedeada em Portugal, podendo o doutoramento ou a investigação científica, ser parcialmente realizado fora do território nacional.

IV. Processo de candidatura

1. Boletim de candidatura

2. Projecto original estruturado numa tese de Doutoramento:

a) Introdução

b) Objectivos

c) Metodologia

d) Previsão do leque de resultados e sua aplicabilidade

e) Bibliografia

f) Cronograma de tarefas e conclusão do trabalho

g) Identificação do Investigador principal

h) Elementos da Equipa de investigação (nome, instituição afiliada, grau académico e email)

i) Plano de trabalho e plano de despesas do projecto

j) Fontes de financiamento asseguradas ou previstas

3. Documentos a apresentar:

a) Curriculum vitae resumido do investigador principal.

b) Documento do Director de Serviço ou Departamento a autorizar a execução do trabalho.

c) Referência do conjunto de artigos ou comunicação científicas da equipa de investigação, sobre o tema ou temas do projecto, publicados ou apresentados nos últimos cinco anos.

d) Documento da Comissão de Ética da instituição

V. Júri

1. Composição

a) Presidente: Presidente da Direcção da SPO.

b) Vogais: 4 elementos a designar pela direcção.

2. Metodologia de avaliação e classificação

a) A estabelecer pelo júri, no respeito pelo presente regulamento.

b) A reunião formal com presença física dos membros do júri poderá ser substituída por metodologias alternativas, a propor pelo presidente.

VI. Prazos

1. As candidaturas serão apresentadas até 31 de Outubro de cada ano.

2. As decisões do Júri serão comunicadas pela Direcção no Congresso Português de Oftalmologia.

3. O projecto deverá iniciar-se durante o início do ano seguinte à divulgação dos resultados.

4. O Investigador Principal comunicará a data de início do projecto à Direcção da SPO.

5. O Investigador Principal obriga-se à apresentação de relatórios semestrais.

6. O relatório final deverá ser apresentado até 36 meses após o início do projecto.

VII. Valor da Bolsa

1. O valor das Bolsas, não ultrapassará um total de 10.000,00€ (dez mil euros) euros anuais, distribuídos por um ou mais projectos aprovados pelo júri ou que este julgue merecedor de bolsa.

2. As bolsas serão decididas anualmente pela Direcção da SPO, sendo publicitadas no congresso anual da SPO.

VIII. Compromissos do Investigador Principal

1. Disponibilidade para apresentação formal do projecto, ou do trabalho final, em Reunião da SPO, a convite da Direcção.

2. Apresentação de um artigo publicado (ou aceite) em revista indexada ou, sugerindo-se ainda a submissão de um artigo à revista “Oftalmologia”.

3. As verbas serão transferidas em 50% no início do projecto sendo os restantes 50% transferidos após prova da aceitação de pelo menos um artigo com os resultados do projecto.

4. A não apresentação dos relatórios leva à suspensão do programa de financiamento.

5. A não conclusão do projecto obriga à devolução de todo o montante anteriormente financiado

6. Referência à Bolsa em publicações ou outras formas de apresentação pública.

IX. Casos Omissos

Os casos omissos serão resolvidos pela Direcção da SPO.

Contactos

Campo Pequeno, 2 – 13º
1000-078 Lisboa

T. (+351) 217 820 443
T. (+351) 924 498 989

geral@spoftalmologia.pt

Sobre a Sociedade Portuguesa de Oftalmologia

A SPO foi fundada em 1939 com o objectivo de promover e contribuir para o desenvolvimento da Oftalmologia nos seus diferentes aspectos: comunitário e profiláctico, assistencial e curativo, científico, pedagógico e de investigação, com respeito pela ética e deontologia profissional; defender os interesses dos seus associados, designadamente no domínio do exercício da profissão; contribuir para a correcta concepção de uma política de saúde no campo da Oftalmologia, com garantia de padrões de qualidade e competência consentâneos com as exigências da Ciência Médica.

Organizações e associações

Concebido por estúdio Ricardo Daniel
Desenvolvido por provider.pt | web artisans