Tear Proteomics in Keratoconus: A Quantitative SWATH-MS Analysis

Maite López-López,[1,2] Uxía Regueiro,[1,2] Susana B. Bravo,[3] María del Pilar Chantada-Vázquez,[3] Rubén Varela-Fernández,[2,4] Paulo Ávila-Gómez,[2] Pablo Hervella,[2] and Isabel Lema[1,2,5]

[1]Department of Surgery and Medical-Surgical Specialties. Faculty of Optics and Optometry, Universidade de Santiago de Compostela, Santiago de Compostela, Spain
[2]Clinical Neurosciences Research Laboratory, Health Research Institute of Santiago de Compostela (IDIS), Santiago de Compostela, Spain
[3]Proteomic Unit, Health Research Institute of Santiago de Compostela (IDIS), Santiago de Compostela, Spain
[4]Department of Pharmacology, Pharmacy and Pharmaceutical Technology. Universidade de Santiago de Compostela (USC), Campus Vida, Santiago de Compostela, Spain
[5]Instituto Galego de Oftalmoloxía (INGO), Hospital Provincial de Conxo, Santiago de Compostela, Spain

Correspondence: Isabel Lema, Instituto Galego de Oftalmoloxía, Hospital Provincial de Conxo, Compostela 15706—Santiago de Compostela (Spain); mariaisabel.lema@usc.es.
Pablo Hervella, Clinical Neurosciences Research Laboratory, Health Research Institute of Santiago de Compostela, 15706—Santiago de Compostela (Spain); pablo.hervella.lorenzo@sergas.es.

MLL and UR have equally contributed to this work.

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Purpose. To elucidate dysregulated proteins in keratoconus (KC) to provide a better understanding of the molecular mechanisms that lead to the development of the disease using sequential window acquisition of all theoretical mass spectra (SWATH-MS) as a protein quantification tool of the tear proteomic profile.

Methods. Prospective cross-sectional study that includes 25 keratoconic eyes and 25 healthy eyes. All participants underwent a clinical, tomographic, and aberrometric exam. Tear sample was collected using Schirmer strips and analyzed by liquid chromatography with tandem mass spectrometry. SWATH-MS was used as a quantification tool of the tear proteomic profile. The expression of the quantified proteins was compared between groups, and the biological and molecular functions of the dysregulated proteins as well as their functional relationships were studied by in silico analysis.

Results. A total of 203 proteins were quantified in tear samples of patients with KC and control participants, of which 18 showed differential expression between groups ($P < 0.05$). An increase in the expression of 7 proteins and a decrease in the expression of 11 proteins were observed. Protein–protein interactions and gene ontology analysis showed the involvement of these dysregulated proteins in structural, inflammatory-immune, iron homeostasis, oxidative stress, and extracellular matrix proteolysis processes.

Conclusions. Tear protein quantification has revealed the dysregulation of proteins involved in biological processes previously associated with KC. Among them, iron homeostasis should be highlighted as a relevant pathway in the KC pathophysiology, and it should be taken into account in the development of therapeutic targets to cope with tissue damage derived from iron accumulation and toxicity.

Keywords: keratoconus, tear fluid, proteomics, mass spectrometry, SWATH-MS

Keratoconus (KC) is a chronic, progressive, and degenerative disease characterized by alterations at the corneo-structural level such as thinning, protrusion, and increased curvature of the corneal tissue.[1]

Generally, KC is an asymmetric condition whose structural changes lead to the appearance of refractive errors, especially irregular astigmatisms, which represent a significant loss of vision and, consequently, a great impact on quality of life.[2] Although KC usually appears in isolation, roughly a quarter of diagnosed patients report a positive family history of this disease, which suggests the influence of heredity in its etiopathogenesis.[3,4]

Keratoconus was considered a noninflammatory disease, but recent studies have shown the alteration in the expression of molecules involved in inflammatory processes, as well as in oxidative stress, extracellular matrix proteolysis, and cellular apoptosis,[5–10] evidencing the participation of all of these biological mechanisms in the KC pathophysiology.

In the keratoconic corneal tissue, a link between oxidative stress and cell damage of stromal keratocytes was found, demonstrated by the increase of free radicals and other reactive species,[11] as well as the decrease in the activity of antioxidant enzymes and other protective molecules against free radicals,[12] which finally result in an increasing thinning of the corneal stroma with the progression of the disease.

In addition, the imbalance between pro- and anti-inflammatory proteins and the increase in matrix metalloproteinase activity reported in KC samples are also associated with the biochemical development of the disease, promoting a decrease in corneal tissue integrity.[13]

Tear fluid is a biological component that covers, protects, and nourishes the ocular surface, especially the corneal

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epithelium. Previous studies have described the exchange of growth factors and other molecules between the tear film, the epithelium, and the corneal stroma, showing that the tear components can affect the phenotype of epithelial cells and stromal keratocytes.[14,15] Thus, tears become an easily accessible biological fluid with direct information from the corneal tissues, allowing the assessment of the underlying tissues and playing a key role in the detection of molecular changes that may interfere with the development of KC.

Previous studies have suggested that changes in the expression of certain proteins in tears may be indicative of an increase of proteolytic activity and the existence of complex chronic inflammatory processes in the pathophysiology of the disease.[16,17] However, most of the studies carried out to date have been focused on the analysis of specific proteins[18,19] with the limitation that isolated biomarkers still lack sufficient specificity and sensibility to be applied in clinical practice.[20] To the best of our knowledge, this is the first study that uses the sequential window acquisition of all theoretical mass spectra (SWATH-MS) as a protein quantification tool of tear samples from patients with KC compared to control participants. SWATH-MS is a data independent acquisition method for simultaneous protein identification and quantification that allows a complete and permanent recording of all fragment ions of the detectable peptide present in a biological sample.

Therefore, the aim of this work is to perform a global quantification of the proteomic profile of the tear fluid, in order to identify differentially expressed proteins in tears of patients with KC, as well as their functional and biological relationships, that could be potential biomarkers of the disease. Moreover, the biological relationships among proteins with differential expression studied by in silico analysis would provide a better understanding of the molecular mechanisms that lead to the development of the disease.

Methods

The present study was carried out following the principles of the Declaration of Helsinki of the World Medical Association. The Ethics Committee for Clinical Research of Galicia approved the ophthalmologic protocol and the biological sample extraction to which all study participants were subjected (2019/623). All participants underwent an ophthalmologic examination that included a common clinical, topographic, aberrometric, and tomographic evaluation. Tear fluid was collected using Schirmer strips. All participants were expressly cited for the purposes of this study and properly informed prior to signing informed consent. All examinations were performed by the same two researchers. The ophthalmologic examination consisted of anamnesis and clinical evaluation, in which the collected data included age, sex, patient´s history of eye disease, patient´s medical history (allergy, eye rubbing, eye itching), positive family history of corneal ectasia, and biomicroscopic exam. Atopic conditions such as asthma, rhinitis, or atopic dermatitis were included as allergic diseases. The ophthalmologic examination also consisted of topographic, aberrometric, and tomographic evaluation, in which dioptric central power, flattest corneal meridian, steeper meridian, maximum dioptric power, inferior-superior dioptric asymmetry, Ambrosio relational thickness, D-index (Belin-Ambrosio deviation index), vertical anterior coma for 3 mm of diameter, minimum thickness point, and posterior elevation were the parameters included in this evaluation. All variables were measured using the PENTACAM tomographer (version 1.6r2031b; Oculus, Wetzlar, Germany).

Study Participants

We have designed a prospective and cross-sectional study in which 25 patients with KC and 25 control participants were enrolled. Patients with KC and control participants were recruited at the Instituto Galego de Oftalmoloxía, Santiago de Compostela, Spain. The main inclusion criteria were the KC diagnosis, supported by slit-lamp examination with the following clinical signs depending on the stage: prominent corneal nerves, Vogt's striae, Fleischer ring, and Munson sign, backed up by topography, aberrometry, and tomography evaluation. All the patients with KC did not present an evolving corneal ectasia, and their age was higher than the usual maximum age of progression of the disease. Inclusion criteria for the control group included normal clinical parameters without alterations in the tomographic evaluation or irregular astigmatisms that could suggest a subclinical state of the disease. Any control participants with family history of KC were also excluded.

The common inclusion criteria for both groups were Schirmer $\geq$15 mm in 5 minutes, conjunctival hyperemia $<$2 (Nathan Efron scale),[21] and at least 1 week with no contact lenses and no instillation of artificial tears or other eye drops. The common exclusion criteria for both groups included previous surgical intervention in the previous segment, corneal trauma or disease, existence of active ocular or systemic inflammation, current treatment with local or systemic anti-inflammatory drugs, infections in the days preceding sample collection, and pregnancy. Renal, hepatic, or hematologic diseases as well as solid tumors were also exclusion criteria for both groups.

Tear Collection, Analysis, and Quantification

Tear Collection. Tear samples were obtained by placing a Schirmer strip over the lower eyelid, approximately 3 mm from the lateral edge, without previous instillation of drugs, vital dyes, or other eye drops. For the collection of tears, the study participants closed their eyes until collecting the same amount of sample, delimited by the scale present on the Schirmer strips. One strip was used for each participant, and the total amount of tear collected corresponded to 15 mm on the strip´s scale. The samples were frozen and stored at -80°C immediately after collection. All samples were collected between January and February to avoid the influence of allergies in the tear analysis.

Tear Analysis. Tear proteins were extracted by cutting and incubating the Schirmer strips in 100 µL of 100 mM ammonium bicarbonate at room temperature for 1 hour. Samples were centrifuged for 20 minutes at 13,000 $\times$ g, and the supernatant was transferred to a new tube. Then the protein was precipitated by the MeOH/$CHCl_3$ method, and the amount of protein was measured using a RC-DC kit (Bio-Rad, Hercules, CA, USA).[22]

In relation to tryptic digestion for mass spectrometry, 100 µg 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.[23,24] 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.,[25] with minor modifications.

SWATH-MS Quantification. Once the proteins were digested, two pools (control and KC) were created using 3 µL of each individual sample. The peptide mixtures (from sample pools) were analyzed using a data-dependent acquisition (DDA) method with micro–liquid chromatography with tandem mass spectrometry (LC-MS/MS) technology to build the MS/MS spectral libraries, as previously described.[26–28] Protein and peptide identification was carried out using the Protein Pilot software (version 5.0.1; Sciex; Framingham, MA, USA) with a Human Uniprot database specifying iodoacetamide as alkylation of the cysteines. The false discovery rate was adjusted to 1% for both proteins and peptides. The MS/MS spectra, the ion data, and the retention time of the identified peptides and proteins were used to generate the spectral library. The spectral library was used to create the spectral window acquisition used in the SWATH-MS method. Then the samples (4 µL) were individually analyzed using three technical replicated by sample to ensure the mass spectometer accuray. The method is based on repeating a cycle that consists of the acquisition of a certain number of scans or time of flight mass spectometry (TOF MS/M) windows. The width of the variable windows was optimized for each set of samples according to the ionic density found in the previous DDA (files used in the library) by means of the SWATH variable window calculator (Sciex, Framingham, MA, USA) spreadsheet.

The data extraction of fragmented ion chromatographic profiles using the SWATH method was performed with the PeakView software (version 2.2; Sciex, Framingham, MA, USA) by the SWATH AcquistionMicroApp (version 2.0; Sciex, Framingham, MA, USA).

Proteins with more than 10 peptides and seven transitions (fragments) were selected based on signal intensity to generate the integrated area peaks for the quantification. Any shared or modified peptides were excluded. Integrated area peaks were exported to the MarkerView software (Sciex, Framingham, MA, USA) for relative quantitative analysis. MarkerView uses processing algorithms that accurately find chromatographic and spectral peaks direct from the raw SWATH data. Data alignment by MarkerView compensates for minor variations in both mass and retention time values, ensuring that identical compounds in different samples are accurately compared with one another. A most like ratio normalization was performed to control for possible uneven sample loss across the different samples during the sample preparation process. Unsupervised multivariate statistical analysis using principal component analysis was performed to compare the data among the samples.[29,30] The average MS peak area of each protein was derived from the biological replicates of the SWATH-MS of each sample, followed by a Student's *t*-test analysis using the MarkerView software for comparison among the samples based on the averaged area sums of all the transitions derived for each protein. The *t*-test was used to indicate how well each variable distinguishes the two groups, reported as a *P* value. The set of differentially expressed proteins ($P < 0.05$) with a 1.13-fold increase or 0.88-fold decrease was selected for each library.

Functional Enrichment and Interaction Network Analysis

In this study, a functional enrichment and interaction network analysis of the dysregulated proteins in the tear proteome between KC and control participants was performed. STRING: Functional protein association network (free access at https://string-db.org)[31] and FunRich: Functional Enrichment analysis tool[32] (open-access software version 3.1.3) were used to develop interaction maps and analyze biological processes and molecular functions. First, differently expressed proteins between KC and control tear samples were filtered by fold change (FC, whenever this was higher or lower than 1) and *P* value (<0.05). Subsequently, the list of the Uniprot codes of the proteins that met both requirements was analyzed using the STRING and FunRich databases.[33]

Statistical Analysis

Statistical analysis was made using SPSS 20.0 software for Windows (IBM, New York, NY, USA). A Kolmogorov–Smirnov test was used to determine the variable distribution. Descriptive statistical analyses were expressed as percentages for categorical variables, mean ± SD values were used for continuous quantitative variables with normal distribution, and discontinuous variables were expressed as median [quartile]. The bivariate comparison of groups was made with the χ^2 test (categorical variables), Student's *t*-test (normal continuous variables), and Mann–Whitney *U* test (no normal continuous variables). Graphic expressions of the comparisons between normal continuous variables were made using error bars, and continuous variables with nonnormal distribution were represented by box plots. Volcano plot was performed using the GraphPad Prism software (GraphPad Software, San Diego, CA, USA), and the resulting graph was generated by plotting the $\log_2$ FC for the identified proteins against their corresponding adjusted $\log_{10}$ *P* value. FC indicates upregulated proteins if FC >1 or downregulated proteins if FC <1. A $P < 0.05$ value was considered statistically significant in all tests.

Results

Clinical Features

In this study, the tear fluid from 25 eyes of 25 patients with KC (60% male; mean age, 44.88 ± 5.01 years) and 25 eyes of 25 control participants (60% male; mean age, 43.96 ± 6.94 years) were analyzed. Both groups were matched in age and sex, so no differences were found for those variables. No significant differences were found regarding the presence of allergic conditions ($P = 0.247$); however, the measurement of visual acuity was significantly lower ($P < 0.01$) in the KC group, while eye rubbing ($P < 0.05$) and ocular itching ($P < 0.05$) were significantly higher in the KC group. In relation to the biomicroscopic examination, within the study group, 48% had visible corneal nerves, 36% Fleischer rings, and 12% Voght striae. Among patients with KC, 12% of the eyes had grade I, 44% grade II, 36% grade III, and 8% grade IV, according to the Amsler–Krumeich classification.[34] As expected, the topographic, aberrometric, and tomographic variables evidenced significant differences between groups. Table 1 shows the clinical and topographic, aberrometric, and tomographic characteristics of the KC and control groups.

Proteomic Analysis

The collected tear samples were analyzed by LC-MS/MS mass spectrometry, and a quantitative analysis was carried out to identify the proteins with differential expression in the tear fluid between groups.

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Table 1. Clinical, Topographic, Aberrometric, and Tomographic Characteristics of KC and Control Groups

Characteristic	Control	KC	*P* Value
Allergic disease (%)	28	44	0.247
Itching (%)	4	72	<0.0001
Rubbing (%)	4	64	<0.0001
DCP (D)	43.30 ± 1.44	51.36 ± 6.72	<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
I-S asymmetry (D)	−0.08 ± 0.60	5.14 ± 3.54	<0.0001
ART	416 ± 60	144 ± 62	<0.0001
D	1.07 ± 0.52	10.35 ± 6.98	<0.0001
$Z_3^{\pm1}$ (µm)	1.52 ± 0.71	9.82 ± 4.15	<0.001
MTP (µm)	534 ± 22	445 ± 67	<0.0001
PE (µm)	9.20 ± 3.73	67.96 ± 31.33	<0.0001

Sample size: KC = 25 participants, 25 eyes; controls = 25 participants, 25 eyes.

ART, Ambrosio relational thickness; D, Belin-Ambrosio deviation index; DCP, dioptric central power; I-S asymmetry, inferior-superior dioptric asymmetry; K1, flattest corneal meridian; K2, steeper corneal meridian; maxDP, maximum dioptric power; MTP, minimum thickness point; PE, posterior elevation; $Z_3^{\pm1}$, vertical coma.

A total of 203 proteins were quantified in the tear preparations of KC and control groups using the SWATH-MS quantification method (available in Supplementary Table S1). Among the identified proteins, 18 showed significant differential expression between both groups. Table 2 shows the proteins with differential expression between KC and control samples, ordered from the most overexpressed (FC > 1.13) to the least expressed (FC < 0.88) in KC tears.

Among the 18 proteins with differential expression, 7 showed upregulation and 11 downregulation in KC tears.

A volcano plot (Fig. 1) was used to represent the global quantification of the tear proteins between healthy participants and patients with KC as well as the dysregulated proteins between groups.

Regarding the seven upregulated proteins in KC samples, we observed an increase in the expression of plastin 3 (FC = 1.736); DNA dC→dU-editing enzyme APOBEC-3A1 (FC = 1.555); tubulin α1C chain (FC = 1.435); 6-phosphogluconate dehydrogenase, decarboxylating (FC = 1.430); cofilin 1 (FC = 1.180); annexin A2 (FC = 1.155); and annexin A1 (FC = 1.139). The remaining 11 proteins were found downregulated in KC samples. This group included serotransferrin (FC = 0.228), serum albumin (FC = 0.255), vitamin D binding protein (FC = 0.414), α1-acid glycoprotein 1 (FC = 0.483), transthyretin (FC = 0.496), α2-HS-glycoprotein (FC = 0.540), hemopexin (FC = 0.547),

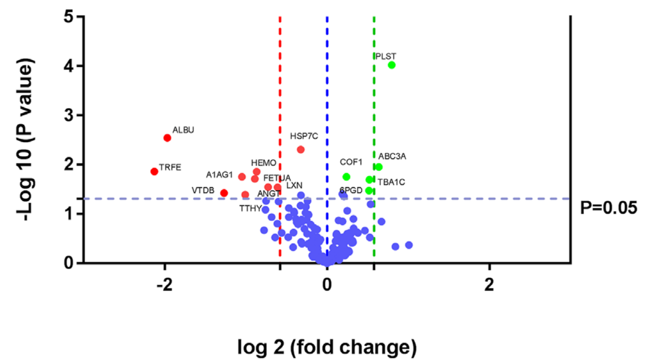

Figure 1. Volcano plot of tear quantitative proteomics data. Volcano plot shows the significantly differentially abundant proteins in tears by quantitative proteomics analysis. Proteins are ranked in a volcano plot according to their statistical *P* value (y-axis) as $-\log_{10}$ and their relative abundance ratio ($\log_2$ FC) between KC and control samples (x-axis). Off-centered spots are those that vary the most between both groups. The cutoffs for significant changes are FC of 1.13 and *P* < 0.05. *Red spots* show the downregulated proteins in KC, *green spots* show the upregulated proteins in KC, and *blue spots* show the nondysregulated proteins between both groups. A1AG1, α1-acid glycoprotein 1; ABEC3A, DNA dC→dU-editing enzyme APOBEC-3A; ALBU, serum albumin; ANGT, angiotensinogen; COF1, cofilin 1; FETUA, α2-HS-glycoprotein; HEMO, hemopexin; HSP7C, heat shock cognate 71-kDa protein; LXN, latexin; PLST, plastin 3; TBA1C, tubulin α1C chain; TRFE, serotransferrin; TTHY, transthyretin; VTDB, vitamin D binding protein; 6PGD, 6-phosphogluconate dehydrogenase decarboxylating.

Table 2. Proteins With Differential Expression Between KC and Control Group Using SWATH-MS

Protein	Uniprot Code	Protein Abbreviation	*P* Value	FC
Plastin 3	sp|P13797|PLST_HUMAN	PLS3, PLST	<0.0001	1.736
DNA dC→dU-editing enzyme APOBEC-3A	sp|P31941|ABC3A_HUMAN	ABC3A, APOBEC3A	0.011	1.555
Tubulin α1C chain	sp|Q9BQE3|TBA1C_HUMAN	TBA1C, TUBA1C	0.020	1.435
6-Phosphogluconate dehydrogenase, decarboxylating	sp|P52209|6PGD_HUMAN	6PGD, PGD	0.033	1.430
Cofilin 1	sp|P23528|COF1_HUMAN	CFL1, COF1	0.017	1.180
Annexin A2	sp|P07355|ANXA2_HUMAN	ANXA 2	0.044	1.155
Annexin A1	sp|P04083|ANXA1_HUMAN	ANXA 1	0.039	1.139
ρ GDP-dissociation inhibitor 1	sp|P52565|GDIR1_HUMAN	ARHGDIA	0.042	0.799
Heat shock cognate 71-kDa protein	sp|P11142|HSP7C_HUMAN	HSP7C, HPSA8	0.004	0.797
Latexin	sp|Q9BS40|LXN_HUMAN	LXN	0.029	0.655
Angiotensinogen	sp|P01019|ANGT_HUMAN	ANGT, AGT	0.028	0.604
Hemopexin	sp|P02790|HEMO_HUMAN	HEMO, HPX	0.014	0.547
α2-HS-glycoprotein	sp|P02765|FETUA_HUMAN	AHSG, FETUA	0.019	0.540
Transthyretin	sp|P02766|TTHY_HUMAN	TTHY, TTR	0.040	0.496
α1-Acid glycoprotein 1	sp|P02763|A1AG1_HUMAN	A1AG1, ORM1	0.018	0.483
Vitamin D binding protein	sp|P02774|VTDB_HUMAN	VTDB, GC	0.038	0.414
Serum albumin	sp|P02768|ALBU_HUMAN	ALB, ALBU	0.003	0.255
Serotransferrin	sp|P02787|TRFE_HUMAN	TRFE, TF	0.014	0.228

FC shown in upregulated proteins if FC > 1 or in downregulated proteins if FC < 1. Sample size: KC = 25 participants, 25 eyes; controls = 25 participants, 25 eyes.

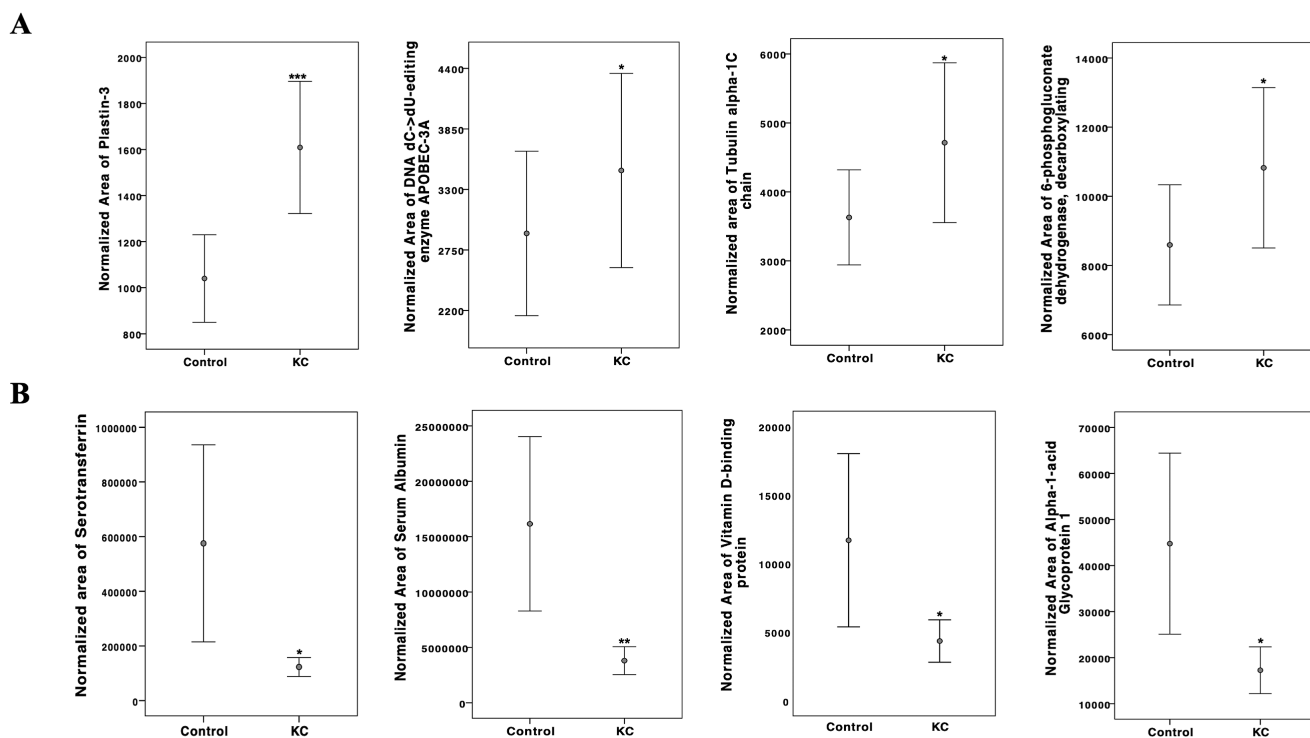

FIGURE 2. (**A**) Representation of the mean ± SD normalized area of the four most overexpressed proteins in the tears of patients with KC. (**B**) Representation of the mean ± SD normalized area of the four most underexpressed proteins in the tears of patients with KC. The normalized area was obtained from the SWATH method for each individual sample. The mean ± SD of the normalized area for each group was represented by error bars for having a normal distribution in the Kolmogorov–Smirnov test. Statistical differences with regard to controls: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (*P* values in Table 2). Sample size: control = 25 eyes; KC = 25 eyes.

angiotensinogen (FC = 0.604), latexin (FC = 0.655), heat shock cognate 71-kDa protein (FC = 0.797), and ρ GDP-dissociation inhibitor 1 (FC = 0.799), cited from most to least underexpressed. Figure 2 shows the four most overexpressed and the four more underexpressed proteins in KC tears in comparison to the control group.

Biological Pathways and Molecular Functions of Dysregulated Proteins in KC Tears

To achieve a better understanding of the biological importance of differential proteins as well as their possible implications in the pathophysiology of the KC, we observed their molecular functions and biological implications performing in silico analysis with STRING, a protein–protein interaction network tool. In this way, a map of the interaction of proteins and their main biological functions was obtained. Figure 3 shows the most representative biological processes involving the overexpressed proteins. These processes included IL-12–mediated signaling pathway, positive regulation of vesicle fusion, regulation of wound healing, actin cytoskeleton organization, and supramolecular fiber organization.

In relation to the 11 downregulated proteins, a decrease in the expression of proteins involved in transport processes, iron transport and homeostasis, extracellular organization, negative regulation of endopeptidase activity, and acute phase and inflammatory response was observed. All these processes are represented in Figure 4.

Complementary, we carried out a search for biological processes and functions of the differentially expressed proteins using the FunRich tool. According to the previous analysis, the results revealed the involvement of overexpressed proteins in the regulation of apoptotic processes, response to hydrogen peroxides, and innate immune response, as well as the participation of downexpressed proteins in iron homeostasis and transport, vitamin D metabolic process, vitamin transport, and inflammatory processes. Figure 5 summarizes the main biological processes and molecular functions of the differentially expressed proteins in tears from patients with KC.

DISCUSSION

In this study, we have focused on the complete analysis of the tear proteome to elucidate proteins with differential expression in KC disease and to study their possible functional relationships.

MS in association with LC has become a commonly used method to study disease markers in different types of samples such as tissues or biological fluids.[35] In this work, we used the LC-MS technique for analyzing proteins associated with KC in tear samples and SWATH-MS as a quantification method. LC combined with MS has been previously used for tear analysis of patients with KC, although with different protein quantification methods.[36,37]

Among the total proteins quantified, 18 showed a differential expression between groups. These proteins were fundamentally related to structural, inflammatory, and oxidative stress and iron transport and homeostasis processes.

In relation to structural processes, three of the most overexpressed proteins in the tears of patients with KC were associated with the organization and composition of the

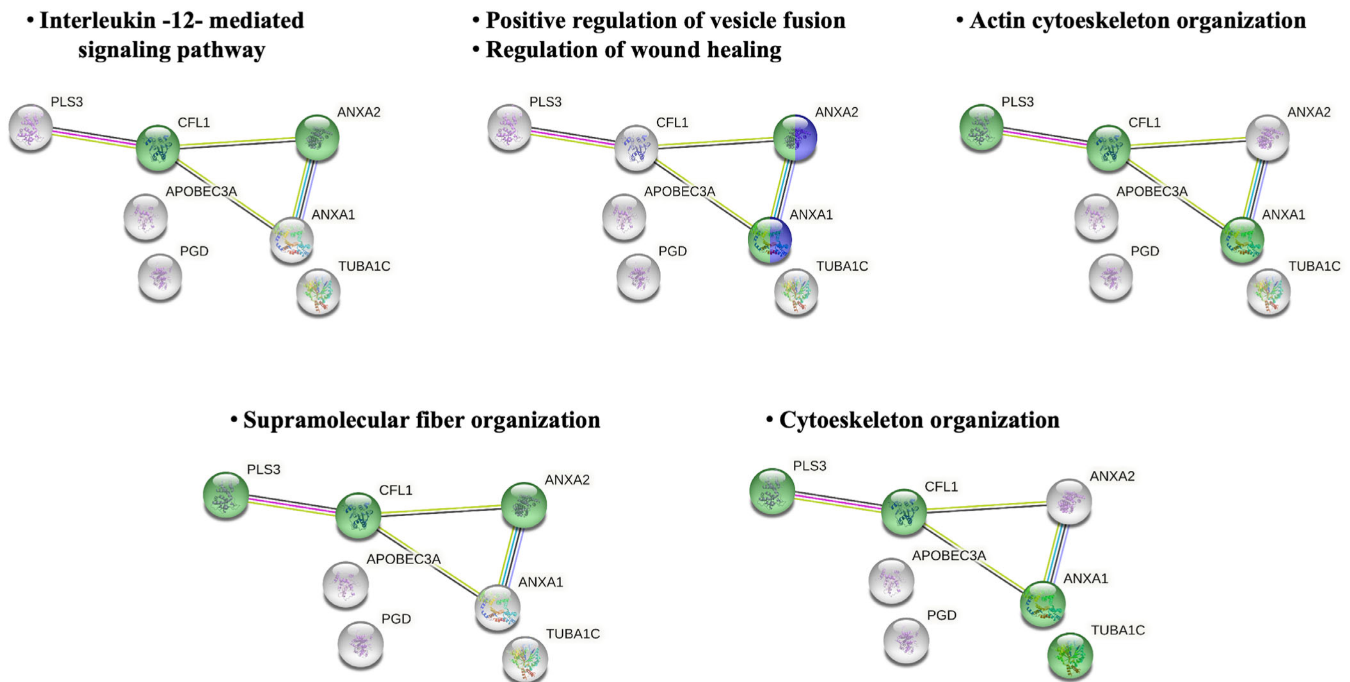

Figure 3. Main biological processes of proteins upregulated in KC samples. Protein–protein interactions were made using the STRING interaction network. Proteins upregulated are represented as a cluster. The *colored* proteins in the clusters are involved in the indicated biological process. Classification of proteins was based on Gene Ontology (GO) biological processes. All the biological processes that are referred to present a false discovery rate (FDR) less than 0.05. ANXA1, annexin A1; ANXA2, annexin A2; APOBEC3A, DNA dC→>dU-editing enzyme APOBEC-3A; CFL1, cofilin 1; PLS3, plastin 3; PGD, 6-phosphogluconate dehydrogenase decarboxylating; TUBA1C, tubulin α1C chain.

cytoskeleton. Plastin 3, an actin clustering protein with an important role in the organization of the cytoskeleton,[38] was the most overexpressed protein in the tears of patients with KC. In the same way, we observed an increase in cofilin 1, an actin binding protein involved in the regulation of actin filament length and organization of the cytoskeleton,[39] and in tubulin α1-C, a protein from the tubulin family that constitutes one of the main components of microtubules. Our results are in line with previous studies that have reported an overexpression of proteins of the tubulin family in epithelial samples of keratoconic corneas[40] and suggest that the accumulation of structural proteins (in tears or in corneal tissues) could be due to an increase in their synthesis to cope with the thinning of the corneal tissue induced by the increase in degradative proteins.[40]

The influence of innate immunity as well as inflammatory processes on KC pathophysiology has already been described in numerous studies. Specifically, previous studies observed an increase in proinflammatory proteins such as IL-6 and TNF-α[19] in tear samples, suggesting the involvement of chronic inflammatory events in KC pathogenesis. A recent study from our group confirmed the overexpression of Toll-like receptors 2 and 4 in corneal and conjunctival epithelial samples of patients with KC, in agreement with the implication of innate immunity in the disease progression.[7,41] In this case, our results showed differences in the expression of some proteins such as annexin 1 (upregulated), α1-acid glycoprotein 1, and α2-HS-glycoprotein (downregulated) in the tears of patients with KC, which are involved in the regulation of the inflammatory and immune response. Previous studies in aqueous humor samples also observed a decrease in α1-acid glycoprotein 1 levels compared to control samples,[42] evidencing an imbalance between pro- and anti-inflammatory proteins in the tissues and the biological fluids of patients with KC. Vitamin D binding protein was the third most underexpressed protein in the tear samples of KC eyes. This is the main vitamin D transport protein in the body, and it is essential for maintaining circulating vitamin D levels and preventing its deficiency. This protein has a wide variety of functions, and at the tear level, it has shown anti-inflammatory properties, playing an important role in the protection of the ocular surface. Previous studies reported a deficit in vitamin D levels in the blood samples of patients with KC, as well as a negative correlation between vitamin D levels and the progression of the disease.[43–45] Under normal conditions, vitamin D levels are higher in tears than in blood[46]; therefore, the reported deficit in blood may be in good agreement with the deficit we have observed in tears.

Serotransferrin was the highest underexpressed protein compared with the control group. Serotransferrin is an essential glycoprotein for iron transport that binds to free iron ions that are toxic and potentially damaging for tissues.[47] Indeed, free iron ions (unbounded to serotransferrin or other iron-transport proteins) may accumulates in tissues, leading to an increase of oxidative stress and reactive species, which promotes cellular damage and tissue injury.[48,49] The decrease in serotransferrin levels was also reported in the corneal stroma of patients with KC, and previous studies associated certain polymorphisms of the transferrin gene with the risk of the appearance of KC.[50] Similar to serotransferrin, some studies have already reported the underexpression of lactoferrin in tears of these patients.[6,18] Lactoferrin is also a protein of the transferrin family that, in addition to iron transport, has antimicrobial, anti-inflammatory, antioxidant, and immunomodulatory

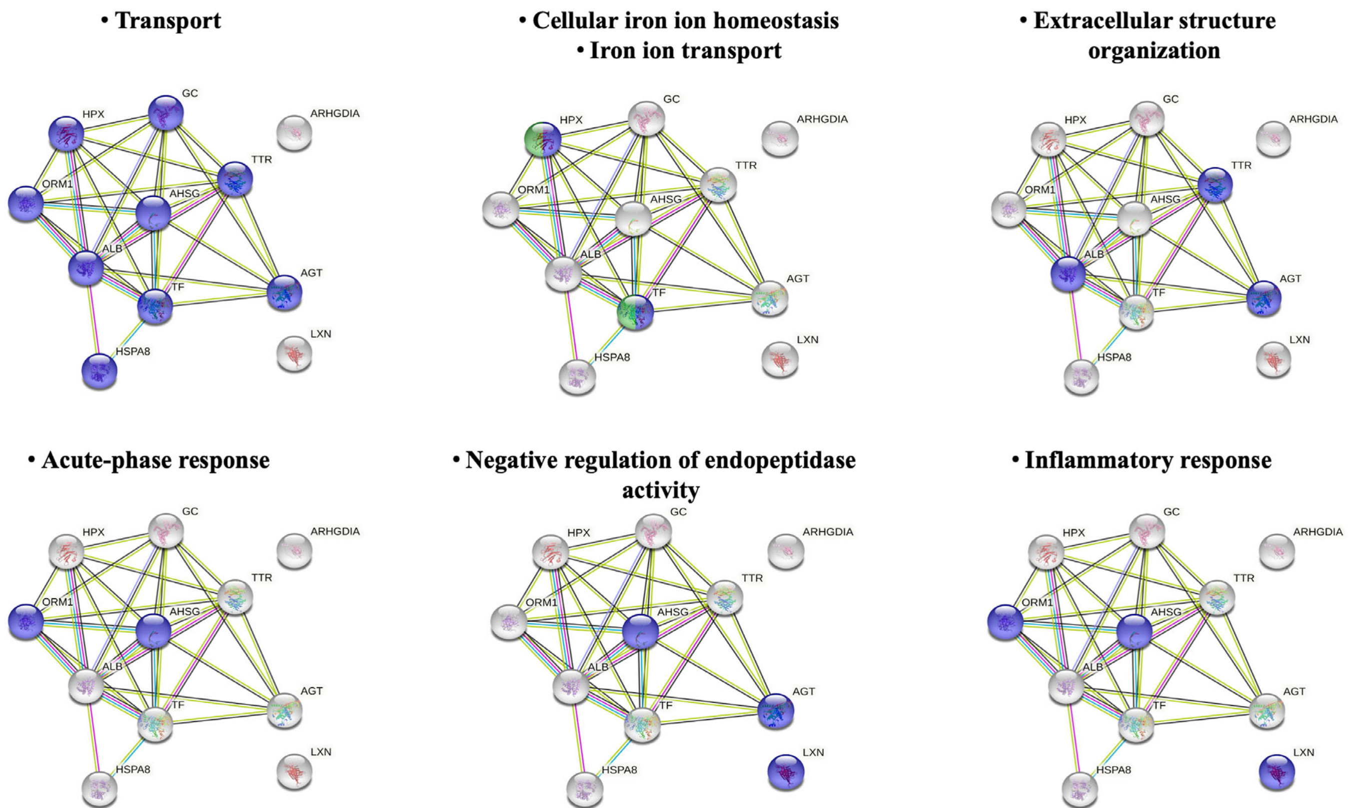

Figure 4. Main biological processes of proteins downregulated in KC samples. Protein–protein interactions were made using the STRING interaction network. Proteins downregulated are represented as a cluster. The *colored* proteins in the clusters are involved in the indicated biological process. Classification of proteins was based on GO biological processes. All the biological processes that are referred to present an FDR less than 0.05. AGT, angiotensinogen; AHSG, α2-HS-glycoprotein; ALB, serum albumin; ARHGDIA, ρ GDP-dissociation inhibitor 1; GC, vitamin D binding protein; HPSA8, heat shock cognate 71-kDa protein; HPX, hemopexin; LXN, latexin; ORM1, α1-acid glycoprotein 1; TF, serotransferrin; TTR, transthyretin.

functions in its apo (iron-free) form.[51] The importance of lactoferrin underexpression in tears of patients with KC has been previously associated with its anti-inflammatory and immunomodulatory properties; however, it is possible that its underexpression contributes to increased oxidative stress due to its function as an iron transporter.

Extracellular iron is usually found in normal tear composition, transported among tissues by proteins such as lactoferrin or transferrin.[49] Taking into account that one of the main histologic characteristics of keratoconic corneas is the iron deposition in the epithelial basement membrane (Fleischer ring), a decrease in the expression of iron-binding proteins in tears could contribute to its filtration and accumulation in the corneal epithelial tissue, whose toxicity would lead to an oxidative microenvironment and cell damage or death cell processes such as ferroptosis.[52] Ferroptosis is an iron-dependent cell death process that has been discovered in recent years and is often accompanied by iron accumulation and lipid peroxidation. Recent research has demonstrated that this type of cell death process plays an important role in the appearance and progression of numerous diseases, such as Parkinson and other neurologic diseases[53,54]; therefore, the study of their biological effects has become a key point for the development of new therapeutic strategies. In the case of KC, the alteration of iron transporting proteins, its accumulation in the epithelial tissue, the increase of reactive species, and the decrease in the expression of proteins with antioxidant activity may indicate the possible implication of this mechanism in the death of stromal keratocytes and in the epithelial degeneration.[12,55–57]

Regarding the increase of oxidative stress levels, in this study, we have found a decrease in serum albumin, hemopexin, and heat shock cognate 71 kDa. Recently, albumin has been associated with antioxidant and antiapoptotic mechanisms as well as vascular integrity. Elevated levels of albumin in tears are used as a marker of active inflammation and ocular damage,[58] but in KC pathology, the decrease in tear albumin levels could affect its antioxidant and antiapoptotic properties, aggravating the processes of cell death and oxidative stress characteristic of the pathophysiology of the disease.[59]

Hemopexin and heat shock cognate 71 kDa are proteins that present antioxidant activity and are part of the response to oxidative stress.[60] Considering that the cornea is potentially susceptible to increased levels of oxidative stress due to constant exposure to ultraviolet light, our results suggest that the antioxidant defense mechanisms and neutralization of reactive species in tears may not be sufficient and contribute to increased oxidative stress in tissues.

Balasubramanian et al.[61] described the dysregulation of five proteins (cathepsin, cystatin S, cystatin SN, α-fibrinogen, and PIGR) in the tears of patients with KC, mainly related to wound healing and proteolysis. Also, regarding cell

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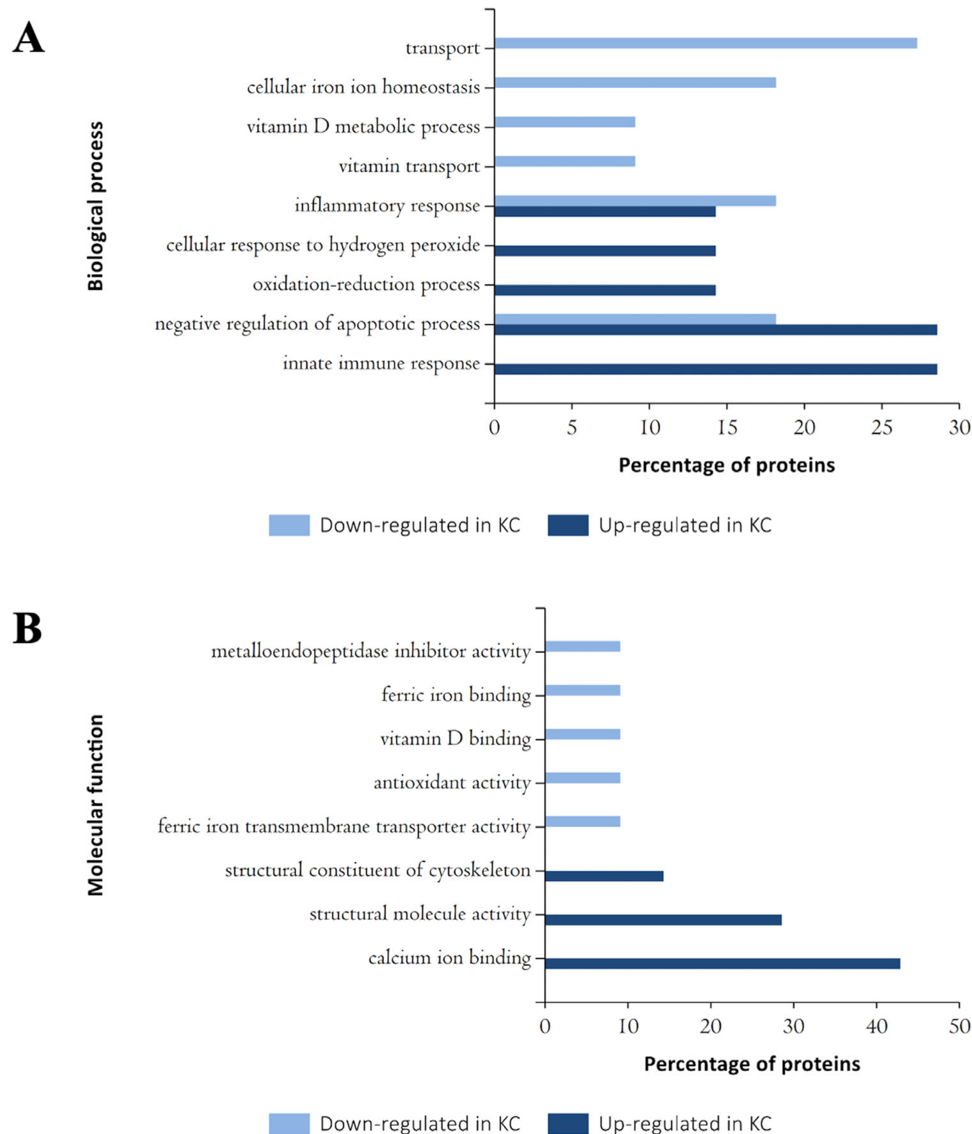

FIGURE 5. (**A**) Biological processes mainly related to the differentially expressed proteins. (**B**) Molecular functions mainly related to the differentially expressed proteins. GO enrichment of dysregulated proteins used the FunRich functional tool. The histograms represent the main categories for each GO term in which differentially expressed proteins were involved ($P < 0.05$). The y-axis shows the biological processes, and the x-axis shows the percentage of proteins involved in this process compared with the total proteins in the database.

damage, we observed a decrease in the expression of latexin, angiotensinogen, and α2-HS-glycoprotein, which belong to the family of protease inhibitor proteins and could compromise the integrity of the corneal tissue due to an increase in the activity of proteases.

Although this study used a novel MS method (SWATH-MS) for analyzing the proteomic profile of tear samples in KC, it has some limitations. The SWATH method is a specific, reproducible, and sensitive approach that allows relative or absolute quantification of proteins. However, the number of proteins identified/quantified is mostly limited by the composition of the spectral library, which was created using a pool of proteins for each condition. In the SWATH technique, seven transitions are quantified for each peptide. Therefore, 10 peptides per protein must be identified to extract the peak areas needed for quantification. Consequently, library proteins in which fewer than 10 peptides are identified will not be quantified by the SWATH method.

Moreover, in this study, the biological functions and relationships between the dysregulated proteins have only been analyzed in silico, so in vitro assays should be developed to confirm the protein–protein interactions as well as validate our results as potential biomarker for the KC disease.

In relation to the sample size, the present study was conducted on a small sample, which did not allow for stratification of the eyes according to the degree of the disease, and in addition, a number of employed KC eyes showed moderated degrees of the disease, but a smaller number of patients had slight or severe degrees.

These weaknesses should be considered in future studies for elucidating the molecular and biological processes

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that lead to the progression of the disease as well as those alterations related to the different stages of the disease.

Overall, our results in tear fluid have shown the implication of several biological processes in the KC pathophysiology, such as oxidative stress, matrix degradation, cellular death, and immune or inflammatory responses, pathways that have also been referred to in the corneal tissues.[9,55,62,63] Therefore, tear fluid becomes a good alternative for the study of the KC pathophysiology, being able to reflect the molecular mechanisms that determine the pathologic conditions of the disease. However, despite the fact that the biological pathways involved appear to be clear, the triggering factor or factors remain potentially unknown.

In conclusion, our findings strengthen the theory by describing keratoconus as a pathology of multifactorial origin involving genetic, environmental, and biomolecular factors. The biological processes that are involved in its pathophysiology are partially understood (inflammatory, immune, oxidative stress, and cell death), and the decrease in proteins that participate in transport and iron homeostasis that we observed at the tear level could make evident another fundamental pathway in the pathogenesis of the disease. These findings may be key to the development of therapeutic strategies to cope with the tissue damage that derives from iron accumulation and toxicity. A better understanding of the cytotoxic mechanisms triggered by the accumulation of iron in corneal tissues is necessary, as well as a comprehensive research related to the alteration of this event in the preclinical stages of the disease.

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