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Preliminary identification of differentially expressed tear proteins in keratoconus

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Purpose: To examine the proteins differentially expressed in the tear film of people with keratoconus and normal subjects.

Methods: Unstimulated tears from people with keratoconus (KC) and controls (C) were collected using a capillary tube. Tear proteins from people with KC and controls were partitioned using a novel in-solution electrophoresis, Microflow 10 (ProteomeSep), and analyzed using linear ion trap quadrupole fourier transform mass spectrometry. Spectral counting was used to quantify the individual tear proteins.

Results: Elevated levels of cathepsin B (threefold) were evident in the tears of people with KC. Polymeric immunoglobulin receptor (ninefold), fibrinogen alpha chain (eightfold), cystatin S (twofold), and cystatin SN (twofold) were reduced in tears from people with KC. Keratin type-1 cytoskeletal-14 and keratin type-2 cytoskeletal-5 were present only in the tears of people with KC.

Conclusions: The protein changes in tears, that is, the decrease in protease inhibitors and increase in proteases, found in the present and other previously published studies reflect the pathological events involved in KC corneas. Further investigations into tear proteins may help elucidate the underlying molecular mechanisms of KC, which could result in better treatment options.

The constituents of the tear film could be vulnerable to changes associated with ocular diseases. A balance between proteases and protease inhibitors is critical to control the cell turnover rates and barrier functions of the cornea. Changes in the ocular environment might interfere with the level of proteases or protease inhibitors, and these changes could be reflected in tear proteins or peptides [1].

Keratoconus (KC) is a disease of the cornea in which its normal sphericity is disrupted. KC is characterized by progressive thinning of the cornea giving rise to a cone-shaped cornea, myopia (near-sightedness), and irregular astigmatism affecting vision. Although the exact pathological mechanisms associated with KC have not been elucidated, studies [2,3] have demonstrated increased collagenase and gelatinase activity of cultured corneal buttons or corneal cells derived from patients with KC [4]. Although subsequent studies showed no difference in the levels of matrix metalloprotease (MMP)-2 and MMP-9 in KC and control corneas [5,6], the tissue inhibitor of metalloprotease (TIMP)-1 decreased, and the MMP/TIMP ratio increased [7].

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In the tears from people with KC, altered expression of zinc-α2-glycoprotein (ZAG), lactoferrin, and immunoglobulin kappa chain [8], several keratins, lysozyme C, lipocalin [9], and the protease-inhibitors cystatins [10] have been reported using proteome techniques involving gel electrophoresis. Using antibody-based techniques, the levels of MMP-1, -3, -7, -13, interleukins (IL)-4, -5, -6, and -8, and tumor necrosis factor (TNF)-α and -β were increased in tears from people with KC [11], and the levels of lactoferrin and secretory IgA were decreased [12].

Modern peptidomic techniques have facilitated the examination of complex mixtures consisting of proteins or peptides. The tear fluid, with its heterogeneous mixture of proteins, salts, and lipids, has been subjected to separation or prefractionation steps to isolate the proteins, before analysis with mass spectrometry (MS) [13]. The MF10 (ProteomeSep; NuSep, French Forest, Sydney, Australia) is a device used for prefractionating and enriching the proteins present in low-volume complex samples [14]. Proteins in particular with low mass that are not captured with techniques such as gel electrophoresis, strong-cation-exchange (SCX), or liquid chromatography (LC), can be enriched by MF10.

First introduced by Horvath and coworkers in 1994, MF10 uses the established principles of electrophoresis to move charged molecules in a solution [15]. In addition to this novel separation technique, the development of a high-powered mass spectrometer such as linear ion trap quadrupole fourier transform mass spectrometer (LTQ-FT MS) has improved the precision of exploring tear proteins [1,16]. The present study for the first time has evaluated the tear proteins in KC by taking a simple gel-free approach using MF10 followed by LTQ-FT MS, and used spectral counting for the quantitative comparison of proteins between tears from people with KC and tears from normal subjects.

METHODS

The study was conducted after approval was obtained from the ethics panel at the University of New South Wales, Sydney, Australia. Each subject gave written informed consent before participating in the study, and all the procedures were conducted complying with the tenets of Declaration of Helsinki.

Subjects: Two groups were included in the study, one consisting of normal subjects (C) who had not been diagnosed with KC and the other subjects with KC. A total of 54 subjects (C=18, KC=36) were enrolled in the study. Demographics of the study subjects are outlined in Table 1. The subjects either gave a history of no or discontinued contact lens wear for at least 1 month before the study began. Subjects with active allergy or history of previous ocular diseases or ocular surgery or who used topical or systemic medications were excluded from the study. The participants with KC gave a history of frequent eye rubbing.

Corneal topography: The corneal curvature was mapped using a Medmont Corneal topographer E300 (Camberwell, Australia) on patients with KC and C subjects. The steepest simulated keratometry reading was recorded. The difference between the mean inferior and superior (I-S) power of the corneal curvature was used to verify the diagnosis of KC [17].

Table 1. Demographics of the study.

Groups	Subjects	
	C	KC
Total	18	36
Men	10	19
Women	8	17
Age (yrs)	36.60±9.85	36.73±13.12

C-Control KC-Keratoconus Age is expressed as mean±standard deviation

Tear collection: Sterile thin glass micro capillary tubes (BLAUBRAND intraMARK, Wertheim, Germany) were used to collect tears. A minimum of 4–6 µl tears were collected from the inferior cul-de-sac of the eye by the same investigator for every subject. Tear flow rate was monitored to ensure that reflex tears were not collected [18], and special care was taken not to touch the ocular surface to avoid reflex tearing. After the tears were collected, they were centrifuged at 2700 ×g for 10 min at 4 °C and stored at −80 °C until used for analysis [19].

Evaluation of tear proteins: An outline of experimental techniques followed in the present study is shown in Figure 1. The total protein concentration of individual tear samples was quantified with bicinchoninic acid (BCA) protein assay [20]. The bovine serum albumin (BSA) standard curve was obtained by preparing serial dilutions of BSA. The BCA solution (Pierce BCA kit, Thermo Scientific, Scoresby, Australia) was added to the standards or tears, and the absorbance was measured at 562 nm using a spectrophotometer (TecanSpectrofluoro Plus; Tecan Group, Männedorf, Switzerland). The total protein levels in both groups were calculated against the standard BSA curve.

Protein fractionation: The MF10 instrument was used to partition the tear proteins. As this technique requires at least 50 µl sample to be added before fractionation, it was decided to use pooled tears as it would have been impractical to collect 50 µl tears from each patient. This resulted in the use of only one pooled sample for the controls and one pooled sample for KC. Thus, we identified the results as preliminary evidence of differences between the two groups. The C group had 36 µl tears, obtained by pooling 2 µl tears from 18 eyes, and the KC group had 72 µl tears, obtained by pooling 2 µl tears from 36 eyes. Both pooled samples were made up to 80 µl before the MF10 was applied. The total protein µg in the pooled C or KC tears were equivalent before fractionation.

The MF10 instrument consists of specific pore-limiting membranes or polyacrylamide membranes (Figure 2). Samples containing complex peptide mixtures can be separated based on their size and charge using the mass restricted polyacrylamide membranes in MF-10 under native, denaturing, or reducing conditions [14]. In the present study, a six-chamber cartridge was assembled using 2.7×2.2 cm polyacrylamide membranes with varying pore sizes of 5, 25, 50, 75, and 150 kDa (NuSep, French forest, Sydney, Australia) and a 1 kDa regenerated cellulose membrane (Millipore, Billerica, MA), arranged in order from anode to cathode (Figure 2). Both ends of the cathode and anode were fitted with 5 kDa membranes to remove the charged contaminants from the sample during the fractionation process. Before use, the

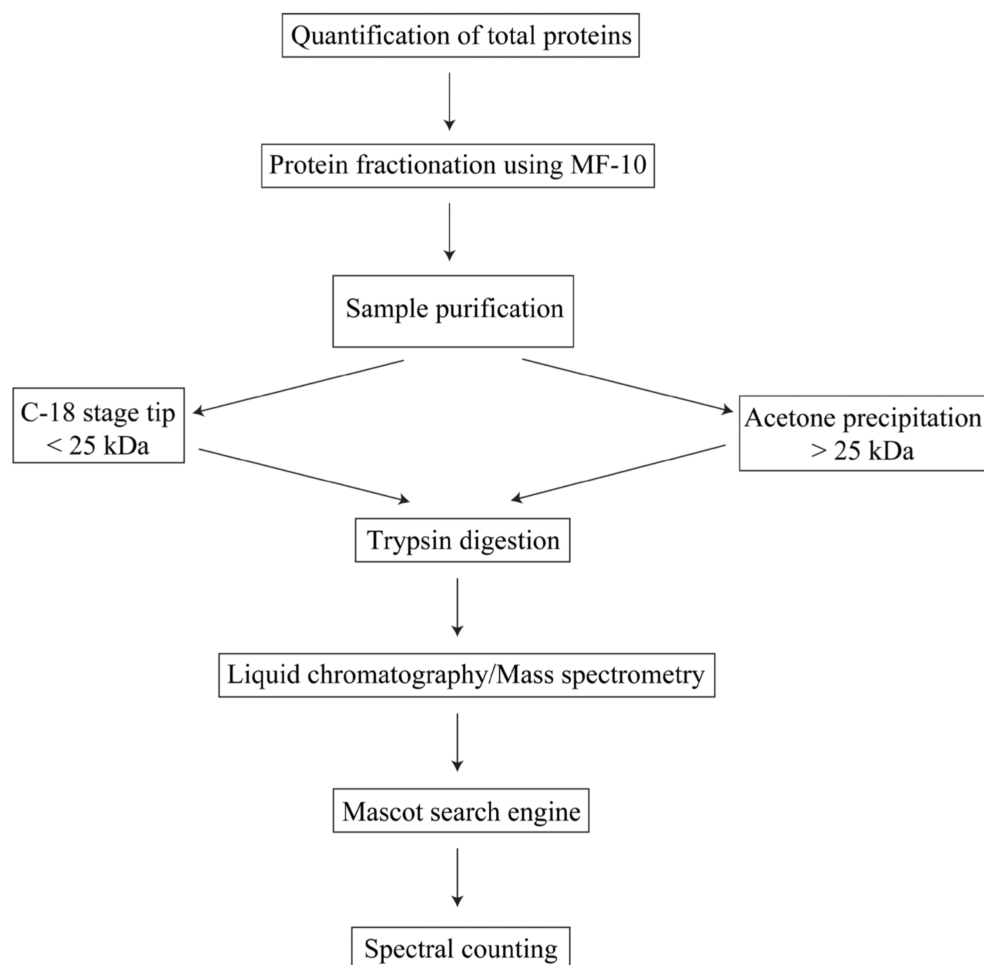

Figure 1. Schematic diagram showing the workflow to analyze tear proteins in the present study.

membranes were washed twice with ultrapure water (H_2O) and then twice with 1X Tris-ε-aminocaproic acid (EACA)/urea buffer (90 mM Tris (hydroxymethyl) aminomethane, 10 mM EACA, 2 M urea). The buffer lines of the MF10 were washed with ultrapure H_2O followed with a 10 min wash with 1X Tris/EACA/urea buffer.

The resulting assembly generated five chambers (F1 to F5) for fractionation (Figure 2). All cartridge assemblies had two lanes of chambers that allowed fractionation of two samples in one run. The chamber cartridge assembly was placed in the MF10 separation unit (Figure 2). A solution containing 80 ml of 90 mM Tris/10 mM EACA/1 M urea (pH 10.2) circulated around the electrodes.

Under native conditions, 35 µL C tears made up to 140 µl sample buffer (90 mM Tris/10 mM EACA/1 M urea, pH 10.2) were loaded into one chamber, and 70 µl KC tears in 280 µl sample buffer were loaded on a separate run. All the remaining chambers of both lanes were filled with an equivalent volume of sample buffer. Fractionation was performed at 50 V for 30 min, 250 V for 2 h (0.5 KvH) at 15 °C. After separation, fractions were collected from the chambers using gel-loading tips (Interpath Services, Heidelberg, Australia).

Sample preparation for liquid chromatography and mass spectrometry: Proteins were recovered for digestion with the C18 Stage tip (Thermo Fisher Scientific, Scoresby, Australia) for the 1 to 5 kDa and 5 to 25 kDa fractions and by acetone precipitation for the remaining fractions to remove salt and buffer contaminants. The stage tips and acetone precipitations were used to concentrate the lower and higher molecular weight proteins, respectively [21,22].

C18 Stage tip: The C18 Stage tip was equilibrated with 25 µl 50% methanol, 5% formic acid (FA) in distilled water (dH_2O; v/v) and washed with 25 µl 5% FA in dH_2O. Samples were slowly passed through the Stage Tip in 25 µl aliquots with elution and reequilibration in between. The tip was washed twice with 25 µl 5% FA in dH_2O (v/v). Peptides were eluted with 10 µl 80% acetone and 5% FA in dH_2O (v/v) into a clean 1.5 ml polypropylene tube. The samples were dried in

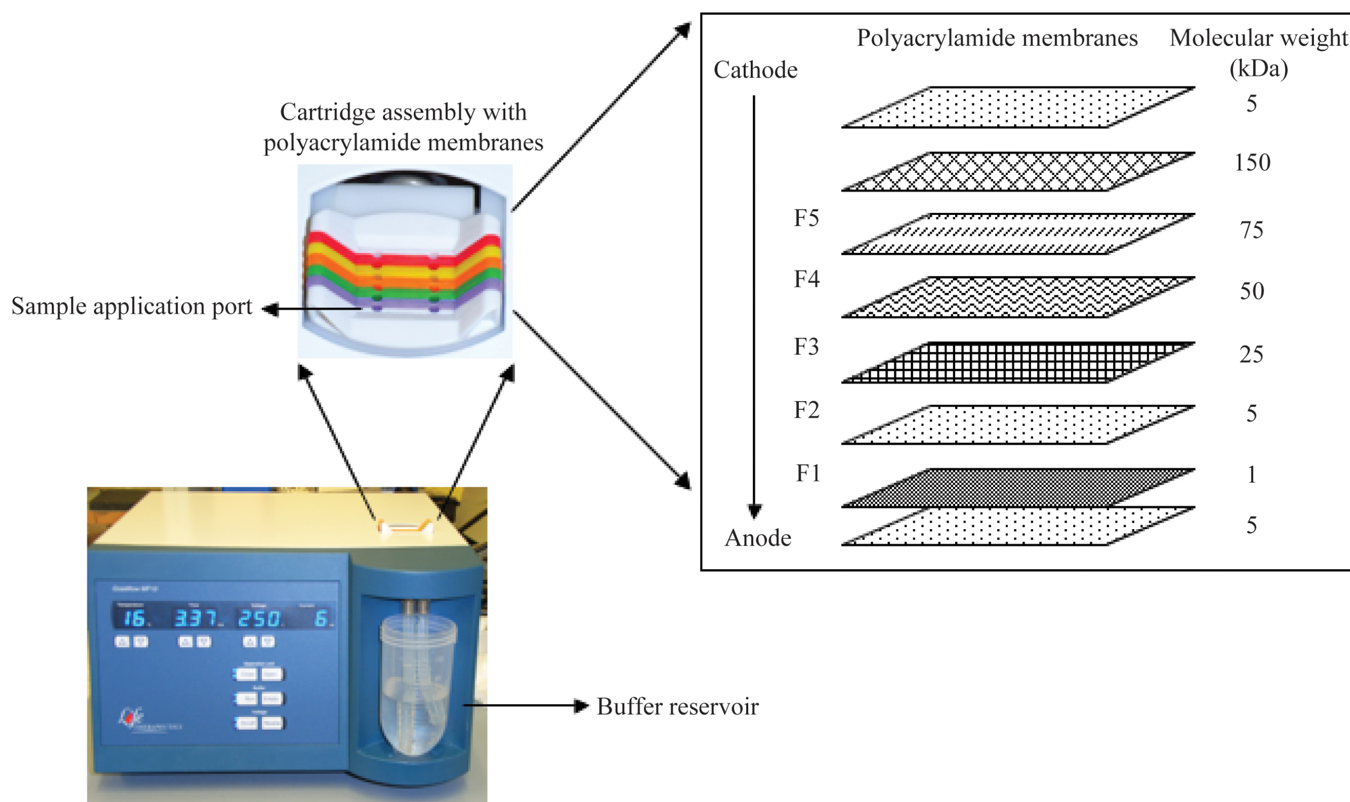

Figure 2. The Microflow 10 separation system, modified from Ly et al. [42]. F represents fraction.

a vacuum centrifuge and frozen at −20 °C until further use. Lyophilized protein samples were reconstituted with 50 µl 50 mM ammonium bicarbonate buffer (pH 8.50) before trypsin digestion.

Acetone precipitation: Ice-cold acetone was added to the fractions (>25 kDa) in the ratio of 4:1. The mixture was incubated at −20 °C for 1 h. The samples were centrifuged in a 5804R centrifuge (Eppendorf AG, Hamburg, Germany) at 15,300 ×g for 12 min at 10 °C. After centrifugation, the supernatant was discarded, and the pellet was air dried for 20 min and stored at −20 °C. Protein samples were reconstituted with 50 µl of 50 mM ammonium bicarbonate buffer (pH 8.50) before trypsin digestion.

Trypsin digestion: The individual fractions were treated with 1 µg trypsin, and the mixture was incubated for 18 h at 37 °C. Reduction and alkylation before trypsin digestion were not performed as has been reported previously [23]. The reaction was stopped by adding 5 µl neat FA. Samples were then dried in a vacuum centrifuge. The lyophilized samples were treated with 5 µl 2% acetic acid and 0.1% FA. Equivalent amounts of fractionated samples were analyzed for each group (C or KC). Fractions were examined with liquid chromatography and mass spectrometry (LC-MS/MS). One to 5 kDa fractions were analyzed at 1 µl from 10 µl, and all the other individual fractions were analyzed at 0.5 µl from 10 µl.

To allow for quantitative comparison, an equal amount of starting material was analyzed. The 1 to 5 kDa fractions were stage tipped to remove buffer components and to concentrate the proteins. The proteins were resuspended into 10 µl buffer A (2% acetonitrile (ACN)/0.1% FA/98% ultrapure H_2O), and equivalent concentrations of total protein were subjected to trypsin digestion and mass spectrometry. This equated to 1 µl of the 1–5 kDa fraction and 0.5 µl of the other molecular weight fractions. These volumes were correctly assessed such that the instrument would be able to provide MS/MS information for up to 10 ions at any given scan cycle rather than being overwhelmed and missing important ions. This also limited saturation of the detector and provided better quality LC separation.

Liquid chromatography and mass spectrometry using linear ion trap quadrupole Fourier transform: The digested MF10 fractions were evaluated using a LTQ-FT Ultra mass spectrometer (Thermo Electron, Bremen, Germany). Peptides were separated with nano-LC using an Ultimate 3000 HPLC and auto-sampler system (Dionex, Amsterdam, Netherlands). Samples were concentrated and desalted onto

a micro C18 precolumn (500 µm × 2 mm) with 0.05% (v/v) heptafluorobutyric acid (HFBA) 20 µl/min. After a 4 min wash, the precolumn was switched (Valco 10 port valve, Dionex, Amsterdam, Netherlands) into line with an in-house built fritless nano C18 column (75 µm × 10 cm) packed with the C18 Magic stationary phase (5 µm, 200 Å pore size; Michrom Bioresources, Auburn, CA). The LC mobile phase comprised buffer A, 2% acetonitrile (ACN)/0.1% FA/98% ultrapure H_2O, and buffer B, 80% ACN/0.1% FA/20% ultrapure H_2O. Peptides were eluted using a linear gradient of 2% to 50% buffer B over 30 min followed by a 100% buffer B wash over 1 min at a flow rate of approximately 300 nl/min. High voltage was applied to low volume tee and a column tip position at approximately 0.5 cm from the heated capillary (T=200 °C) of the LTQ-FT. Positive ions were generated by electrospray, and the LTQ-FT operated in the data-dependent acquisition (DDA) mode. An MS survey scan was acquired (m/z 350–1700) in the Fourier transform ion cyclotron resonance cell (resolution=100,000 at m/z 400) up to seven of the most abundant ions (>2,000 counts) with charge states of +2 or +3 were sequentially isolated and fragmented within the linear ion trap using collision-induced dissociation (CID). Mass to charge ratios selected for MS/MS were dynamically excluded for 45 s. Peak lists were generated using MASCOT Daemon/extract_msn software, version 2.2 (Matrix Science, London, England) using default parameters and submitted to the database search program MASCOT.

Identification of peptides: All MS/MS spectra were searched using the MASCOT search engine. A minimum MASCOT score of 50 was used to identify peptides. MF10 protein/peptide fractions were searched against the Swiss-Prot database release 15 with the following criteria: precursor tolerance and the product ion tolerances were at 6 ppm and ± 0.6 Da, respectively, variable modification of methionine oxidation, and no enzyme.

Although trypsin was used, MASCOT was searched using the “no enzyme” setting. While trypsin was used to cleave the larger proteins and peptides, we decided to look for other possible peptides. This allowed for identification of peptides that had undergone proteolysis with other endogenous enzymes allowing the potential to identify proteins that were degraded in the tears. Thus, selecting “no enzyme” within MASCOT increased the potential number of peptides that could be identified as it was not reliant on the peptides having an arginine or lysine terminus. Because there were some proteins with low mass, restricting the search to only trypsin-derived peptides may have resulted in fewer peptides identified for each protein as there may not have been sufficient tryptic cleavage sites in smaller proteins.

Analysis was performed using Scaffold proteome software version 3 (Proteome Software, Portland, OR). Scaffold is a bioinformatics tool designed to compare the spectral counts obtained between multiple samples for the purposes of relative quantitation. A peptide probability threshold of 95% with at least two peptides required for confirmation. This gave a 1.50% false positive rate for identifications.

We chose not to merge the peak files. However, we combined the analysis within Scaffold. Our aim was first to define and enrich for the low mass component within the tears and then to compare the data sets. Thus, determining which peptides partition to which mass fraction before quantitative analysis was essential.

Statistical methods: Significant differences in total tear protein levels between the groups were analyzed using the Student *t* test. The differential expression of proteins across the samples was compared between the C and KC groups based on peptide spectral counts using the Scaffold software. The relative abundance of individual proteins across the groups was calculated with spectral counting. Spectral counting compares the MS/MS spectra assigned to each protein and is considered sensitive for detecting the proteins that undergo changes in abundance [24]. The spectral count correlates with the protein abundance [25]. Standard deviations were automatically calculated across replicates, and the spectral counts were normalized between the groups to remove statistical errors created from repeated measurements [26]. The fold change in the Scaffold analytical software was used to test the significance of the ratio of spectral counts between the C and KC groups.

RESULTS

Corneal topography: The mean steepest keratometry reading (K2) in the C and KC groups was 43.7±1.2 D and 57.3±10.5 D, respectively. The KC subjects recruited had mild (<45 D), moderate (45–52 D), and severe (>52 D) stages of the disease. The proportion of the KC subjects with mild, moderate, and severe stages of KC were 13.9%, 38.8%, and 47.2%, respectively. However, for the analysis, the tears of all KC subjects were pooled.

Total tear protein level: The differences in age or gender were not statistically significant between the C and KC groups. The patients with KC had a significantly lower level of total tear protein (3.24±0.70 µg/µL) compared to the C (non-keratoconic) group (6.35±1.24 µg/µl; $p<0.0001$), and thus, twice as much tears were analyzed for the KC group to normalize this difference.

Tear protein profile: Pooled C (36 µl) and KC group (72 µl) tear samples had equal total protein amounts before protein fractionation. A total of 75 tear proteins in the C and KC groups (Appendix 1) were identified confidently. The proteins were also identified in the individual tear fractions of the C and KC groups, i.e., 1–5 kDa (Appendix 2), 5–25 kDa (Appendix 3), 25–50 kDa (Appendix 4), 50–75 kDa (Appendix 5), and 75–150 kDa (Appendix 6).

Differentially expressed proteins in keratoconus compared to controls: Based on the spectral counts, cathepsin B was increased in abundance in the KC group compared to the C group (Figure 3A). Polymeric immunoglobulin receptor (PIGR), fibrinogen alpha chain or α-fibrinogen, cystatin SN, and cystatin S were decreased in the KC group compared to the C group (Figure 3B–E). The tear proteins found only in the tears from the KC group were keratin type-1 cytoskelatal-14 (K1C14) and keratin type-2 cytoskeletal-5 (K2C5).

Fold change is the ratio of spectral counts in the KC group compared to the spectral counts in the C group of a particular protein. Larger fold change values imply larger differences in a particular protein between the groups. The corresponding molecular weight fractions and the fold change values of the differentially expressed proteins in the KC group are shown in Table 2. The MS/MS spectra of the altered proteins in the C group compared to the KC group are illustrated in Appendix 7.

Ontology analysis: The proteins listed in Table 2 were grouped based on location (Figure 4A), biologic process (Figure 4B), and functions performed (Figure 4C) by using the gene ontology (GO) term analysis in Scaffold. Several proteins belonged to more than one category within each group. The altered proteins identified in the KC group are mainly intra- and extracellular proteins (Figure 4A), implicated in cellular or developmental processes (Figure 4B), and carry out important enzyme regulatory or molecular functions (Figure 4C).

DISCUSSION

The use of tear proteomics to understand the pathophysiology of KC has been a topic of considerable interest in recent years. This study is the first to use a novel in-solution electrophoretic device to partition tear proteins and enrich for the low mass (<25 kDa) proteins and peptides in KC. Due to tear protease activities, the larger proteins might exist as snippets and thus can enriched in the lower mass partition of MF10. The MF10 coupled with LTQ-FT showed a relative increase in the abundance levels of cathepsin B and decreased levels of PIGR, α-fibrinogen, cystatin SN, and cystatin S in the tears of people with KC. Keratins K1C14 and K2C5 were present only in the KC tears. The protocol required that tear samples be pooled so that sufficient volume and concentration of tears could be used with the MF10 separation instrument. Thus, any differences seen should be considered preliminary evidence of actual differences in the tears of the people with KC and the control subjects. Further analysis to confirm differences should be conducted in future studies, using, for example, mass spectrometry techniques such as multiple reaction monitoring that can identify and quantify individual proteins in small volumes.

Electrophoresis using one-dimensional (1D) or two-dimensional (2D) gels is the most common technique used to fingerprint tear proteins. Although this technique is considered simple and effective, many proteins go undetected or can be lost during the various steps involved in gel-based techniques [27]. Recent advances in the field of proteomics have improved the investigation of lower abundance and low mass proteins or peptides. Fractionation strategies are essential to improve the isolation and enrichment of lower molecular weight proteins or peptides from complex samples. The ability of the MF10 instrument to fractionate, concentrate, and desalt the samples before MS analysis enhances the identification of complex tear proteins present in low volumes [13].

The tear proteins identified in this study were present in more than one molecular weight fraction using MF10. This might be due to the inherent ability of proteins to form complexes with other proteins or due to the influence of protease activity. Importantly, the altered tear proteins between the C and KC groups were identified in their native molecular weight fractions according to this study (Table 2), which shows the reliability of MF10 protein separation.

Cathepsin B belongs to the lysosomal group of proteases. It has been localized in the corneal epithelium [28] and known to degrade extracellular matrix proteins [29]. Although cathepsin B has been shown to be elevated in KC corneas [6], its expression in KC tears has not been studied. Overexpressed cathepsin B in KC corneas might be the reason for the increased expression in the tear film reported in this study. These proteases may play a vital role in the apoptosis of keratocytes, which is the major form of cell death in KC [30].

The keratins (K1C14 and K2C5) are normally found in the outer or epidermal layer of human skin and not necessarily present in the tear film or cells from the eye. A possible explanation for the presence of keratins in KC tears could be the habit of frequent eye rubbing seen among people with KC in the present study. This result is consistent with elevated keratin levels in the corneal epithelium and tears of

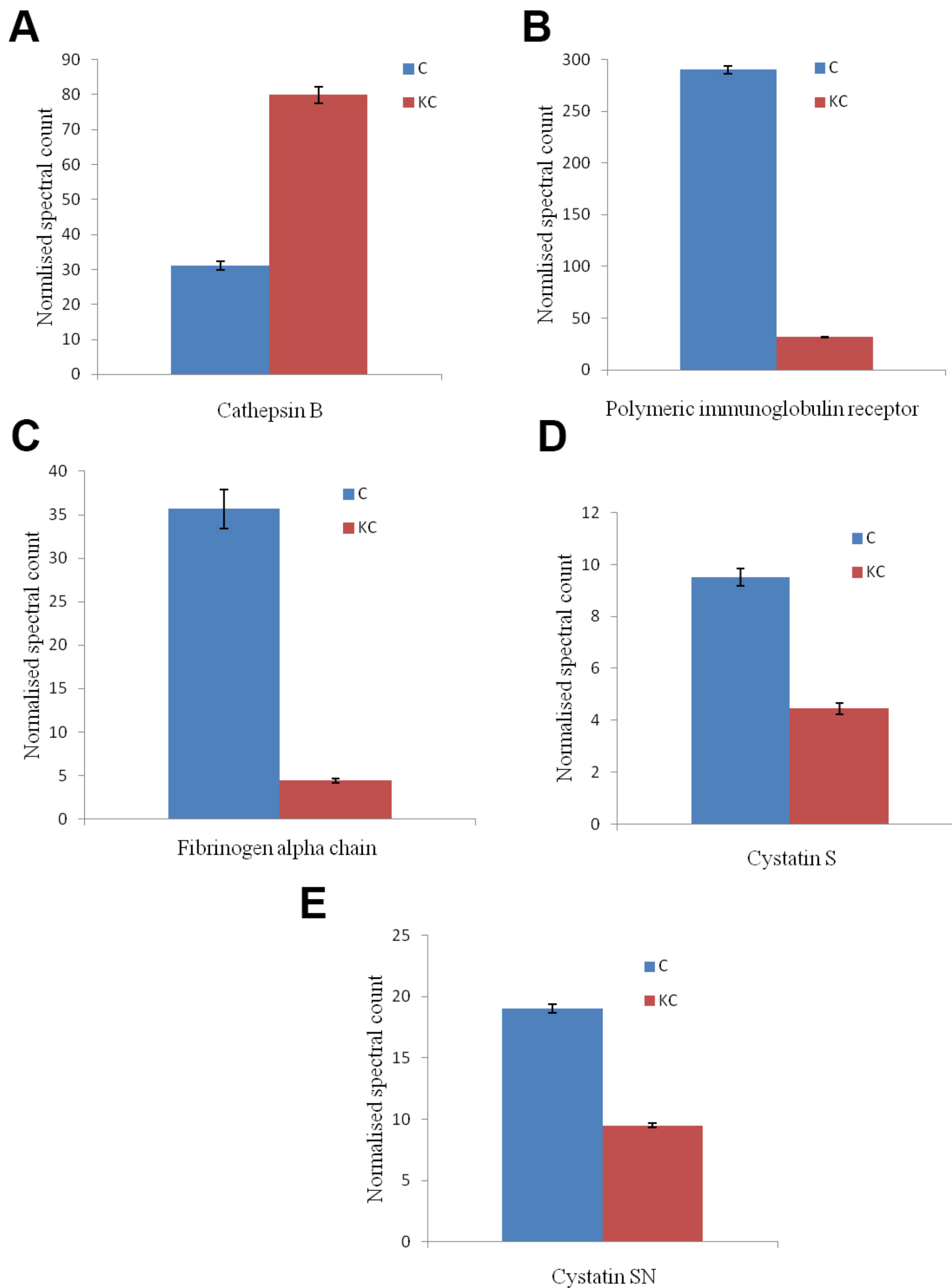

Figure 3. Altered tear protein levels. **A**: Protein that was increased in abundance in tears from people with keratoconus (KC) compared to the controls. **B-E**: Proteins that were decreased in abundance in tears from people with KC compared to the controls.

patients with KC reported previously [9,31]. The eye rubbing seen in KC is abnormal and disease-related [32]. Abnormal eye rubbing seen in patients with KC might have a profound impact on their tear protein profile. Our recent study showed that eye rubbing increases the concentration of MMP-13, IL-6, and TNF-α in the tear film [32].

The epithelial cells of the lacrimal gland express PIGR [33]. These receptors are essential for transporting secretory immunoglobulin A (sIgA) into the tear film. Binding of PIGR to sIgA protects the sIgA from proteolytic degradation [34]. Tear fluids are rich in sIgA, and low levels of PIGR might affect the concentration of sIgA in the tears of patients with KC, thus disrupting the normal immune function of the tear

Table 2. Tear proteins altered in KC compared to C.

Accession number	Identified proteins	Theoretical mass (kDa)	Indentified Fraction (kDa)	Spectral count*		Fold change
				C	KC	
P07858	Cathepsin B	38	25–50	31.05	79.90	+2.6
P01833	PIGR	83	75–150	290.19	31.65	−9.4
P02671	α-Fibrinogen	95	75–150	35.68	4.43	−8.2
P01037	Cystatin SN	16	5–25	9.51	4.45	−2.2
P01036	Cystatin S	16	5–25	19.03	9.5	−2.1
P02533	Keratin, type I cytoskeletal 14	52	50–75	-	5.7	1#
P13647	Keratin, type II cytoskeletal 5	62	50–75	-	3.8	1#

PIGR- Polymeric immunoglobulin receptor * p<0.0001 # Fold change is 1 as K1C14 and K2C5 were not found in tears from the C group.

film. Low levels of tear sIgA in patients with KC have been reported previously [12].

Fibrinogen is an important glycoprotein present in the epithelial basement membrane of the cornea. This complex protein consists of α, β, and γ chains [35]. Degradation of the epithelial basement membrane is an important feature of KC, and studies have demonstrated weak expression of fibrinogen in the epithelial basement membranes of KC corneas [35,36]. Fibrinogen is involved in corneal wound healing by assisting the adhesion and migration of epithelial cells [37]. This study is the first to report the downregulation of α-fibrinogen in KC tears and the role of α component of fibrinogen in KC needs further investigation.

Cystatins are a group of lysosomal or cysteine protease inhibitors [38]. Cystatin S and cystatin SN are extracellular proteins belonging to this family. These levels were significantly lower in the KC tears compared to the C group tears. Our results are in agreement with the decreased levels of cystatin SN and cystatin S reported previously in KC tears [10]. Low levels of protease inhibitors cystatin S and cystatin SN might increase the level of tear proteases and its activity in KC.

Due to the nature of the low volume of basal tears, the tear samples were pooled to generate adequate tear volume to examine the tear proteins using mass spectrometry. Although pooling did result in any differences being only preliminary in nature, pooling reduced the diversity of changes in individual samples such that extremes in individual tear samples would be diluted within the pool and only commonalities within the pool were used to determine differences across the two groupings. Quantitation of total protein in individual samples before pooling allowed for equalization in concentrations between the two groups and the final concentration of total protein in each pool was equivalent. Samples were run in duplicate on ProteomSep and in triplicate for MS analysis. Previous studies have shown little variation between the parallel sample runs and the technical replicates [14,39,40]. Thus, the fold changes seen in pooled tears were an observation of overall trends. The data are reported as a ratio and therefore are an acceptable measure of differences between two pooled groups. The majority of proteins did not change in abundance between the groups.

Spectral counting, a label-free quantitation method, was used to compare individual tear proteins between the samples in this study. This method has been shown to be linear over a broad range for the majority of proteins. The method assumes that the number of times a protein acquires MS/MS information is correlated with the abundance of that protein within a sample. There are limitations in this method, such as a reduction in sensitivity to measure proteins of low abundance or single peptide proteins. However, the experimental protocol is enriched for lower mass components, thus reducing the effect of this limitation. The data indicated that despite having some single peptides, there was a low false positive rate of 1.50% at the protein and peptide levels.

Proteins of lower molecular mass may result in a small number of tryptic cleavage sites per protein, and they may not be quantified using spectral counting. Therefore, single peptide matches were recorded as evidence for the presence of particular proteins. As stated previously, further validation work using multiple reaction monitoring MS for absolute quantification should be performed in future experiments. Many of the proteins identified likely were fragments/breakdown products, as they partitioned to the lower mass fractions within the MF10 and not according to the molecular weight of the full length protein. Indeed, some were not even tryptic digestion products, suggesting that proteolytic cleavage had

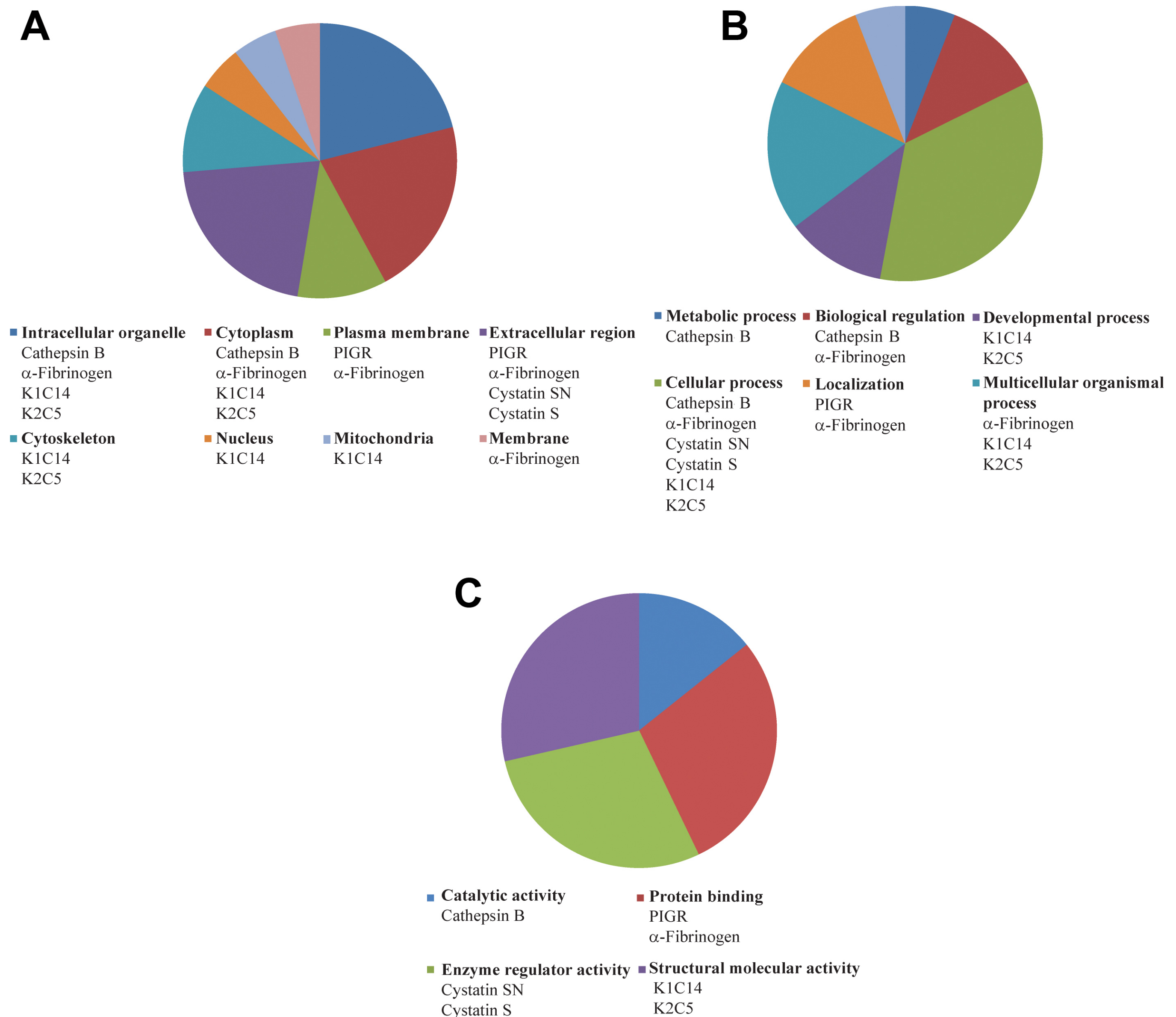

Figure 4. Ontology analysis of tear proteins. **A**: Cellular compartments of the proteins altered in tears from people with keratoconus (KC). **B**: Probable role in biologic processes of the proteins altered in tears from people with KC. **C**: Functions of the proteins altered in tears from people with KC.

occurred in the tear film before collection and addition of trypsin for MS analysis.

MF10 has been used to identify proteins in plasma [39], urine [40], and tear film [13], but the instrument is still in the developmental stage and is not used routinely for separating tear proteins. Although low abundant proteins, proteases, and protease inhibitors were identified in this study, strategies that could be used to deplete the high abundant proteins [40] during MF10 were not used. The masking effect of one or more high abundant tear proteins such as lactoferrin, secretory IgA (sIgA), serum albumin, lysozyme, and lipocalin might reduce the chances of detecting other tear proteins which are less abundant in tears [41]. Tear proteins present at low concentrations might provide valuable information about the ocular environment [42], and future studies are essential to further uncover the tear proteins present in low concentrations in KC by depleting high abundant proteins.

In summary, the tear proteins differentially expressed in KC were various but included increased protease and decreased protease inhibitors. Further comprehensive studies

investigating tear proteases and protease inhibitors are vital to understand the course of the disease and formulate new therapeutic interventions.

APPENDIX 1. LIST OF TOTAL TEAR PROTEINS IDENTIFIED IN C AND KC.

To access the data, click or select the words "Appendix 1."

APPENDIX 2. PROTEINS IDENTIFIED IN THE 1 TO 5 KDA FRACTION.

To access the data, click or select the words "Appendix 2."

APPENDIX 3. PROTEINS IDENTIFIED IN THE 5 TO 25 KDA FRACTION.

To access the data, click or select the words "Appendix 3."

APPENDIX 4. PROTEINS IDENTIFIED IN THE 25 TO 50 KDA FRACTION.

To access the data, click or select the words "Appendix 4."

APPENDIX 5. PROTEINS IDENTIFIED IN THE 50 TO 75 KDA FRACTION.

To access the data, click or select the words "Appendix 5."

APPENDIX 6. PROTEINS IDENTIFIED IN THE 75 TO 150 KDA FRACTION

To access the data, click or select the words "Appendix 6."

APPENDIX 7. SPECTRA OF THE ALTERED PROTEINS IN KC COMPARED TO C.

To access the data, click or select the words "Appendix 7."

ACKNOWLEDGMENTS

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