# Shotgun Proteomics for the Identification and Profiling of the Tear Proteome of Keratoconus Patients

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**Purpose.** The qualitative approach followed in this study aims to obtain an extensive view of the keratoconus (KC) tear proteome, which could highlight proteins previously undetected and enlarge our knowledge of the disease's pathophysiology.

**Methods.** Twenty-five patients diagnosed with KC and 25 control subjects were studied in a prospective, cross-sectional study. KC screening examinations, including clinical and tomographic examinations, were performed on all participants. Tear samples were collected using Schirmer strips and analyzed by liquid chromatography-tandem mass spectrometry in a data-dependent workflow. A spectral count was used as a semiquantification tool. The tear proteomes of both groups were identified and profiled, and the functional interactions and biological characterization of differential proteins were analyzed using in silico tools.

**Results.** We identified a total of 232 proteins, of whom 133 were expressed in both groups' samples; 41 were observed only in control samples and 58 were identified just in tears of patients with KC. A semiquantitative analysis showed the dysregulation of 17 proteins in the KC samples. An in silico analysis linked proteins only expressed in KC samples to oxidative stress, skin development, and apoptosis. The dysregulation of proteins involved in iron transport, inflammation, oxidative stress, and protease inhibition was observed in the semiquantitative results.

**Conclusions.** A shotgun analysis showed that the tear proteome of patients with KC differed from controls by more than one-third of the total proteins identified, highlighting the relationship of the proteins only expressed in KC tears with processes of cell death, oxidative damage, and inflammation. The underexpression of proteins involved in iron pathways might support the iron imbalance as a contributing factor to cellular damage and death in KC disease.

Keywords: keratoconus, mass spectrometry, proteomics, shotgun, tear fluid

Keratoconus (KC) is a chronic and degenerative condition marked by pathological weakening, thinning, and protrusion of the corneal tissue.[1] KC leads to visual impairment because of irregular astigmatism and high-order aberrations, resulting from thinning and an increased curvature of the cornea. KC may occur either in isolation, in combination with other clinical diseases, or as a complement of another syndrome.[2] In most cases, it is a bilateral and asymmetric disorder that begins in adolescence and progress up to 30 to 40 years of age. Currently, KC is described as a multifactorial disease in which mechanical–environmental factors coexist with a genetic predisposition to its development.[3,4]

In recent years, many studies have been carried out to determine the biochemical and biomolecular changes that may impact the KC onset and progression.[5–8] In this regard, an analysis of the tears and corneal tissue from patients with KC has revealed overexpression of inflammatory cytokines such as IL-1, IL-1ß, IL-6, IL-17, TNF-$\alpha$, and TNF-ß.[9–11] In addition, previous studies from our group have shown the involvement of innate immunity in the pathophysiology

of KC, observing an increase in the expression of Toll-like receptors 2 and 4 in corneal and conjunctival epithelial cells, as well as in blood monocytes and neutrophils.[5,12,13] Oxidative stress also seems to play a crucial role in the pathophysiology of KC, as evidenced by numerous studies that have reported higher levels of damaging free radicals and lower levels of antioxidant molecules in the KC samples.[14–16] The extracellular matrix degradation and the apoptosis of corneal stromal and epithelial cells are triggered by both an increase in metalloproteinases levels and a decrease in the levels of their inhibitors,[17–20] which may be aggravated by the intense and frequent eye rubbing characteristic of patients with KC. Based on these characteristics, biological pathways such as inflammation, the innate immune response, oxidative stress, matrix proteolysis, and apoptosis have been brought to the spotlight in the pathophysiology of KC.

Biomolecules expressed in tears are vulnerable to changes associated with ocular diseases, providing information about the corneal tissue's conditions.[21] Tear fluid has become an easily accessible biological fluid that could play a key role in the detection of molecular changes that may be involved in the development of KC.

Recently, our group carried out a quantification approach of the tear proteome of patients with KC and control participants, using for the first time the sequential window acquisition for all theoretical mass spectra to identify the main dysregulated proteins in the tears of patients with KC.[22] In this way, results from the quantitative study revealed that dysregulated proteins were involved in the central processes of the KC pathophysiology, such as inflammation, oxidative stress, matrix proteolysis, and iron homeostasis.[22] However, the qualitative approach followed in this study aims to complement our previous results, trying to overcome the main handicap of quantitative studies (in which it is necessary to recognize a critical number of peptides to achieve protein quantification), and to obtain an even more extensive view of the tear proteomic profile of the patients with KC. Based on these factors, we performed a shotgun analysis to massively identify the tear proteome of patients with KC, which could highlight proteins undetected in the quantitative analysis and enlarge our knowledge of the disease's pathophysiology.

## Methods

The current study was carried out following the standards of the Declaration of Helsinki of the World Medical Association (as revised in Brazil 2013). The Ethics Committee for Clinical Research of Galicia (2019/623) approved the clinical, topographic, and tomographic protocol, including the biological sample extraction for all subjects. Each participant gave written informed consent after a thorough description of the methods and tests. All tests were performed by the same researchers. Regarding the data collected, variables included age, gender, patient's ocular history, patient medical history (allergy and other conditions such as eye itching and eye rubbing), and family history of corneal ectasia. Atopic conditions such as asthma, rhinitis, and atopic dermatitis were included as allergic diseases. Regarding the ophthalmological examination, topographic, and tomographic examinations were performed, including maximum dioptric power, flattest corneal meridian, steeper meridian, inferior–superior dioptric asymmetry, minimum thickness point, and posterior elevation. All variables were measured using PENTACAM (Oculus, Wetzlar, Germany).

### Study Subjects

This prospective and cross-sectional study included 50 subjects divided into two groups: 25 patients diagnosed with KC (study group) and 25 control participants (control group). The inclusion criteria for the study group was the diagnosis of KC, supported by slit-lamp examination and corneal topography and tomography examinations. Conjunctival hyperemia of less than 2 (Nathan Efron scale),[23] Schirmer test of more than 15 mm in 5 minutes, and at least 5 days without contact lenses, artificial tears, or eye drops were the common inclusion criteria for all groups. The common exclusion criteria for both groups included corneal trauma or disease, previous surgical intervention in the anterior segment, active ocular or systemic inflammation, current treatment with local or systemic anti-inflammatory drugs, and/or pregnancy. Renal, hepatic, or hematologic diseases, as well as solid tumors, were also exclusion criteria for both groups. Control subjects with a family history of corneal ectasia were excluded. This study involved the same participants who were enrolled in our previous sequential window acquisition for all theoretical mass spectra analysis.[22]

### Tear Sample Extraction and Analysis

Tear samples were collected using Schirmer Strips. Strips were removed when the sample reached the 15 mm on the Schirmer scale (9 µL). Samples were extracted without previous instillation of drugs, artificial tears, or vital dyes, and immediately after collection they were frozen and stored at −80°C until their analysis.

### Sample Preparation

Proteins were extracted by cutting and incubating Schirmer strips for 1 hour (h) at room temperature in a 100-µL solution of 100 mM ammonium bicarbonate. Samples were centrifuged for 20 minutes at 13,000x*g*, and the supernatant was transferred to a new tube. Subsequently, 600 µL of acetone were added to the new Eppendorf with the supernatant and incubate at –20°C overnight. After this time, samples were centrifuged 20 min at 13,000x*g* and the supernatant was removed. The pellet was dried at room temperature for 1 h to evaporate the acetone and 100 µL of miliQ was added. Finally, the amount of protein was measured using a RC-DC kit (BioRad, Hercules, CA).[24] These samples were used both for tryptic digestion and micro-liquid chromatography with tandem mass spectrometry (LC-MS/MS) analysis as well as for the quantification by ELISA.

### Tryptic Digestion

Trypsin digestion was performed as described previously by our group.[22] Briefly, 100 µg of protein was concentrated on a 10% SDS-PAGE gel. The gel was allowed to run until the front entered 3 mm of the separator gel.[25,26] The protein band was visualized with Sypro-Ruby fluorescent staining (Lonza, Porriño, Pontevedra, Spain), excised and subjected to tryptic digestion following the standard protocol of Shevchenko et al.,[27] with minor modifications. The peptides were extracted and stored at –20°C until their use.

### Protein Identification by Shotgun (LC-MS/MS)

Digested peptides were resuspended in 20 µL of mobile phase A (2% acetonitrile, 0.1%, formic acid), by sonication for

10 minutes to obtain 1 µg/µL peptide solution. We injected 4 µg (4 µL) of each sample and separated by reverse phase chromatography using a ChromXP C18CL column (150 µL × 0.3 mm, 120ª, s-3 µL) (Sciex, Framingham, MA).

Data acquisition was performed on a Triple-TOF 6600 system (Sciex) using a data-dependent workflow with micro-LC-MS/MS technology. After MS/MS acquisition, the files were processed with ProteinPilot 5.0.1 software (Sciex) using the Paragon algorithm for database searching and Progroup for data clustering. Searches were performed using a human-specific Uniprot database (Swiss-Prot), trypsin as enzyme of digestion, and cysteine alkylation as fixed modification. The search was performed allowing a fragment ion mass tolerance of 0.100 Da and a parent ion tolerance of 0,050 Da. The false discovery rate was obtained by a nonlinear adjustment that considers correctly identified proteins with a false discovery rate of less than 1%.

### Semiquantitative Analysis by Spectral Count

The Scaffold program was used for semiquantification by spectral counting (version Scaffold_5.0.1, Proteome Software Inc., Portland, OR). The total samples of both groups (control and KC) were subgrouped for quantification.

MS/MS-based peptide and protein identifications performed in ProteinPilot software were validated by Scaffold. Peptide identifications were accepted if they could be established at greater than 95.0% probability by the Percolator posterior error probability calculation.[28] Protein identifications with a probability of greater than 99.0% and at least two identified peptides were accepted. Protein probabilities were assigned by the Protein Prophet algorithm.[29] Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Proteins sharing significant peptide evidence were grouped into clusters. The Fisher's exact test and the Benjamini–Hochberg multiple correction test were used for quantification and fold change determination.

### ELISA Quantification

ELISA on human tears were performed using commercial kits from FineTests for the following four proteins: albumin (ELISA kit catalog number EH2613), transferrin (ELISA kit catalog number EH0385), peroxiredoxin-1 (ELISA kit catalog number EH2166), and mammaglobin-B (ELISA kit catalog number EH4335). All assays were carried out following the manufacturer's protocols and were optimized and used with the following dilution factors: 1:50 for peroxiredoxin-1 and transferrin, 1:100 for albumin, and 1:200 for mammaglobin-B. All samples were assayed in duplicate, and the mean values were reported as nanograms per milliliter. Curve Expert 1.4 was used for curve and data analysis and box plots were performed using GraphPad Prism 8.0 (San Diego, CA) software.

### Interaction Networks and Gene Ontology Enrichment Analysis

Protein–protein interactions and gene ontology (GO) enrichment analysis were performed to determine the interactions and functional characteristics of the identified proteins.

To create protein interaction maps, we used STRING (Functional Protein Association Network, free access at https://string-db.org) program, and to analyze biological processes and molecular functions we use the following tools: function enrichment interaction analysis (FunRich) and the Panther Classification System. We use Uniprot codes for the identification and functional characterization of each protein.

### Statistical Analysis

SPSS 20.0 for Mac (IBM, Armonk, NY) and GraphPad Prism 8.0 were used for statistical analysis. The Kolmogorov–Smirnov test was used to confirm the normality of the quantitative variables. For categorical variables, the results were represented as percentages, and for continuous quantitative variables with a normal distribution, the mean ± standard deviation was used. Error bars were used to represent comparisons between normal continuous variables and box plots were used to represent continuous variables with non-normal distributions. The $\chi^2$ test (for categorical variables), Student *t* test contrasts (for normal continuous variables), and the Mann–Whitney *U* test (for non-normal continuous variables) were used to compare groups bivariately. The Fisher exact test and Benjamini–Hochberg multiple correction test were used for quantification and FC determination in the spectral counting semiquantitative analysis. FC indicates upregulated proteins if the FC was more than 1 or downregulated proteins if the FC was less than 1. A *P* value of less than 0.05 was considered statistically significant in all tests.

## Results

### Clinical Features

In this study, we included 25 healthy eyes from 25 control subjects (60% male; mean age, 43.96 ± 6.94 years) and 25 eyes from 25 patients with KC (60% male; mean age, 44.88 ± 5.01 years). No differences were found regarding age and sex because both groups were previously matched according to these conditions. Allergic disorders showed no statistical differences between groups ($P = .247$). Patients with KC reported a higher rate of eye itching (72%) and, as a result, a higher rate of eye rubbing (64%) than the control subjects (4% for each condition) ($P < .0001$). Predictably, we found differences between the groups for all topographic and tomographic parameters. The clinical features as well as the topographic and tomographic parameters of each study group are displayed in Table 1.

**Table 1.** Clinical and Topographic–Tomographic Characteristics of Each Study Group

| | Control | *KC* | *P* Value |
|---|---|---|---|
| Allergic disease (%) | 28 | 44 | 0.247 |
| Itching (%) | 4 | 72 | **<0.0001** |
| Rubbing (%) | 4 | 64 | **<0.0001** |
| K1 (D) | 42.70 ± 1.39 | 47.14 ± 5.31 | **<0.0001** |
| K2 (D) | 43.73 ± 1.48 | 50.47 ± 5.28 | **<0.0001** |
| maxDP (D) | 44.40 ± 1.25 | 55.56 ± 7.10 | **<0.0001** |
| MTP (µm) | 534.00 ± 21.69 | 445.00 ± 66.65 | **<0.0001** |
| PE | 9.20 ± 3.73 | 67.96 ± 31.33 | **<0.0001** |

K1, flattest corneal meridian; K2, steeper corneal meridian; maxDP, maximum dioptric power; MTP, minimum thickness point; PE, posterior elevation.

Size sample: KC = 25 subjects, 25 eyes; controls = 25 subjects, 25 eyes.

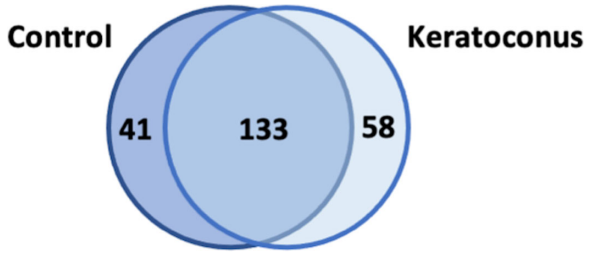


FIGURE 1. Venn diagram shows the number of unique and overlapping proteins identified in the qualitative proteomic analysis of the tear samples of patients with KC and control subjects.

## Data-dependent Workflow Shotgun Analysis of the Tear Proteome

Using micro-LC-MS/MS technology in data-dependent workflow mode, we performed a shotgun analysis to identify the complete profile of proteins expressed in the tear samples of patients with KC and control participants. We identified 232 proteins with a false discovery rate of less than 1%. Of the total identified proteins, 133 were expressed in both groups' samples, 41 were observed only in the control samples, and 58 were identified just in the tears of patients with KC. The list of the 232 proteins identified (expressed in both groups and only found in KC or control samples) is available in supplementary data (Supplementary Table S1), including the main functional protein classification. A Venn diagram (Fig. 1) presents the distribution of proteins identified in both tear profiles.

## Protein–Protein Interaction Network and Functional Pathway Enrichment Analysis

The proteomic tear profile of the control group and the KC group was analyzed and categorized. To clarify the differences in protein composition between the two protein profiles, we performed a global categorization of all the proteins found in the shotgun analysis according to the protein class ontology. Moreover, proteins only expressed in the tears of the controls (absent in KC) or only expressed in the KC profile were analyzed separately to detail the differences between the two profiles. For this purpose, we used the PANTHER tool (free access at http://pantherdb.org), inserting the Uniprot code of each protein of interest and selecting the type of GO enrichment analysis categorization.

The proteomic profile of both groups showed a large percentage of immunomodulatory and defense proteins. Moreover, proteins with metabolic, cell signaling, cellular adhesion, binding, transport, transfer or carrier, adaptor, transcriptional regulator, or cytoskeletal activity were also detected (Fig. 2.$A_1$ and 2.$B_1$). Figures 2.$A_2$ and 2.$B_2$ depicts the main categories in which proteins only expressed in controls or in the KC group were involved. In this regard, tear samples from patients with KC showed a higher percentage of structural, metabolic, and chaperone proteins, expressing transcriptional proteins that were not present in the control group.

In line with the previous GO analysis according to the protein type, we carried out an analysis of the functional interactions over proteins only expressed in the tears of patients with KC, which could evidence critical biological processes in the pathophysiological development of the disease. We used the STRING Interaction Network to explore the protein–protein interactions (Fig. 3).

Keratins (KRT), peptidyl-prolyl cis-trans isomerases (PPIases), and aldehyde-dehydrogenases (ALDH) formed three different clusters in the interaction network analysis. The KRT cluster included 13 cuticular and cytoskeletal keratins, the PPIases cluster was made up of 5 proteins from the PPIase family, and the last cluster consisted of 10 ALDH family members. Supplementary Table S1 presents the complete names and Uniprot codes for each of the proteins.

In silico functional and biological characterization using STRING and FunRich tools determined that proteins only expressed in the KC samples were mainly related with processes such as skin–epidermis development, epithelial cell differentiation, aldehyde cell metabolic processes, and oxidation–reduction processes. The graphs show the differences between the main biological processes (Fig. 4A) and molecular functions (Fig. 4B) related to the proteins of KC and control tear proteome. Oxidative stress, hydrogen peroxide catabolic processes, oxidation–reduction processes, aging, and epidermis development were the most enriched process related to the KC proteome.

In the same way, a molecular function analysis reflected an increase in the KC proteomic profile of proteins related to structural and oxidative stress-related activities (such as antioxidant, peroxiredoxin, and aldehyde dehydrogenase activity) and, conversely, a decrease in zinc- and iron-binding proteins as well as in protease inhibitors.

## Semiquantitative Analysis by Spectral Count and ELISA Validations

After the qualitative analysis, we performed a semiquantification to determine proteins with differential expression between groups using the Scaffold Proteome Software. In this way, the dysregulation of 17 proteins in the KC samples was observed in the semiquantification. Of these proteins, 13 showed an overexpression in KC tears, whereas the remaining four were underexpressed. Table 2 lists the proteins with different expressions between the groups.

In the GO analysis targeting differentially expressed proteins, upregulated proteins were related to skin development and cell proliferation processes (KRT type 1 cytoskeletal 9, KRT type II cytoskeletal 1), inflammatory response (S100-A4 protein, Zymogen granule protein 18 homolog B [ZG16B], and lysozyme C), and defense to oxidative stress (peroxiredoxin-1). Instead, downregulated proteins were associated with iron binding and transport, zinc and cooper binding, antioxidant activity, and protease binding and inhibition. Figures 5A and 5B present the main biological functions and processes involving differentially expressed (upregulated or downregulated) proteins.

Four of the most dysregulated proteins were quantified by ELISA analysis as a validation of the semiquantification results shown elsewhere in this article. In this regard, the concentrations of serotransferrin (TF) and albumin (downregulated in KC samples in the semiquantification) and mammaglobin-B and peroxiredoxin-1 (upregulated in KC samples) were measured. Figure 6 presents the ELISA quantification of these proteins in the KC and control groups.

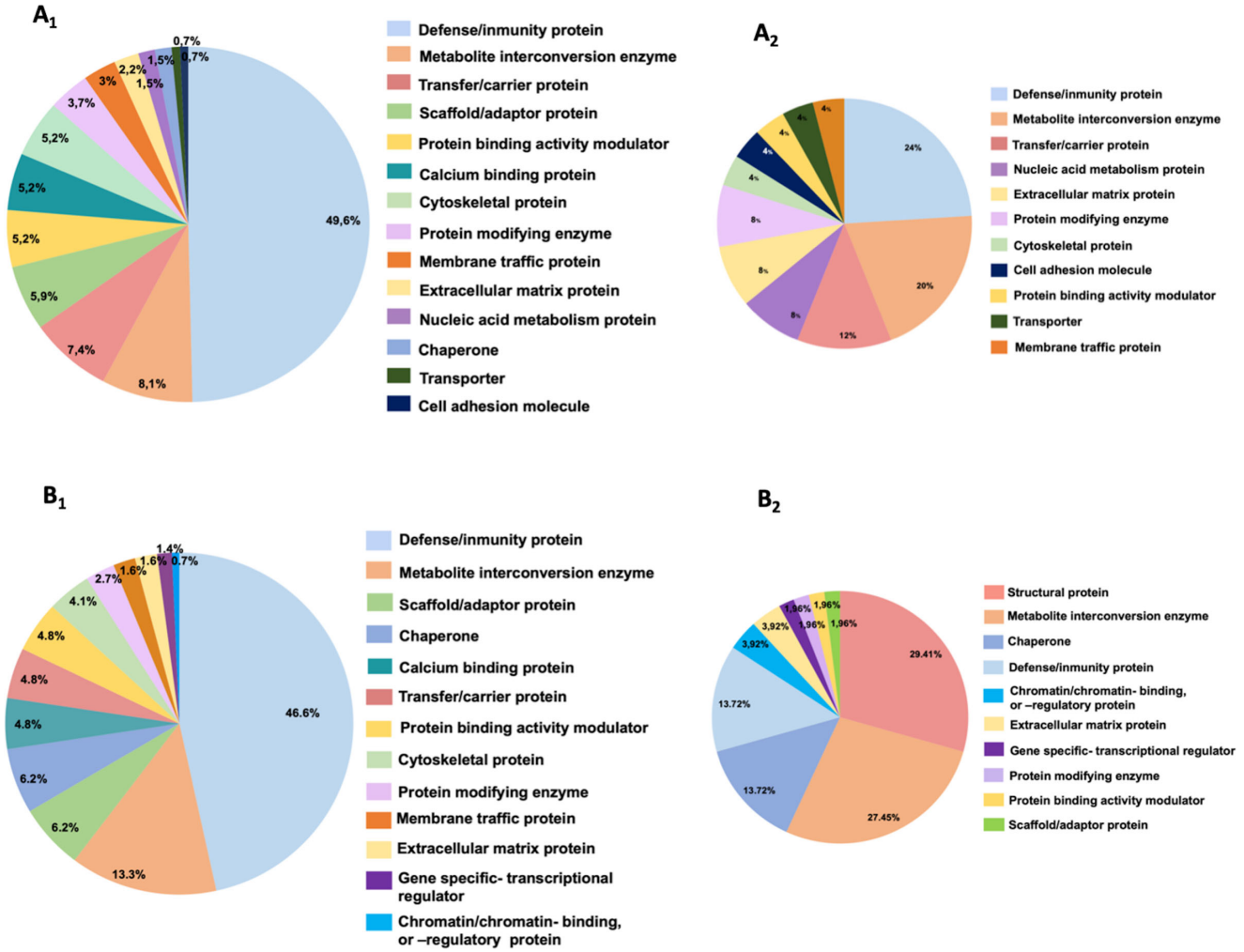


**Figure 2.** Charts integrated into this figure represent the types of proteins that make up the tear profile of the control ($\mathbf{A_1}$ and $\mathbf{A_2}$) and participants with KC ($\mathbf{B_1}$ and $\mathbf{B_2}$). ($\mathbf{A_1}$) Different types of proteins that composed the control tear profile. ($\mathbf{A_2}$) The categories in which the 41 proteins exclusively found in control participants' tears were involved. ($\mathbf{B_1}$) The types of proteins that composed the proteomic profile of the tear of patients with KC. ($\mathbf{B_2}$) The main categories for the proteins only expressed in the KC tear samples.

## Discussion

Shotgun proteomics approaches have achieved great popularity and acceptance in recent years for offering additional insight into the molecular pathways involved in the pathophysiology of many diseases. Quantitative analyses provide valuable information about specific targets; however, qualitative approaches are essential in the search for a global characterization that does not leave behind events overlooked by quantitative limitations. In this way, we performed a shotgun proteomic approach to obtain an even more extensive view of the tear proteomic profile of the patients with KC, as well as to observe the differences regarding the proteomic profile of control subjects. We also performed a semiquantification analysis by spectral count to determine the differentially expressed proteins between groups, and several of the most dysregulated proteomic candidates were validated by ELISA.

In silico tools were used to study the interaction networks and to characterize the biological and functional profile of the proteins. A GO analysis of the tear protein composition of both groups revealed that a large percentage of the identified proteins had immunomodulatory and defense properties, mainly related to the pivotal role of the tear fluid against external pathogens and damage.[30] Metabolic, cell signaling, cellular adhesion, transport, or transfer or carrier were other of the most enriched molecular functions.

The tear composition of patients with KC showed remarkable differences concerning the control group, differing from the control tear proteome in more than one-third of the total proteins identified. An interaction network analysis between proteins expressed only in KC tear samples identified three protein clusters with specific functions. First, we identified a cluster composed of 13 cuticular and cytoskeletal keratins. KRT constitute the main component of the outermost layer of the epidermis and are also expressed in the corneal epithelium cells, but they are not present in tears under normal conditions. Previous studies reported the presence of keratins in the tear samples of patients with KC, relating this finding as a possible consequence of the eyelid filtration owing to ocular rubbing, a frequent habit of the patients with KC.[31] Nevertheless, six keratins that we have identified in the tears of patients with KC are expressed in the corneal epithelium (KRT Hb6, KRT14-KRT17, and KRT19),[32]

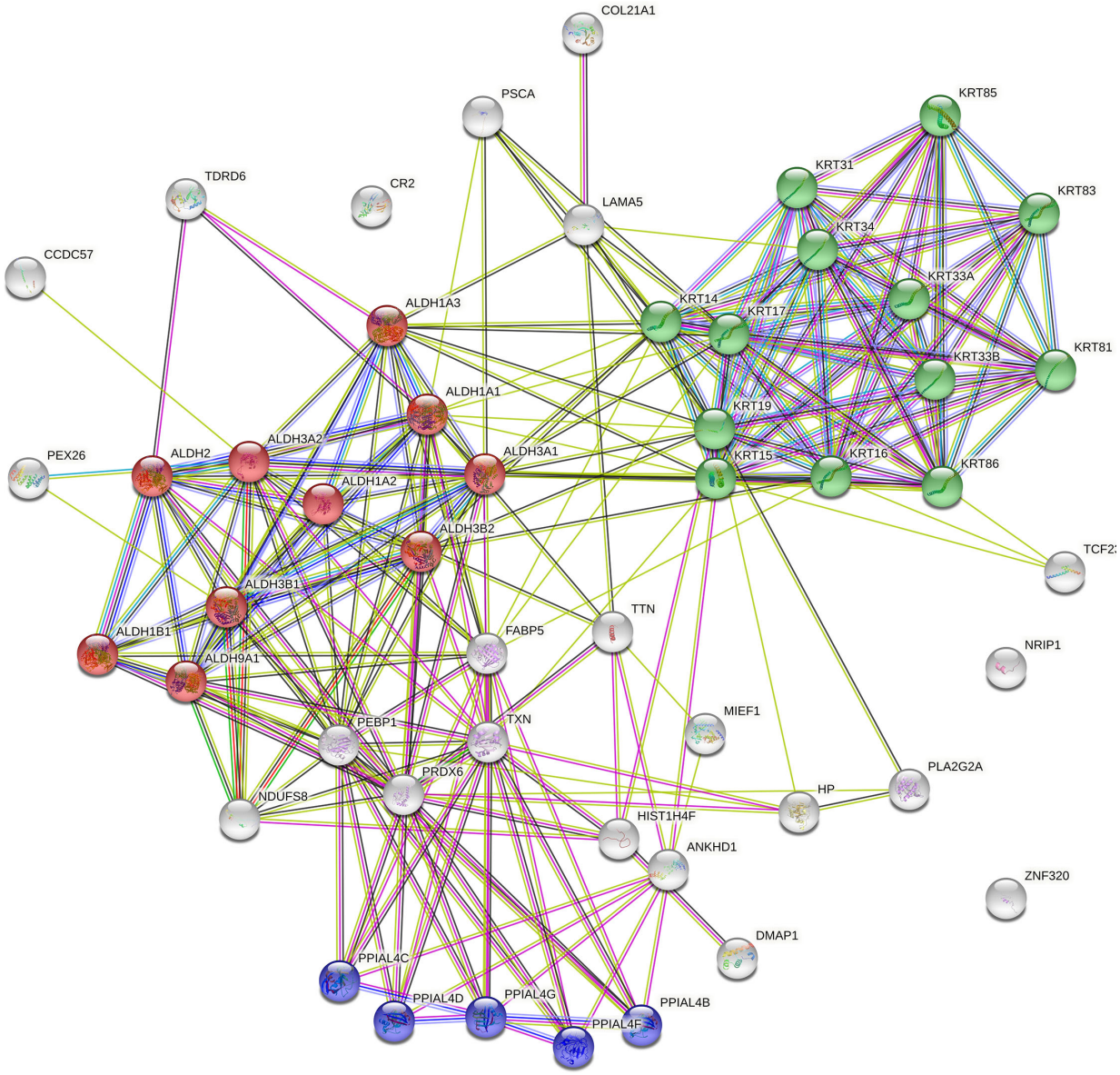


**Figure 3.** STRING Interaction Network of proteins only identified in KC tears. Three clusters were observed in the interaction network, formed mainly by three types of proteins, so that the green balloons point to the group of cuticular and cytoskeleton keratins, blue balloons to the PPIases, and red balloons refer to the proteins of the aldehyde dehydrogenase family.

suggesting that its presence in the tear fluid may be associated with epithelial cell death and epithelial desquamation, that altogether result in the overall thinning of the cornea seen in KC pathophysiology.

Five members of the PPIases were assembled in the second cluster. PPIases are a protein folding enzymes that catalyzes the cis–trans isomerization of proline imidic peptide bonds in oligopeptides.[33] Besides that, these proteins have and important role in the cellular response to UV radiation as well as in the regulation of UV-induced apoptosis.[34] The involvement of PPIases in the pathogenesis of KC is broadly unknown, but, considering that the cornea is subjected to high levels of UV radiation, PPIases properties as regulators of UV-induced apoptosis could be related to the degeneration and death of the corneal epithelium and stroma cells, as a result of the redox imbalance in the KC cornea.

Finally, an enrichment of processes such as aldehyde oxidation-reduction and response to reactive oxygen species was observed, which was predominantly related to the expression of 10 proteins from the ALDHs in KC tear samples. Owing to their critical role in neutralizing and removing aldehyde radicals, ALDHs are extensively expressed in the corneal tissue, providing important protection against oxidative stress.[35–37] Research about the ALDHs in tears is scarce and there is no previous information about its dysregulation in the tear samples of patients with KC. However, the increased expression of ALDH proteins has been observed in the tear fluid of patients with other ocular surface diseases, such as the dry eye.[38,39]

Despite the lack of data on KC tear samples, the upregulation of ALDH3A1 has also been reported in the KC corneal stroma.[40] Therefore, our results in tear samples are in agreement with these studies and suggest that ALDH expression

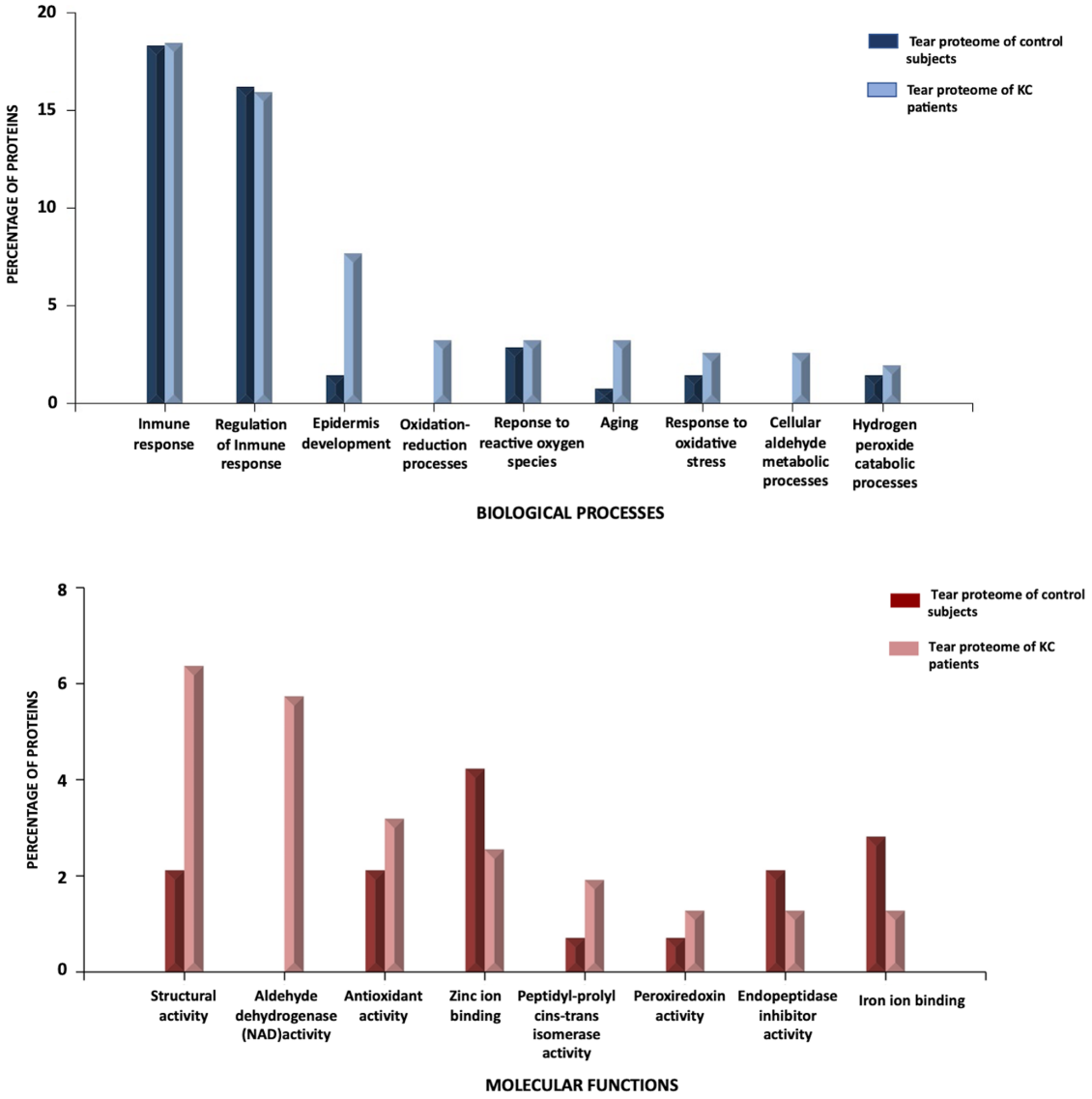


**Figure 4.** GO enrichment of tear proteomic profile of KC and control subjects, using the FunRich tool. (**A**) The differences in the main biological processes related to the tear proteome of patients with KC and control subjects, and (**B**) the differences in the main molecular functions associated with them. The histograms represent the main categories for each GO term in which proteins were involved ($P < 0.05$). The *x* axis shows the biological processes and molecular functions, and the *y* axis shows the percentage of proteins involved in this process compared with the total proteins in the database.

**Table 2.** Proteins With Different Expression Between KC and Control Samples

| Protein Name | Protein Code | *P* Value | FC |
|---|---|---|---|
| Peroxiredoxin-1 | Q06830 | 0.0007 | 5.8 ↑ |
| ZG16B | Q96DA0 | 0.024 | 4.3 ↑ |
| KRT, type I cytoskeletal 9 | P35527 | 0.0066 | 2.4 ↑ |
| Mammaglobin- B | O75556 | 0.00063 | 2.1 ↑ |
| Heat shock protein $\beta$- 1 | P04792 | 0.0053 | 2.0 ↑ |
| Protein S100- A4 | P26447 | 0.026 | 2.0 ↑ |
| Polymeric immunoglobulin receptor | P01833 | 0.0027 | 1.6 ↑ |
| Secretoglobin family 1D member | O95968 | 0.027 | 1.5 ↑ |
| Cluster of cystatin-S | P01036 | 0.039 | 1.5 ↑ |
| KRT, type II cytoskeletal 1 | P04269 | 0.034 | 1.4 ↑ |
| Extracellular glycoprotein lacritin | Q9GZZ8 | <0.0005 | 1.4 ↑ |
| Lipocalin-1 | P31025 | 0.0048 | 1.3 ↑ |
| Lysozyme C | P61626 | 0.043 | 1.3 ↑ |
| TF | P02768 | <0.0005 | 0.5 ↓ |
| Serotransferrin | P02787 | <0.0001 | 0.4 ↓ |
| Immunoglobulin constant mu | P01871 | 0.040 | 0.2 ↓ |
| Alpha 1-antitrypsin | P01009 | 0.0042 | 0.2 ↓ |

FC shown upregulated proteins if FC > 1 or downregulated proteins if FC < 1.

Size sample: KC = 25 subjects, 25 eyes; controls = 25 subjects, 25 eyes.

may be related to the tear's redox imbalance and the greater demand for oxidative protection in patients with KC' tears and tissues.

In relation to the oxidative stress cycle involving PPIases and ALDHs, semiquantification analysis also revealed the dysregulation of some stress-related proteins. For instance, we observed a five-fold increase of peroxiredoxin-1 expression in KC samples compared with the control samples, which was subsequently validated by ELISA. Peroxiredoxin-1 belongs to the family of ubiquitinated peroxiredoxins and plays a crucial role in cell proliferation as well as in catalyzing peroxide reduction; in this sense, its overexpression has been described as a key factor in the protection against oxidative stress.[41] Changes in the expression of these proteins have been previously observed in some systemic and ocular surface conditions like psoriasis, pterygium and dry eye,[42–44] all of them, on a different etiological basis, characterized by lesions and alterations at the skin or epithelial level. In the case of KC, the overexpression

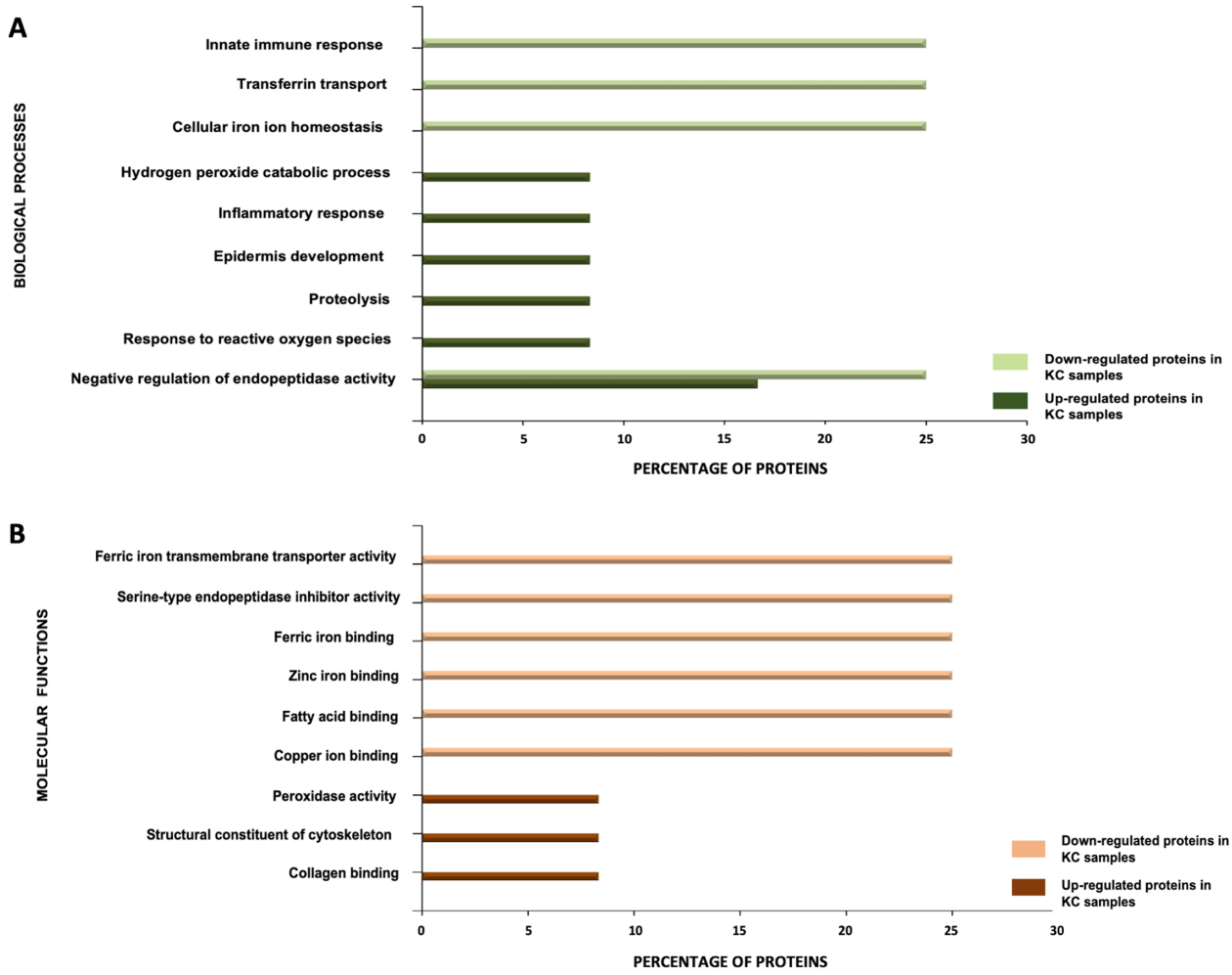


**Figure 5.** (**A**) Main biological processes associated with dysregulated (upregulated and downregulated) proteins in samples from patients with KC. The graph was made using the Functional Enrichment Analysis tool (FunRich). (**B**) Main molecular functions associated with dysregulated (upregulated and downregulated) proteins in samples from patients with KC. The graph was made using the Functional Enrichment Analysis tool (FunRich). Percentages reflect our study proteins involved in the different biological processes and molecular functions, in relation to the total proteins in the database.

of this protein could be related to its pathophysiology in several ways; first, with increased levels of oxidative stress and consequently with increased antioxidant demand, and, in a complementary pathway to corneal epithelial desquamation and corneal tissue injury. In the same way, heat shock protein beta-1 (HSPB1) was also overexpressed in the KC samples. Enrichment of the HSPs has been described previously in the corneal and tear samples of patients with KC.[45] In particular, HSPB1 comes to the HSP family, which is expressed in a variety of cells and tissues under normal conditions.[46] HSPB1 is inducibly increased in stressful conditions, such as pH shift, heat shock, or hypoxia, to cope with and prevent cell death triggered by different pathways. In addition, HSPB1 has been considered an important regulator of the ferroptosis, a possible pathway of cell death in the pathophysiology of KC.[22,47]

One of the main sources of redox imbalance and increase in reactive species could be the disruption of iron transport and homeostasis in the tear samples of patients with KC. As such, our results, validated by ELISA, evidenced a decreased expression of iron related proteins such as TF and albumin. TF is a glycoprotein with specific functions in the control of iron homeostasis and the prevention of the harmful effects of free unbound iron.[48] Free unbound iron is potentially toxic owing to its ability to produce reactive species by different reactions. Moreover, free iron ions have high susceptibility to accumulate in the tissues by pathways not fully described, contributing to cell damage and tissue injury.[49]

The internalization, storage, and export of cellular iron is controlled in a coordinated manner by very strict mechanisms contributing to the proper maintenance of intracellular iron levels. Therefore, an imbalance in some of the components involved in iron homeostasis (such as lactotransferrin (LTF), TF, and TF receptor, among others) triggers potentially detrimental effects. In addition, a downregulation in TF expression has been reported in inflammatory and iron overload conditions.[49] Our results in tear samples are in agreement with previous studies, in which TF levels are decreased in the corneal stroma of patients with KC; in addition, some TF gene polymorphisms have been linked to the risk of developing KC.[50] Similarly, albumin is an antioxidant protein that can bind weakly to iron ions at high concentrations, contributing to a decrease in the levels of oxidative stress caused by free iron.[51,52] Previous studies

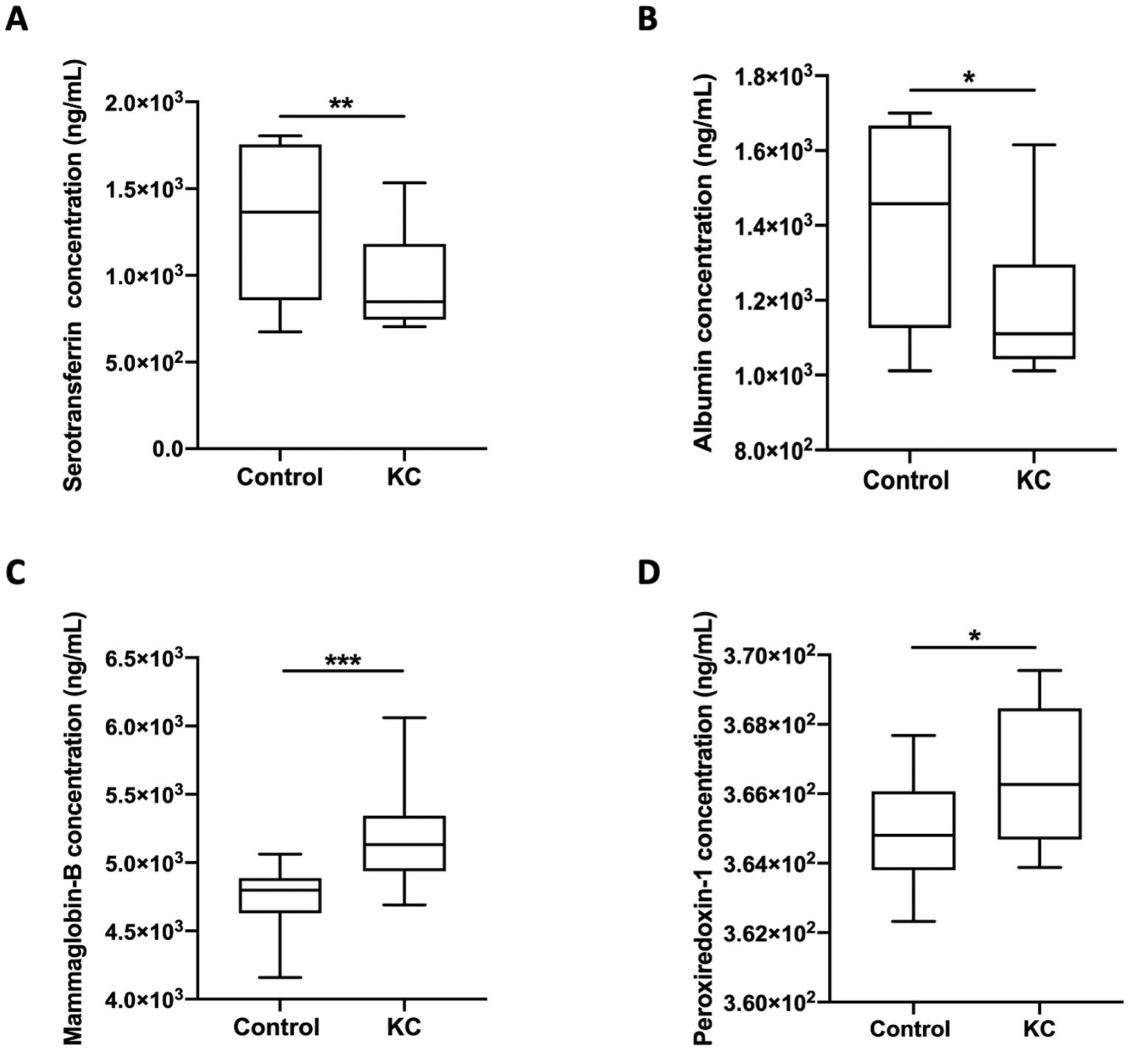


**Figure 6.** Box plot representation of the (**A**) TF, (**B**) albumin, (**C**) mammaglobin-B, and (**D**) peroxiredoxin-1 concentrations (ng/mL) in the tear samples of control and KC participants, measured by ELISA. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

have suggested that the depletion of different proteins such as lactoferrin and albumin disrupts iron homeostasis during KC pathophysiology.[22,53,54] In this context, decreased transferrin and albumin expression could indicate a detrimental and chronic oxidative state in corneal tissues, caused by the underexpression of proteins necessary for iron transport and homeostasis.

Ongoing oxidative stress drives the activation of chronic inflammatory pathways that are involved in a wide range of degenerative diseases.[55–57] In this regard, the upregulation of proteins with inflammatory implication as S100-A4 protein, ZG16B, or lysozyme C was observed in the KC tear samples.

S100 proteins have been suggested as molecular targets in ocular surface inflammatory diseases.[58] These proteins are commonly known as alarm- or damage-associated patterns and are frequently exported to the extracellular space to fight oxidative damage and increased stress levels. Apoptosis, inflammation, angiogenesis, and cell differentiation are just a few of the biological processes in which S100-A4 is involved. It plays a fundamental role in the activation of proinflammatory pathways, promoting the release of cytokines, growth factors, and other inflammatory molecules.[59] Specifically, S100-A4 can promote the expression of matrix metalloproteinases,[60] whose overexpression has been observed in KC tissues and tears, linking to the progressive weakening of the corneal tissue. Our results in tears are consistent with previous studies, which showed that elevated levels of S100-A4 in the corneal epithelium of patients with KC, confirming the pathophysiology's inflammatory processes.[61]

In contrast with the S100s, the functions of ZG16B and mammaglobin-B in tears are potentially unknown. A role in maintaining the inflammatory state in cancerous tissues has been postulated for ZG16B,[62] and mammaglobin-B has been proposed as a prognostic biomarker of uterine endometrial cancer.[63] At the tear level, the overexpression of ZG16B has been found in patients with dry eye,[39] and previous studies have suggested the possible involvement of mammaglobin-B in the lipid tear film structure. Moreover, the dysregulation of mammaglobin-B was previously described in the KC tear samples.[64] However, further studies are needed to determine the possible implications of these proteins in the tear fluid and thus in the KC pathophysiology.

Lysozyme C, an essential antimicrobial and inflammatory regulator in the tear fluid, was also upregulated in KC. The

dysregulation of this protein was previously observed in tear samples from patients with low-grade KC, suggesting that this protein may play a key role from the early stages of the disease, but its specific function has not yet been clarified.[65]

In addition to proteins involved in the tight circle of oxidative stress and inflammation, the downregulation of alpha 1-antitrypsin and the upregulation of lipocalin 1 were also observed. Thus, the antiproteolytic properties of the first one and the close relationship with lipid peroxidation of the second one could also compromise corneal tissue integrity.

Although this is an innovative study with new findings that shed some light on the biological mechanism on the KC physiopathology, there are also several limitations. We recognize that the small sample size did not for allow the subcategorization of patients according to the severity of KC, so our research was unable to detect the proteins altered at different stages of the disease. As a result, further studies are needed to detail the intertwining events as the disease progresses.

In conclusion, this shotgun analysis revealed that the KC tear proteome differed from the control tear proteome in more than one-third of the total proteins identified. These differences have highlighted the importance of oxidative damage, inflammation, and cell death processes in the KC tear samples. In this sense, the alteration of iron homeostasis and its link with oxidative stress and inflammation could be postulated as contributing factors to the cellular damage characteristic of the disease. Moreover, the discovery of the biological pathways and molecular mechanisms that describe and characterize the pathophysiology of KC brings us closer to an in vitro model, a promising step toward developing diagnostic and therapeutic targets.

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