

Proteomic analysis of the tear film in patients with keratoconus

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Purpose: To identify proteins differentially expressed between the tear film of keratoconus (KC) patients and control subjects using two dimensional electrophoresis (2-DE) and mass spectrometry-based techniques.

Methods: Twenty two patients (44 eyes) diagnosed with bilateral KC and 22 control subjects (44 eyes) were studied in a prospective case-control study. Keratoconus screening programs and Orbscan II topographies were performed on all participants. Tear samples were collected by the Schirmer I method using filter paper. Proteins were extracted from the Schirmer strips and separated by 2-DE. Comparison of protein patterns was performed using PDQuest Software and protein differences were identified by mass spectrometry. Finally, results were validated by western-blot.

Results: Four spots were identified to be differentially expressed between KC patients and control subjects. Three of them were more expressed in healthy subjects and they were identified as zinc- α 2-glycoprotein (ZAG), lactoferrin, and IGKC (immunoglobulin kappa chain). The other spot was more expressed in KC patients and it was identified as ZAG. Differences in ZAG seem controversial in two different spots because different posttranslational modifications, however, analysis of both spots revealed that globally, ZAG is overexpressed in healthy subjects. Found differences in ZAG, lactoferrin, and IGKC expression were subsequently validated by western blot.

Conclusions: IGKC protein, ZAG, and lactoferrin are under-expressed in the tears of patients diagnosed with bilateral KC compared with healthy subjects. These differences could contribute to the knowledge of the pathophysiology of this disease.

Keratoconus (KC) is a primary corneal ectasia, generally bilateral and progressive, with a conical shape as a result of the thinning of the stroma. This thinning induces irregular astigmatism, myopia, and protusion, leading to mild to marked impairment in the quality of vision [1]. KC has its onset at puberty, and it progresses until the third or fourth decade of life. The calculated incidence of KC is between 1/500 and 1/2,000 in the general population [1,2]. KC affects young persons in a very productive period of their lives. Yet the cause of this disorder, considered to be the most common indication for penetrating keratoplasty in developed countries [3], remains unknown.

KC may be isolated or may be associated with various clinical conditions, or even form part of a syndrome. Its etiology remains poorly understood, but it has been reported to arise as a consequence of biochemical alterations to corneal collagenase, which breaks down collagen leading to stromal thinning [4]. Other authors have suggested that it may arise as a consequence of keratocyte loss [5], which is on average about 19% lower in contact lens users [6]. In some atopic diseases, KC may be due to apoptosis of corneal cells, possibly

mediated by interleukin-1 [7]. Rahí et al. [8], found atopic diseases in 35% of patients with KC, in contrast to 12% of control subjects. KC is frequently associated with ocular itchiness and rubbing, provoking chronic epithelial lesions [7]. The cornea is part of an integrated system - the ocular surface - which contains specific and non-specific immune molecules. Tissue degradation in thinning disorders, such as KC, involves the expression of inflammatory mediators, such as proinflammatory cytokines, cell adhesion molecules, and matrix metalloproteinases [9-12]. Moreover, previous studies from our group have demonstrated that the levels of proinflammatory cytokines (interleukin-6 [IL-6] and the tumor necrosis factor alpha [TNF- α]) and matrix metalloproteinase-9 (MMP-9) are significantly increased in the tears of patients with KC [13]. Despite these advances, there are currently no diagnostic markers of KC and the pathophysiology of this disease is largely unknown.

Proteomics is a discipline that study complex mixtures of proteins. Two-dimensional electrophoresis (2-DE) and mass spectrometry are very useful techniques to find out protein differences between different types of samples. Tear proteomics has been used to find out potential cancer markers [14], to study responses in patients with fungal keratitis [15] and for identification of tear proteases [16].

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TABLE 1. CLINICAL CHARACTERISTICS OF PATIENTS WITH KERATOCONUS AND CONTROL SUBJECTS.

Variables	Keratoconus (n=22)	Control (n=22)	p
Age (years)	31.3±7.8	30.8±7.6	0.876
Males, n (%)	11 (50.0)	12 (54.5)	0.763
Atopic disease, n (%)	10 (45.5)	5 (22.7)	0.052
Eye rubbing, n (%)	12 (54.5)	3 (13.6)	0.005
K ₂ , diopters	54.8±7.1	43.7±1.6	<0.0001
Thinnest P (µm)	386.2±71.6	542±23.9	<0.0001

Table 1 shows clinical characteristics of patients with keratoconus and control subjects. No age-related or sex-related statistical differences were detected between KC patients and control subjects. A family history of KC was reported for 3 patients (13.6%) and 1 control (4.5%). Ten KC patients (45.5%) and five controls (22.7%) were diagnosed as having atopic disease (most commonly to pollen or acaroids). Twelve KC patients (54.5%) admitted frequent and vigorous eye rubbing and 3 control eye rubbing (13.6%).

In this study we used proteomic tools, such as two dimensional electrophoresis (2-DE) and mass spectrometry, to find out protein differences in tear samples that allow the differentiation between eyes of KC patients and normal controls.

METHODS

Subjects and clinical examination: We have designed a prospective, case-controlled study in which 22 bilateral KC patients (44 eyes) and 22 normal control subjects (44 eyes) were recruited from the Contact lens Unit, Instituto Galego de Oftalmología, Complejo Hospitalario Universitario de Santiago de Compostela, Spain. KC patients (50.0% male; mean age, 31.3±7.8 years) and control subjects (54.5% male; mean age, 30.8±7.6 years) had not worn contact lenses for a week prior the study. All patients were expressly cited for the purposes of this study, and all examinations were performed by the same researcher between September 2007 and April 2008. Data collected included gender, age, patient's ocular history (eye rubbing), medical history (atopy), and family history of KC. This research was performed in accordance with the Declaration of Helsinki of the World Medical Association (2000). Protocol was approved by the Hospital's Research Ethics Committee, and informed consent was given by all patients and control subjects.

The biomicroscopy exam was performed to detect signs of KC and the corneal topography study was performed to quantify topographic parameters. The modified Rabinowitz-McDonnell test was used to confirm the diagnosis of KC, and the severity of KC was classified according to the steepest simulated keratometry reading on the keratometric map (K₂ <45 D, mild; K₂ between 45 and 52 D, moderate; K₂ >52 D, severe KC) [17].

Inclusion criteria were; 1) Bilateral KC patients, severe in both eyes or severe and moderate, with biomicroscopic signs in both eyes. 2) Subjects in the control group with normal corneal topography maps.

Exclusion criteria included; 1) Previous surgical intervention in the anterior segment, or childhood corneal trauma or disease. 2) Existence of active or systemic inflammation, or ocular disease, or current treatment with systemic or local anti-inflammatory drugs.

Instrumental: Basic examination instruments were a Topcon biomicroscope and Topcon refraction column (Topcon, Esseebaan, Netherlands). As specific examination instruments we used a Keratron Scout (Opticon 2000; SpA, Rome, Italy) and an Orbscan II corneal topographer (Orbtek, Salt Lake City, UT).

Two-dimensional electrophoresis was performed in IPGphor (Bio-Rad, Hercules, CA) for the first dimension and ETTAN-DALTSIX for second dimension (GE, Uppsala, Sweden). Western blot was performed using Miniprotean 3 system and Trans blot SD semi-dry transfer cell (Bio-Rad). Gels and blots were digitized in FX-Proplus (Bio-Rad). Finally, MALDI-TOF mass spectrometer (MS) analyses were performed using a 4700 Proteomics Analyzer MALDI TOF/TOF MS (Applied Biosystems, Carlsbad, CA).

Tear analysis: Tear samples were obtained by placing a Schirmer strip over the lower lip, approximately 3 mm from the lateral canthus, without previous instillation of drugs or vital dyes. The subject was instructed to close the eyes for the 5 min test duration, the wet length was not recorded but was observed to be within normal ranges in all cases (>10 mm). We collected four Schirmer strip for subject; samples were frozen and stored at -20 °C immediately. Proteins were extracted from the Schirmer strips by incubating with 200 µl of 100 mM ammonium bicarbonate at room temperature for 1 h. Then 600 µl cold acetone were added and incubated for 2 h at -20 °C. Precipitation of proteins was performed by centrifugation at 18,000× g. Precipitated proteins were resuspended in solubilization buffer (9 M urea, 2% CHAPS). Proteins were quantified by modified Bradford assay using BSA as protein standard.

Two-dimensional electrophoresis: Two replicate gels were performed for 12 patients (24 eyes) and 12 control subjects

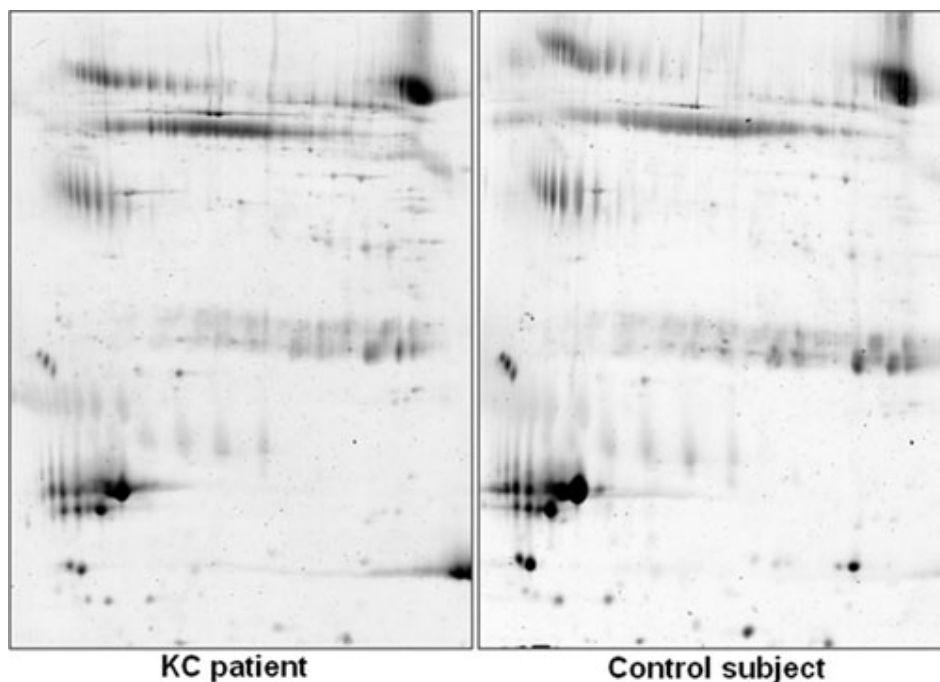


Figure 1. Master gel created by combining spots from all gels of keratoconus patients and control subjects.

(24 eyes). Protein (20 μ g) was applied on 17 cm 3–10NL IPG strips and first dimension was performed until 45 kV h was achieved. Second dimension of gel electrophoresis was performed on 12.5% polyacrilamide gels on ETTAN-DALTSIX (GE) at 5 W per gel for 15 min, and then at 17 W per gel until the dye front reached the bottom of the gel. Analytical gels were stained with Flamingo Fluorescent Stain and visualized on Molecular Imager FX Pro-plus (Bio-Rad). Preparative gels were stained in 0.1% Coomassie Blue Brilliant R-250.

Gels were digitalized using a Bio-Rad Molecular Imager FX Pro-plus. Protein patterns were analyzed using PDQuest 7.0 from Bio-Rad. Gel spots of interest were manually excised from preparative gels and digested according to Shevchenko et al. [18].

MALDI-TOF MS analyses were performed. The obtained peptide mass fingerprints were used for protein identification. The non-redundant NCBI or SwissProt database was searched using MASCOT 1.9 through the Global Protein Server v3.5 from Applied Biosystems. All determinations were performed without knowledge of the corresponding clinical data (blind test).

Western blot: Tears collected from 10 patients (20 eyes) with KC and 10 control subjects (20 eyes) were used to subsequent validation of expression differences found using proteomics. Ten μ g of total protein extracted from the Schirmer strips were loaded and size-separated in a 10% sodium dodecyl sulfate-polyacrilamide gel electrophoresis (95 V). The proteins were blotted in semi-dry conditions using polyvinyl difluoride membranes (Millipore, Billerica, MA) and incubated with

primary antibodies raised in mouse against lactoferrin (1:2,000; Abcam, Cambridge, UK), immunoglobulin kappa constant (IGKC) (1:2,000; Abcam) and zinc- α 2-glycoprotein (1:700; Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4 °C. For detection, a goat anti-mouse Cy3-labeled secondary antibody (1:3,000; GE) was used and images were acquired using Molecular Imager FX Pro-plus (BioRad). QuantityOne software (BioRad) was used to analyze the expression of the different bands of protein. To verify equal protein loading, membranes were stripped at 50 °C for 30 min in stripping buffer containing 62.5 mM Tris, 2% SDS and 100 mM β -ME, subsequently re-blocked and probed with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (1:3,000; Abcam). Experiments were performed in duplicate.

Statistical analysis: Descriptive statistical analyses were performed with percentage for categorical variables. Discontinuous variables were expressed as median [quartiles]. Statistical significance for intergroup differences was assessed by the χ^2 test for categorical variables. One-way ANOVA and the Mann–Whitney test were performed for comparison between groups. A value of $p < 0.05$ was considered to be statistically significant. On the other hand, protein patterns were analyzed using PDQuest 7.0 from Bio-Rad. The reproducibility of the gels was calculated by Pearson's coefficient. Differences were considered when spot intensity was significantly different between patients and controls ($p < 0.01$) and at least two times greater in one group respect to the other. The statistical analysis was conducted

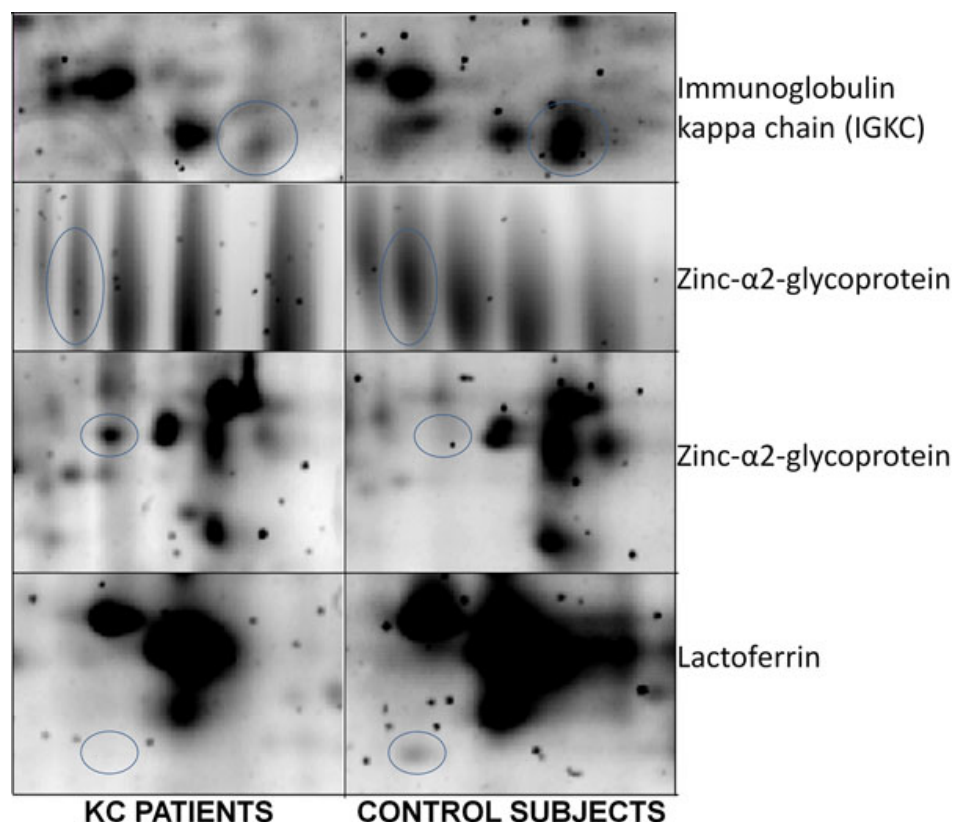


Figure 2. Two-dimensional gel fragments showing differentially expressed proteins between keratoconus patients and control subjects. Three of them were more expressed in control subjects and they were identified as zinc- α 2-glycoprotein (ZAG), lactoferrin, and IGKC (immunoglobulin kappa chain). The other spot was more expressed in KC patients and it was identified as ZAG.

TABLE 2. PROTEIN EXPRESSION VALUES FOR THE DIFFERENTIALLY EXPRESSED PROTEINS BETWEEN PATIENTS WITH KERATOCONUS AND CONTROL SUBJECTS. THE VALUES OF THE PROTEIN EXPRESSION ARE SHOWN AS MEAN SPOT INTENSITY (A.U.).

Variables	Keratoconus (n=12)	Control (n=12)	p
IGKC*	113.7 \pm 99.6	370.3 \pm 96.2	<0.001
ZAG†	819.1 \pm 247.2	2939.7 \pm 1700.9	<0.001
ZAG	63.5 \pm 23.4	16.8 \pm 2.9	<0.001
Lactoferrin	6.4 \pm 4.0	23.1 \pm 18.5	<0.001

*IGKC: immunoglobulin kappa chain. †ZAG: zinc- α 2-glycoprotein.

using SPSS 16.0 (IBM, Chicago IL) for Windows XP (Microsoft Corporation, Redmond, WA).

RESULTS

Clinical Features: All of the KC eyes showed either moderate or severe stage of progression: 18 eyes (40.9%) presented moderate KC (K_2 between 45 D and 52 D), 26 eyes (59.1%) had severe KC ($K_2 > 52$ D).

Mean K_2 was 54.8 \pm 7.1 D in the KC eyes, versus 43.7 \pm 1.6 D in the control subjects ($p < 0.0001$). Mean of the thinnest point pachymetry was 386.2 \pm 71.6 μ m in the KC eyes, versus 542 \pm 23.9 μ m in the control subjects eyes ($p < 0.0001$).

Proteomic analysis: An average of 532 spots was observed in both groups (Figure 1). Gels were highly reproducible ($r^2 = 0.95$) between groups. No qualitative differences have been found, however three spots were found to be expressed

at least twofold in healthy subjects than in KC patients and one spot was found twofold in KC patients than in healthy subjects (Figure 2). The expression levels are shown in Table 2. Furthermore these differences were statistically significant ($p < 0.001$). Mass spectrometry analysis revealed that quantitative differences more expressed in healthy subjects were; IGKC (Immunoglobulin Kappa Chain) protein, zinc- α 2-glycoprotein (ZAG; Figure 3), and lactoferrin and quantitative difference more expressed in KC patients was identified as ZAG.

Western blot: Western blot analysis for IGKC protein, ZAG, and lactoferrin showed a statistically significant decrease in protein expression (optic mean intensity) in KC patients compared to healthy controls ($p < 0.05$; Figure 4).

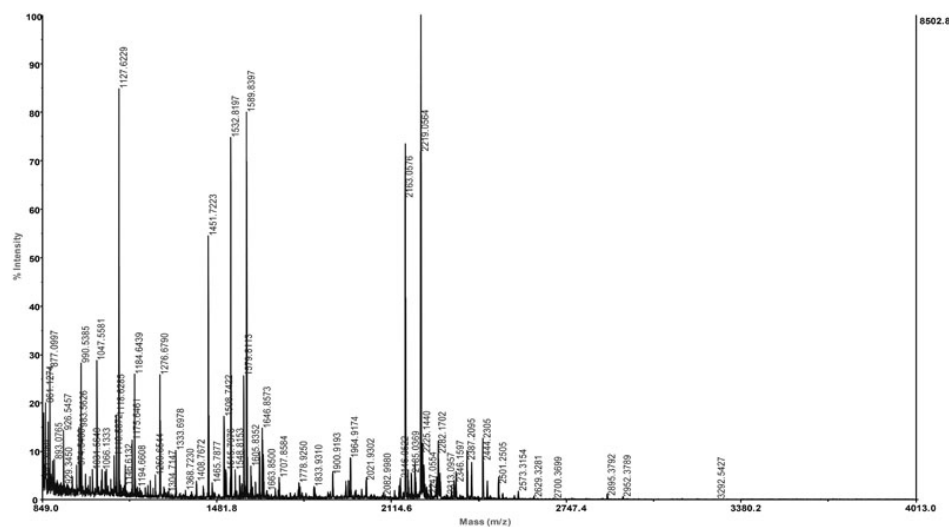


Figure 3. A typical profile of molecular mass of tryptic fragments of zinc- α 2-glycoprotein (ZAG) during analysis by MALDI-TOF mass spectrometry.

DISCUSSION

Despite extensive basic and clinical studies of keratoconus in recent years, the precise mechanisms underlying this pathology still remain largely unknown. In this study, we performed a detailed proteomic analysis of both eyes in patients with bilateral KC and control subjects. Our results show the usefulness of proteomics to find out protein differences between KC patients and healthy subjects in tear samples. By comparing the global protein expression in tears, we found three spots whose expression was greater in the healthy subjects (IGKC protein, ZAG, and lactoferrin) and one spot was found to be more expressed in KC patients (ZAG). It is not strange to detect two or even more spots that belong to the same protein in a 2-DE gel. This is an indication that the protein has different posttranslational modifications. In our case, we have found two different spots for ZAG, one of them is threefold less expressed in KC patients respect to the controls, while the second spot is fourfold more expressed in patients than in control subjects. This is not a contradiction; one posttranslational modification could be more expressed in one group while the other could be just in the opposite way. Proteomics papers have described differences in posttranslational modifications in tears, specially homopolymers formation, protease degradation and glycosilation. Since ZAG is a glycoprotein, we hypothesized that posttranslational modification should be a glycosilation [19]. Nevertheless, what it is important is that overall the protein is more expressed in controls than in patients, as reflected in the western-blot results, where each band includes all posttranslational modifications (spots in 2-DE gels) of a protein. Therefore, we conclude that ZAG is under-expressed in KC patients respect to healthy subjects.

Tear lactoferrin is an 82 kDa protein produced in the acinar cells of the lacrimal gland. Lactoferrin is present in normal tears of humans and was found to be invariant through age and independent of sex. It has been reported reduced levels

in certain diseases such as Sjögren syndrome, idiopathic dry eye, vernal conjunctivitis, contact lens induced giant papillary conjunctivitis, trachoma, herpes simplex keratitis, chronic irritative conjunctivitis keratoconjunctivitis sicca, and clinical dry eye with a marked keratopathy [20]. In tear film lactoferrin is thought to be antimicrobial and antiinflammatory. In particular, it has been demonstrated that Lipopolysaccharide-induced production of IL-6, TNF- α , IL-1- β , and IL-8 is inhibited by lactoferrin, in various human monocytic cell lines [21-24]. Previous studies from our group have demonstrated that the levels of proinflammatory cytokines (interleukin-6 [IL-6] and the tumor necrosis factor alpha [TNF- α]) are significantly increased in the tears of patients with KC [13]. Likewise, it has been postulated that reactive oxygen and nitrogen species are raised in keratoconus that, in turn, induce collagenases and gelatinases responsible for the corneal thinning process in this disease [13,25]. Consequently, it is tempting to postulate that lactoferrin might be protective in keratoconus because of its role in decreasing the expression of proinflammatory cytokines and collagenases.

ZAG is a 41-kDa protein component of plasma, saliva, and tears. It stimulates lipid breakdown in adipocytes and is associated to the extreme weight loss that occurs in some cancers [26]. On the other hand, structural organization and fold is similar to MHC class I antigen-presenting molecule; hence, ZAG may have a role in the expression of the immune response [27]. We have demonstrated that the tears of patients with keratoconus contain a smaller amount of ZAG. Contradictory results were found in patients with Graves ophthalmopathy where the expression of ZAG is increased [28]. However, the specific role of ZAG in tears is not known.

Finally, we found a significant decrease in protein expression for IGKC in KC patients. IGKC and immunoglobulin kappa light chain variable region constitutes the light chains of an antibody and is related with immune response. In recent years, several clinical studies of KC

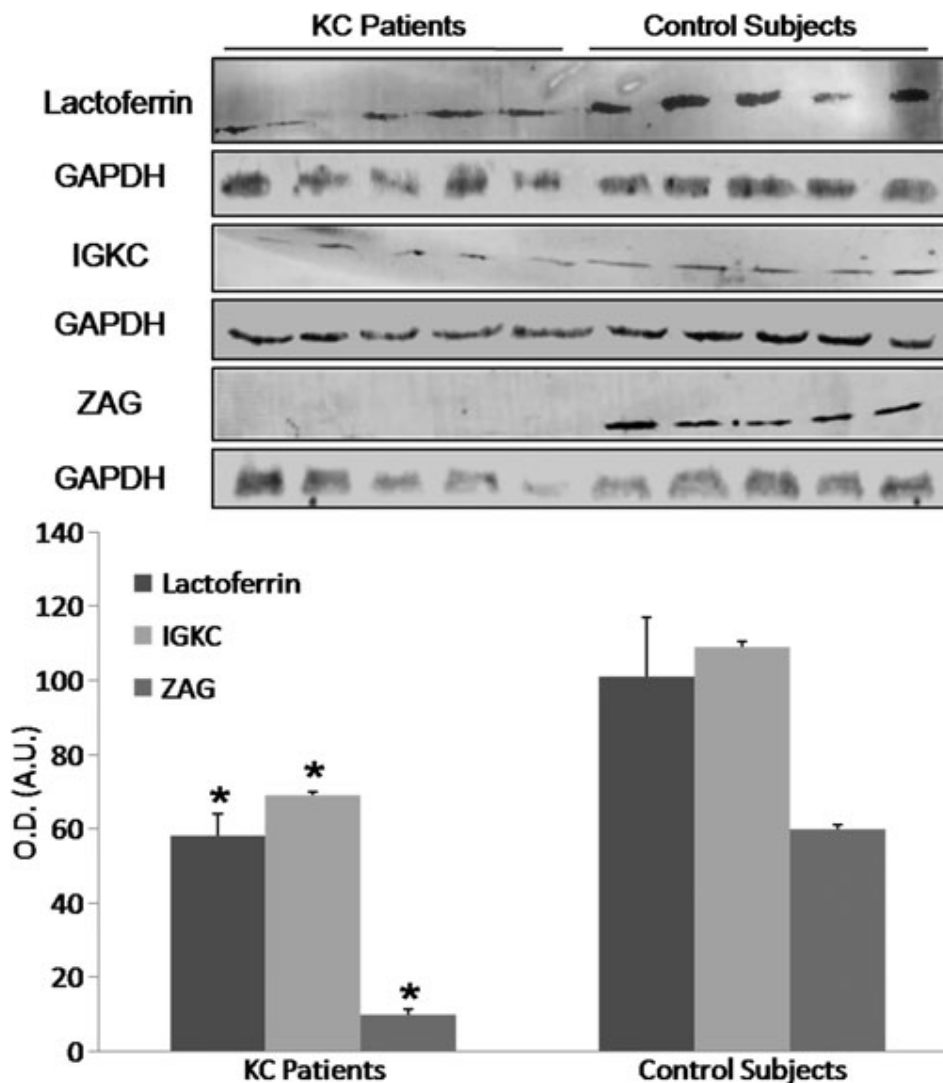


Figure 4. Western-blot analysis of the identified proteins showing a significant decrease in protein expression of zinc- α 2-glycoprotein (ZAG), lactoferrin, and IGKC (immunoglobulin kappa chain) in KC patients compared to control subjects. O.D.: optical density; A.U.: arbitrary units; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; * $p < 0.05$ versus control subjects.

support the idea that its pathogenesis involves an immune and inflammatory component [29,30]. Indeed, previous studies from our group, together with others, have shown that the levels of proinflammatory cytokines are significantly increased in the tears of patients with KC [10,13,31].

The limited sample size of the present study weakens the strength with which we may draw our conclusions. However, the robustness of the results gives support to the need to explore the role of these molecular markers as a new therapeutic or diagnostic tool in the KC. Although sample size is not so large, similar proteomic studies have used similar sample sizes [32]. Another limitation is that proteomics based on two-dimensional gels has a sensitivity in the order of magnitude of nanograms. Therefore, proteins whose expression is below the nanogram scale cannot be detected on the gels. Consequently, we do not rule out the possibility that other differences (not identified because of the sensitivity of staining methods) could exist between these two groups.

In conclusion, this study reveals that IGKC protein, zinc- α 2-glycoprotein, and lactoferrin are under-expressed in the tears of patients diagnosed with bilateral KC compared with healthy subjects. However, the precise role of each of these molecular factors still needs to be defined, but the results suggest that the pathogenesis of KC might involve immune and inflammatory processes.

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