Mediators of Corneal Haze Following Implantation of Presbyopic Corneal Inlays

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Purpose. To identify protein mediators of corneal haze following presbyopic corneal inlay surgery.

Methods. Tears were collected from eyes with corneal haze following surgery with a shape-changing corneal inlay. Samples were subjected to quantitative proteomic analysis using iTRAQ and proteins significantly increased or decreased (1.3-fold or more) in haze eyes relative to fellow eyes were identified. Expression ratios were compared to postoperative eyes without corneal haze to identify proteins selectively increased or decreased in corneal haze eyes.

Results. Inlay-associated haze occurred in 35% of eyes (6 of 17). Of 1443 unique tear proteins identified, eight proteins were selectively reduced in tears from postoperative haze eyes and one protein selectively increased. Proteins reduced in haze eyes included complement 4a (level relative to nonhaze eyes 0.464, $P = 0.037$), complement factor H (0.589, $P = 0.048$), immunoglobulin kappa variable 2–29 (0.128, $P = 0.006$), immunoglobulin kappa variable 2D-28 (0.612, $P = 0.025$), immunoglobulin lambda variable 7–46 (0.482, $P = 0.007$), S100 calcium binding protein A4 (0.614, $P = 0.048$), Shootin-1 (0.614, $P = 0.048$), and tissue inhibitor of metalloproteinase-1 (0.736, $P = 0.023$). The Xaa-Pro aminopeptidase 1 was increased in haze eyes relative to nonhaze eyes (1.517, $P = 0.023$).

Conclusions. Corneal haze following corneal inlay surgery is associated with reduction in levels of known inflammatory and immune mediators. These findings represent a starting point for elucidation of pathways involved in corneal haze following synthetic inlay implantation and may enable development of targeted therapies that modulate the haze response.

Keywords: corneal inlay, tear proteomics, haze, refractive surgery, corneal wound healing

Population aging in developed countries has led to a growing desire for spectacle-free near vision among patients with presbyopia. Recently corneal inlay surgery for presbyopia correction[1] has been adopted by refractive surgeons to address this demand. Corneal inlay surgery is a relatively new treatment option for presbyopia that involves the implantation of a synthetic implant beneath a stromal flap or within a stromal pocket created using a femtosecond laser.[2,3] The inlays employ refractive power, small aperture optics, or corneal reshaping to achieve improvements in near vision.[1,4–8]

Despite increasing adoption by refractive surgeons, corneal inlay surgery carries similar risks to other laser refractive surgeries, including dry eyes, infectious keratitis, and flap-related complications such as epithelial ingrowth. Corneal haze, defined as a decrease in tissue transparency or as a subepithelial stromal opacity, has also recently surfaced as a potentially important complication related to corneal inlay surgery.[9] Corneal haze is often asymptomatic, but may cause starburst effects, reduced visual acuity, and refractive regression.[10] We recently reported on the development of corneal haze several years after implantation of KAMRA inlays in a series of three patients that ultimately required explantation.[9] Histopathologic examination of the explanted inlays showed fibrotic membranes engulfing the inlays associated with chronic inflammatory cells. Other centers have also reported variable rates of corneal haze of up to 73% associated with corneal inlays.[5,11,12] Though corneal inlays are foreign bodies that would be expected to elicit some degree of host response, it remains unclear why corneal inlays induce corneal haze after prolonged postoperative periods, and why the haze response only affects certain eyes. Given the increasing interest of such surgical approaches to address presbyopia and the great potential of corneal inlays as a spectacle-free solution, it is clearly important to address this apparent gap in knowledge.

Work in our center and elsewhere has previously employed tear proteomics to identify tear proteins involved in ocular

Table 1. Study Subject Characteristics

	No Postoperative Haze (n =11)	Postoperative Haze (n = 6)	Significance, P
Age, ±SD	49.1 ± 2.98	52.7 ± 4.22	0.058
Female sex	8 (72.7%)	3 (50%)	0.332
Preoperative distance VA*	0.26 ± 0.38	0.12 ± 0.11	0.300
Postoperative distance VA*	0.20 ± 0.19	0.38 ± 0.22	0.159
Preoperative near VA*	12.3 ± 7.3	13.2 ± 10.8	0.851
Postoperative near VA*	5.5 ± 0.93	7.2 ± 9.3	0.505
Preoperative CCT, μm	561.5 ± 37.6	569.2 ± 36.5	0.712
Postoperative CCT, μm	565.9 ± 37.7	572.6 ± 22.5	0.680
Tear collection time, mo postoperative	15.2 ± 11.0	13.8 ± 9.6	0.728

* Visual acuities (VA) are expressed in LogMAR units (distance) or Moorfields near vision reading chart units (near). Values are for uncorrected monocular visual acuity.

surface diseases including dry eye disease,[13,14] thyroid eye disease,[15] Sjögren syndrome,[16] vernal keratoconjunctivitis,[17] and postoperative LASIK eyes.[18] This approach opens the possibility of rapid and noninvasive screening for protein mediators of ocular surface disease responses, and has already proven useful for the development of clinical assays for ocular disease.[14] We reasoned that a tear proteomics approach would enable identification of the extracellular proteins that signal cellular changes underlying the corneal haze response.

In the current work, we sought to identify mediators of the corneal haze response following presbyopic corneal inlay surgery. Using a tear proteomics approach, we examined a patient cohort following surgical implantation of the Raindrop near vision corneal inlay to identify proteins preferentially up- or downregulated in eyes with postoperative inlay-associated haze.

Methods

Subject Selection

Institutional review board approval for this study was obtained from our local ethics committee and the work adhered to the tenets of the Declaration of Helsinki. Informed written consent was obtained from 17 prospectively recruited patients prior to ocular surgery and their tears collected for proteomic analysis postoperatively. Patient characteristics are summarized in Table 1.

Surgical inclusion criteria included symptomatic and clinically significant presbyopia with age between 41 and 65 years, manifest refractive spherical equivalent of –0.50 to +1.00 D, less than or equal to 0.75 D of cylinder, no requirement for distance correction, and requirement of near reading addition of +1.50 to +2.50 D. Photopic pupil size of >3 mm and mesopic pupil size of <7 mm was ensured by clinical examination, in accordance with the manufacturer's recommendation (Revision Optics, Inc., Lake Forest, CA, USA). Exclusion criteria included patients with corneal thickness preventing a stromal bed of >300 μm below the flap, abnormal corneal topography, active ocular infection or inflammation, autoimmune diseases, severe dry eye syndrome, keratoconus or keratoconus suspects, uncontrolled diabetes, corneal disease secondary to recent ocular infection, and uncontrolled glaucoma.

Surgical Techniques

Implantation of the Raindrop Near Vision Inlay was performed according to the manufacturer's protocol (Revision Optics, Inc.), by a single surgeon (JT). Briefly, patients were pretreated with topical difluprednate 0.05% four times daily for 2 days preoperatively and the surgeries were performed under topical amethocaine analgesia. Corneal flaps of 160 μm thickness were created using a 500 kHz femtosecond laser (Visumax; Carl Zeiss Meditec AG, Jena, Germany) and the eye irrigated with chilled balanced salt solution. Flap interfaces were irrigated with 0.02% mitomycin C for 15 seconds prior to washout with balanced salt solution. One patient from the corneal haze group and one patient from the nonhaze group did not have this treatment. Corneal inlays were prepared and inserted beneath the corneal flap using the manufacturer's inlay inserter instrument. Briefly, the inserter containing the corneal inlay was assembled and the patient positioned with the operative eye centered under the microscope and instructed to fixate on the light. The tip of the Inlay Inserter was positioned above the stromal bed and centered over the light-constricted pupil. The inlay was transferred from the inserter to the stromal surface using a disposable 30-gauge cannula and centered on the light-constricted pupil. Inlays were allowed to adhere to the stromal bed for one minute prior to flap repositioning. Inlay positioning was assessed by slit lamp biomicroscopy immediately after the procedure and the patient started on a tapering course of topical difluprednate 0.05% with moxifloxacin hydrochloride 0.5% antibiotic coverage. Difluprednate was tapered from four times daily for 1 week, to three times, two times, and finally once daily, each for 1 week. Patients were then switched to loteprednol etabonate 0.5% at twice daily dosing for 2 months, followed by once daily dosing for the third month. Patients were reviewed at 1 day, 1 week, 1 month, 6 months, and 12 months postoperatively. Postoperative corneal haze was graded as 0 (no haze), 1 (mild peripheral edge haze), 2 (prominent peripheral edge haze), or 3 (central haze). Representative clinical photos for each grade of haze are shown in Figure 1.

Tear Sample Collection

Tear samples were collected postoperatively after onset of corneal haze (median of 2 months after haze onset) using a Schirmer Type 1 tear test without local anesthesia as described elsewhere.[14] Briefly, a strip of filter paper (Sno strips; Bausch and Lomb, Rochester, NY, USA) was placed into the inferior fornix and the patient instructed to close their eyes without moving their eye under the lid for 5 minutes, after which time the strip was removed and the wetted area measured prior to freezing the strips at –80°C. Total protein content was assayed using a protein assay kit (RC/DC, Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions. Tear samples were lyophilized and stored at –80°C until required. Corneal haze was managed with topical steroids that were commenced following tear sample collection.

FIGURE 1. Postoperative corneal haze following Raindrop Near Vision Inlay surgery. Corneal haze was scored as grade 0 (no haze), grade 1 (mild peripheral edge haze), grade 2 (prominent peripheral edge haze), or grade 3 (central haze).

Quantitative Proteomic Analysis of Tear Fluid by Liquid Chromatography and Tandem Mass Spectrometry (LC-MS/MS)

Aliquots of lyophilized tear protein extracts (25 μg total protein) were used for isobaric tags for relative and absolute quantitation (iTRAQ) labeling (AB Sciex, Framingham, MA, USA) followed by LC-MS/MS analysis as previously described.[18] Liquid chromatography was performed using a commercial LC system (Dionex Ultimate 3000 Nano LC; Thermo Fisher Scientific, Inc., Sunnyvale, CA, USA) and mass spectrometry with a commercial device (TripleTOF 5600; AB Sciex, Framingham, MA, USA) according to procedures described elsewhere.[18]

Proteomics Data Analysis

Tear proteomics data obtained from LC-MS/MS proteomics (iTRAQ; AB Sciex) described above were processed with protein identification software (ProteinPilot 5.0; AB Sciex) using the October 2014 version of the UniProt protein database.

Statistical Analysis

The average ratios of nonhaze-to-control and haze-to-control from iTRAQ sets were calculated using geometric means. Data were initially sorted according to proteins that were increased or decreased by >1.3-fold in operated eyes compared to the nonoperated fellow eyes using log-transformed median expression values. After this initial exclusion, we performed paired two-tailed student *t*-tests (heteroscedastic) on expression ratio values obtained for haze and nonhaze eyes with nonoperated eyes, and between the haze and nonhaze groups, to identify significantly increased and decreased proteins (Fig. 2).

Post-hoc power calculations were performed following identification of significant protein hits using an alpha = 0.05 with standard statistical methods (ClinCalc Post-Hoc Power Calculator).

RESULTS

For this study we analyzed 34 eyes, including 17 eyes that underwent refractive surgery with the Raindrop Near Vision Inlay and 17 fellow control eyes. Among the test eyes, six developed varying degrees of postoperative corneal stromal

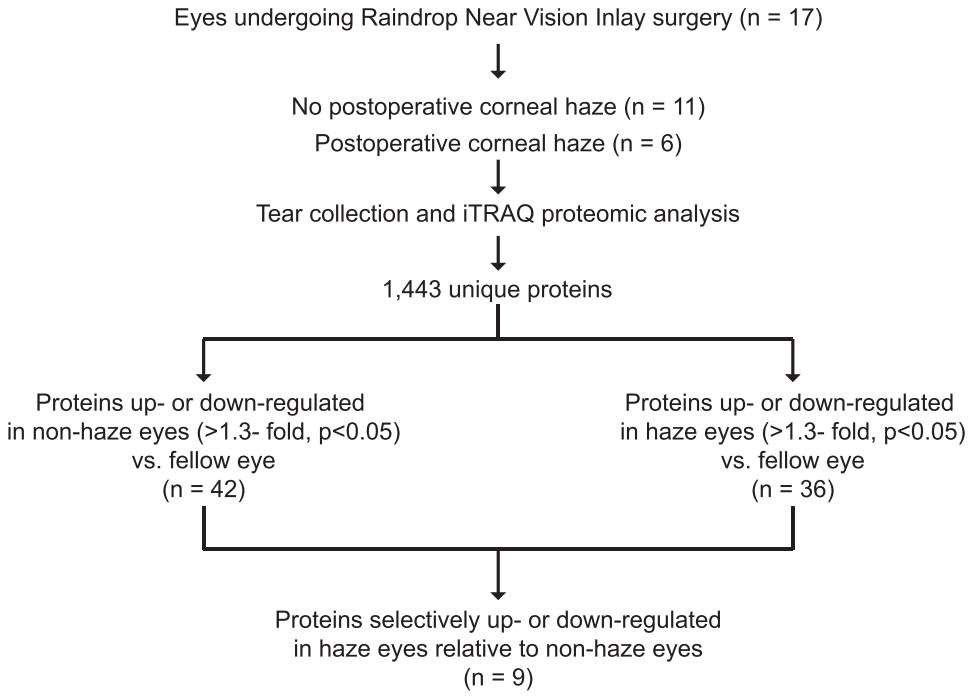

FIGURE 2. Experimental approach for identification of proteins specifically associated with corneal inlay-associated haze.

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Table 2. Gene Ontology Analysis of Differentially Regulated Proteins in Tears From Eyes With Corneal Haze After Inlay Surgery

Biologic Process	Haze-Associated Tear Proteins	Enrichment (fold)*	*P* Value†
AMP biosynthesis	ASL, ADK	>100	0.049
De novo IMP biosynthesis	ASL, GARS	>100	0.049
Regulation of vesicle fusion	ANXA2, CORO1A, ANXA1	69.22	0.030
Negative regulation of protein ubiquitination	OTUB1, VPS28, RPS3, ISG15	31.64	0.021
SRP-dependent cotranslational protein membrane targeting	OTUB1, VPS28, RPS3, ISG15	23.56	0.034
Translational initiation	RPS21, RPS20, RPS8, DENR, RPS3	18.96	0.022
Nonsense-mediated mRNA decay	RPS21, RPS20, RPS8, RPS3	18.61	0.049
Regulation of wound healing	ANXA2, ANXA1, S100A9, PLG	17.58	0.050
Platelet degranulation	TIMP1, TLN1, PLG, ORM1	17.30	0.048
Regulation of inflammatory response	CFH, ANXA1, S100A9, IGKV2D-28, PSMB4, C4A	8.37	0.050
Symbiont process	ANXA2, CFH, VPS28, DENR, PSMB4, TLN1, ISG15, PLG	6.73	0.028
Immune effector processes	ANXA2, IQGAP1, CFH, CORO1A, S100A9, IGKV2D-28, C4A, ISG15, ORM1	4.71	0.049
Immune response	ANXA2, OTUB1, IQGAP1, CFH, IGLV7-46, CORO1A, ANXA1, S100A9, IGK2D-28, C4A, ISG15, ORM1	3.75	0.046

Tear proteins significantly up- or downregulated in haze eyes relative to the unoperated fellow eye were used to interrogate the biological process section of Gene Ontology Consortium database[70] with a false discovery rate threshold of <0.05.

* Enrichment of protein query set relative to the expected number of proteins expected to be present in a randomly sampled group of proteins.

† The *P* values were determined using Fisher exact test with false discovery rate multiple test correction; output from the Gene Ontology Consortium database.[70]

haze (two each of grades 1, 2, and 3; see Fig. 1) in the region adjacent to the inlay at various times postoperatively (median 7.5 months; interquartile range: 6–9 months). Clinical characteristics of the two cohorts, including pre- and postoperative distance and near visual acuity, and central corneal thickness were statistically similar (Table 1).

This initial analysis identified a total of 1443 discrete proteins identified (false discovery rate < 1%) from the tear samples. Expression ratios were then determined for the operated eye compared to the unoperated fellow eye for each patient, with a *P* value of < 0.05 and expression ratio of greater than 1.3 (i.e., increased) or less than 0.769 (i.e., decreased) being considered significant. This step identified 42 regulated proteins in the nonhaze group and 36 proteins in the haze group (Fig. 2 and Supplementary Tables S1, S2). Gene ontology analysis of the proteins identified in the nonhaze group did not reveal any significant enrichment for proteins involved in a particular biologic process, while the haze group showed enrichment for proteins from several pathways, including protein and energy metabolism, immune functions, platelet degranulation, and wound healing (Table 2). Comparison of the haze and nonhaze hits revealed two proteins, ISG15 (haze expression 0.544 [$P = 0.042$]; nonhaze expression 0.594 [$P = 0.047$]) and Talin-1 (haze expression 0.522 [$P = 0.047$]; nonhaze expression 0.587 [$P = 0.005$]) that were significantly different between operated and unoperated eyes for both the haze and nonhaze groups (see Supplementary Tables S1, S2).

We next identified proteins that were significantly increased or decreased in eyes with postoperative corneal haze relative to eyes without corneal haze. This process yielded a total of nine discrete proteins, eight of which were decreased in the haze group and one increased (Table 3, Fig. 3). Haze-specific proteins were immune- and inflammation-related, including two complement proteins (C4A and complement factor H) and three immunoglobulin variable chains (IGKV2-29, IGKV2D-28, IGLV7-46). The tissue inhibitor of metalloproteinase TIMP1, neurite outgrowth modulator Shootin-1, and angiogenesis stimulator S100A4 were also among the depleted proteins, while aminopeptidase P1 was the only identified protein selectively increased in the hazy cornea group. We did not observe a clear dose response relating corneal haze grade to tear protein expression for the haze-specific proteins with the exception of S100A4, whose levels were progressively

Table 3. Identities of Proteins Significantly Up- or Downregulated in Tears From Patients With Postoperative Corneal Haze

Gene Symbol	Protein Name	No Haze Expression	Haze Expression	Fold Difference	*P* Value	Post-Hoc Power, %
C4A	Complement C4a	1.130 (0.571–2.235)	0.525 (0.270–1.019)	0.464	0.037	65.4
CFH	Complement factor H	0.935 (0.449–1.948)	0.551 (0.342–0.888)	0.589	0.048	58.8
IGKV2-29	Immunoglobulin kappa variable 2-29	3.715 (3.458–3.991)	0.476 (0.296–0.766)	0.128	0.006	100
IGKV2D-28	Immunoglobulin kappa variable 2D-28	0.809 (0.395–1.656)	0.495 (0.311–0.789)	0.612	0.025	70.8
IGLV7-46	Immunoglobulin lambda variable 7-46	1.068 (0.649–1.757)	0.514 (0.360–0.734)	0.482	0.007	91.5
S100A4	S100A4	0.987 (0.371–2.622)	0.606 (0.342–1.074)	0.614	0.048	57.8
SHTN1	Shootin-1	1.130 (0.992–1.287)	0.766 (0.678–0.865)	0.678	0.011	98.2
TIMP1	Metalloproteinase inhibitor 1	1.013 (0.760–1.352)	0.746 (0.590–0.943)	0.736	0.023	70.2
XPNPEP1	Aminopeptidase P1	0.893 (0.643–1.241)	1.355 (1.079–1.702)	1.517	0.023	78.3

Expression values are geometric means (geometric standard deviation range in parentheses) for expression ratios of proteins in tears from eyes with and without haze relative to the nonoperated fellow eye. Statistical significance was determined by paired two-tailed Student's *t*-tests comparing expression ratios in the two groups of eyes. Post-hoc power analysis for individual proteins was performed using alpha = 0.05.

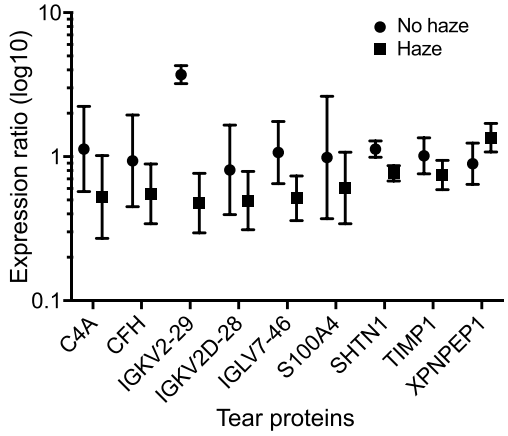

FIGURE 3. Comparison of protein expression patterns in tears collected from near-vision implant patients with postoperative corneal haze. Expression ratios are geometric means of protein expression ratios for eyes with (*squares*) and without (*circles*) postoperative corneal haze, with *error bars* indicating the geometric standard deviation ranges.

diminished with increasing grades of corneal haze (Supplementary Fig. S1).

The apparent overrepresentation of immune- and inflammation-associated proteins was confirmed using gene ontology analysis to identify overrepresentation of the nine proteins in biologic processes. This revealed 83-fold enrichment of the proteins involved in the regulation of complement activation, 47-fold enrichment in immunoglobulin production, and 43-fold enrichment in activation of the classical complement pathway (Table 4). The overall findings of the study are summarized in Figure 4.

DISCUSSION

Increasing demand for convenient alternatives to spectacle correction for presbyopia have driven the development of corneal inlay surgery, with several inlays now available.[2] However, a series of papers have identified corneal haze as an important complication of inlay surgery.[9,11,12] In this work, we have used tear proteomics to identify a series of inflammation- and immunity-related proteins that are differentially regulated in the tears of patients with corneal haze following inlay surgery with the Raindrop Near Vision Inlay.

Corneal haze represents the final stage of a complex pathway that commences with disruption of the epithelial basement membrane and the apoptosis of central keratocytes that progresses to migration and transformation of keratocytes and abnormal collagen deposition.[10,19] Although haze following refractive procedures can be effectively managed with topical anti-inflammatory medications such as steroids and mitomycin C, these medications have numerous well-characterized side effects.[20,21] Recent work with novel antifibrotic drugs such as histone deacetylase inhibitors suggests that selective targeting of protein mediators of the corneal haze response may circumvent these side effects while effectively blocking haze formation following keratorefractive surgery.[19] It is also important to note that the haze-associated proteins identified here were differentially regulated in spite patients receiving intra- and postoperative mitomycin C and topical steroids, respectively, suggesting that components of the haze response may not necessarily respond to these common anti-inflammatory agents.

We identified two proteins, ISG15 and Talin-1, that were differentially regulated in tears from postoperative eyes relative to unoperated fellow eyes. This was observed in both the haze and nonhaze eyes. ISG15 is a ubiquitin-like protein that binds numerous proteins in a process termed ISGsylation and serves as a central immune regulator that modulates the activation of lymphocytes, monocytes, and natural killer cells.[22,23] ISG15 is expressed and secreted by corneal cells[24] and was demonstrated to function as an immune modulator that plays a prominent role in the host response to fungal keratitis.[25] The second protein, Talin-1, activates integrins during the process of neutrophil recruitment to inflammatory sites, leading to free radical production, phagocytosis, and degranulation.[26,27] In keeping with this role, Talin-1 was previously identified in injured corneal tissues but not normal cornea.[28]

Our previous work on tear proteomics in post-LASIK eyes did not identify either of these two proteins as being differentially regulated in after LASIK surgery.[18] It would be of particular interest to determine if the regulation of these proteins was specific for the corneal inlay surgery, although the central role of these proteins in general inflammatory responses makes this seem unlikely.

Among the tear proteins we identified as being specific to the corneal haze response, C4a and complement factor H were decreased. In addition to mediating interactions between antigen-antibody complexes, C4a can be cleaved to release the C4 anaphylatoxin, which mediates local inflammation.[29,30] Previous work with human donor corneas did not show significant increase of C4a following excimer laser treatment.[31] In our study, C4a levels also remained largely unaffected among the nonhaze cornea group relative to unoperated fellow eyes but were significantly reduced in the hazy cornea group. Similarly, we saw a reduction of complement factor H, a complement regulator that limits the action of complement to activating surfaces.[32] Diminished factor H is known to result in

TABLE 4. Haze-Associated Proteins Form Part of the Complement and Immune Response Pathways in Gene Ontology Analysis

Biologic Process	Haze-Associated Tear Proteins	Enrichment (fold)*	*P* Value†
1. Regulation of complement activation	C4A, CFH, IGKV2-29, IGKV2D-28	82.76	0.002
1a. Regulation of acute inflammatory response	C4A, CFH, IGKV2-29, IGKV2D-28	60.73	0.001
1b. Regulation of proteolysis	C4A, CFH, IGKV2-29, IGKV2D-28, TIMP1	14.40	0.015
2. Immunoglobulin production	IGKV2-29, IGKV2D-28, IGLV7-46	47.07	0.036
3. Complement activation (classical pathway)	C4A, IGKV2-29, IGKV2D-28	43.30	0.042
3a. Adaptive immune response	C4A, IGKV2-29, IGKV2D-28, IGLV7-46	17.92	0.043

The eight identified tear proteins associated with haze were used to interrogate the biological process section of Gene Ontology Consortium database,[70] with a false discovery rate threshold of <0.05.

* Enrichment of protein query set relative to the expected number of proteins expected to be present in a randomly sampled group of proteins.

† The *P* values were determined using Fisher exact test with false discovery rate multiple test correction; output from the Gene Ontology Consortium database.[70]

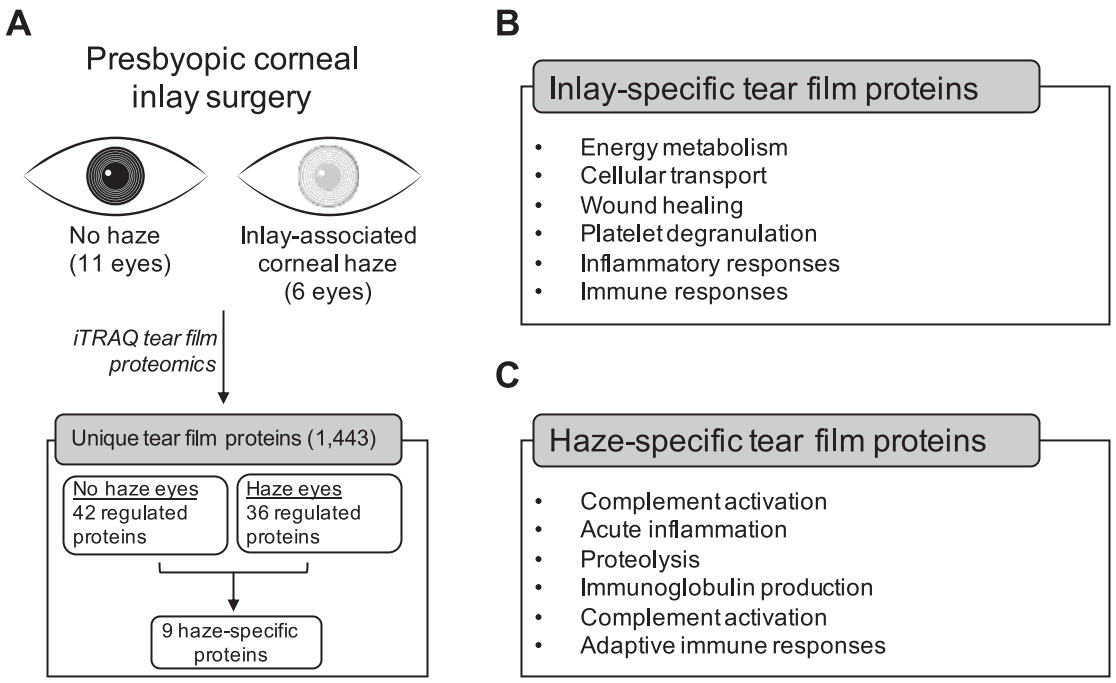

Figure 4. Summary of the experimental findings in the current study. (**A**) Outline of the experimental approach and identification of corneal inlay haze-associated tear film proteins. (**B**) Gene ontology findings of inlay surgery-associated tear film proteins. (**C**) Gene ontology findings of inlay haze-associated tear film proteins.

increased complement activity on healthy host cells, resulting in autoimmune disease.[33] This is consistent with the expectation that patients experiencing corneal haze have aberrant inflammation. Interestingly, C4a protein expression is also diminished in lung tissues under conditions of experimental lung injury and idiopathic pulmonary fibrosis,[34] while both C4a and complement factor H are reduced in hepatitis C-induced liver fibrosis,[35] suggesting a common fibrosis pathway between cornea other tissues.

In addition to the two complement proteins, we also saw reduction in the presence of three immunoglobulin variable chains, namely kappa variable 2–29, kappa variable 2D–28, and lambda variable 7–46, in corneal haze patients. Similar reductions tear immunoglobulin chains were seen in previous work on dry eye disease[36] and a rabbit model of Sjögren syndrome,[16] although the biologic significance of these findings remains unclear. The tear film is known to contain readily detectable IgA and its concentration increases in a number of ocular surface diseases including conjunctivitis, although interestingly its level was observed to decrease in cases of acute bacterial keratitis.[37] Similar to our findings for complement factor H and C4a, this may also represent depletion of immunoglobulins from the tear film during active corneal inflammation.

The calcium binding protein S100A4 was reduced in corneal haze tears. This protein has been studied mainly for its direct role as an intracellular mediator of tumor metastasis,[38] although it is now also regarded as a marker for fibroblast activity.[39] Expression of S100A4 is increased following corneal wounding and its expression level appears to reflect keratocyte activity and motility.[40–42] We previously identified S100A4 in a tear proteomics screen of dry eye patients,[14] whereby its expression was upregulated. However, previous work using corneal limbal tissue harvested from normal eyes and from eyes with ocular inflammation secondary to endophthalmitis demonstrated that S100 proteins, including S100A4, were readily detected in normal eyes but virtually undetectable in inflamed eyes.[43] Further work is required to elucidate the expression pattern of S100A4 in the cornea over time following trauma or other inflammatory stimuli.

The TIMP-1 protein also plays an important role in corneal scar formation following keratorefractive surgery, and is found at increased concentrations at the sites of radial keratotomy incisions.[44] Moreover, adequate expression of TIMP-1 was found to be necessary to protect against stromal degradation and basement membrane destruction during bacterial infections of the cornea.[45] This would appear to lend a biologic rationale to our finding that TIMP-1 levels were decreased in corneal haze patients.

Shootin-1, also identified here as being decreased in postoperative haze eyes, is a widespread intracellular protein that appears to function in dendritic outgrowth and cell migration.[46–50] It was recently uncovered in a tear proteomics screen for age-related macular degeneration-associated biomarkers,[51] although more work is needed to determine what role it might play at the ocular surface.

Aminopeptidase P1 was the only protein identified in our screen that was increased in tears from corneal haze patients. This protein is a soluble cytosolic peptidase expressed in human leukocytes and platelets,[52,53] that plays a role in neurodevelopment, with knockout mice exhibiting growth retardation and microcephaly.[54] Increased levels of aminopeptidase P1 (Xaa-Pro aminopeptidase 1) were found in healed combat wounds compared to dehisced wounds in a proteomics study of skin injuries,[55] although there is limited data on its role at the ocular surface.[56]

Corneal wound healing after refractive surgery occurs via a series of inflammatory cascades that result in the transformation of stromal keratocytes into fibroblasts and myofibroblasts.[57] In conventional refractive surgery such as LASIK, this process is typically transient and active inflammation usually resolves within a month.[58] In contrast, the development of stromal haze following corneal inlay surgery is considerably more delayed, with various reports showing the development

of corneal haze many months and even years after surgery.[8,9,12] Previous work from our group demonstrated that even at this late stage, corneal inlays that were explanted due to stromal haze showed evidence of chronic inflammation.[9] The pathophysiologic changes that trigger haze development after inlay surgery have not yet been fully elucidated, although other groups have suggested several possible mechanisms, including inadequate transfer of nutrients and subsequent stromal degeneration, chronic mechanical irritation of keratocytes, and foreign body responses to the inlay material itself.[12,59] The previous finding that greater flap depth dramatically reduces the appearance of stromal haze[8] favors the presence of an immune response against either the inlay itself or degenerating keratocytes around the inlay, which becomes less effective with increasing stromal depth.

While the small number of haze-associated proteins identified here certainly seem to show a clear biologic pattern of immune and inflammatory mediation, we acknowledge that the limitations of our experimental approach may have prevented identification of all proteins involved in the development of corneal haze. For the purposes of statistical analysis many proteins were excluded from our final hit list because they were undetectable in tear samples, preventing the identification of proteins that are dramatically depleted in haze or nonhaze groups. A second limitation is the nonuniformity of sample collection timing following onset of corneal haze. However, it is reasonable to assume that the onset of corneal haze would occur with a stepwise alteration in inflammatory mediators that would vary over the course of disease. Due to clinical limitations, our samples were taken at varying times following the onset of haze, which varied among individuals and this would have precluded the identification of certain proteins at varying stages of biologic regulation at the time of sampling. This issue would need to be addressed for our identified proteins using a tear sampling time course experiment following either the initial inlay surgery or the onset of corneal haze to elucidate the kinetics of each protein. A related shortcoming of this work is the likely substantial underestimation of the number of proteins that contribute to the corneal haze response. Tissue inflammatory responses involve coordinated regulation of hundreds of immune and inflammatory mediators[60,61] and it is likely that the haze response elicited by corneal inlays involves a similar level of complexity. Although a sizable number of proteins were differentially regulated in haze eyes and unoperated eyes, only nine discrete proteins were identified as being haze-specific. The lack of serial sampling would likely have prevented the identification of numerous transiently regulated tear film proteins involved in the haze response. Additionally, many potentially relevant proteins failed to meet the stringent exclusion criteria used, though we felt it was important to use strict cutoffs because of the small number of patients recruited for the study.

Subgroup analysis of the differentially regulated tear film proteins did not show a clear dose response for most of the haze-specific proteins in terms of corneal haze grade. This may relate to the variability in tear sampling times and the limitations of the haze grading system used. Ideally corneal densitometry would be needed to properly quantify corneal haze and serial sampling would be needed to confirm that the identified proteins do indeed show follow a predictable increase (or decrease) in concentrations that relates to the appearance of corneal haze.

An additional methodologic limitation to our study was the absence of intraoperative mitomycin C treatment for one eye from the haze group and one eye from the nonhaze group. Anecdotal reports suggest that mitomycin C circumvents corneal haze following inlay surgery[62] and it is reasonable to assume that the absence of mitomycin C in these two eye may have introduced bias into our analysis. That said, after excluding these two patients from our analysis, the list of haze-related proteins remained significantly associated with corneal haze (data not shown).

The current study involved a small sample size and although our screening approach identified several significantly regulated haze-related tear film proteins, a post-hoc power analysis indicated that the sample size for several of the proteins was insufficient to reliably detect differences between the haze and nonhaze groups (i.e., power $< 80\%$; see Table 3). Among the nine haze-related proteins, assays for IGKV2-29 (100%), IGLV7-46 (91.5%), and Shootin-1 (98.2%) were adequately powered, while the remainder had discriminating power between 58.8% and 78.3%. Subsequent analyses indicated that 10 patients per group would be required to obtain sufficient statistical power to reliably detect differences between groups for the majority of tear film proteins identified in this study. Subsequent prospective studies with larger cohorts will be required to address this shortcoming.

Use of the tear proteome as a surrogate for corneal biological responses is limited by our incomplete understanding of how well the tear proteome reflects biologic processes within corneal tissues.[63–65] The current work is also limited in this regard and ideally corneal tissue would be better for proteomics analysis to determine the difference biological responses that occur following the development of corneal haze. That said, recent work by the Human Eye Proteome Project, which utilized a similar methodology for protein identification as that described in our work, provides some insight into the degree of overlap between tear film and corneal proteomes.[66] Current estimates place the total number of proteins expressed by the human genome at 20,500 proteins, while the Human Eye Proteome Project identified 3708 corneal proteins and 1698 tear film proteins.[67] Among the corneal and tear film proteomes, 521 proteins are common to both, yielding a representation factor of 1.7.[68] This indicates that there are 1.7-times more proteins in common between cornea and tear film than would be expected if the overlap occurred by chance. It is therefore likely that the tear film does share a significant proteomic overlap with the cornea, although without the benefit of corneal tissue for comparison it is difficult to conclude how many of the proteins identified in the current work are present within the corneal stroma of postoperative eyes.

In spite of these limitations, it appears clear from this work that corneal haze following corneal inlay surgery involves differential regulation of a series of inflammatory and immune mediators, several of which have known roles in corneal disease and tissue fibrosis. Future directions for this work would include functional assessment of the haze mediators in tear and corneal samples, and also a longitudinal analysis of tear samples beginning from the preoperative stage and continuing through the early and later postoperative stages to determine the kinetics of the haze protein responses. Additionally, it will be informative to determine whether the haze responses described here are specific for the Raindrop inlay or represent a more general pathway applicable to other corneal inlays and corneal inflammatory disorders in general.

As an addendum to this work, the authors would like to point out that following completion of this research, the manufacturer of the Raindrop Near Vision Inlay, ReVision Optics, had ceased operations. Additionally, the FDA has recently issued a safety communication to medical providers stating that the due to the high incidence of corneal haze (42%) observed after five years of follow-up, the Raindrop Near Vision Inlay is no longer recommended for use and the remaining inlays are now being removed from circulation.[69]

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References

1. Moarefi MA, Bafna S, Wiley W. A review of presbyopia treatment with corneal inlays. *Ophthalmol Ther*. 2017;6:55–65.
2. Konstantopoulos A, Mehta JS. Surgical compensation of presbyopia with corneal inlays. *Expert Rev Med Devices*. 2015;12:341–352.
3. Lindstrom RL, Macrae SM, Pepose JS, Hoopes PC Sr. Corneal inlays for presbyopia correction. *Curr Opin Ophthalmol*. 2013;24:281–287.
4. Vukich JA, Durrie DS, Pepose JS, Thompson V, van de Pol C, Lin L. Evaluation of the small-aperture intracorneal inlay: three-year results from the cohort of the U.S. Food and Drug Administration clinical trial. *J Cataract Refract Surg*. 2018;44:541–556.
5. Moshirfar M, Desautels JD, Wallace RT, Koen N, Hoopes PC. Comparison of FDA safety and efficacy data for KAMRA and Raindrop corneal inlays. *Int J Ophthalmol*. 2017;10:1446–1451.
6. Binder PS. Intracorneal inlays for the correction of presbyopia. *Eye Contact Lens*. 2017;43:267–275.
7. Beer SMC, Santos R, Nakano EM, et al. One-year clinical outcomes of a corneal inlay for presbyopia. *Cornea*. 2017;36:816–820.
8. Whitman J, Dougherty PJ, Parkhurst GD, et al. Treatment of presbyopia in emmetropes using a shape-changing corneal inlay: one-year clinical outcomes. *Ophthalmology*. 2016;123:466–475.
9. Ong HS, Chan AS, Yau CW, Mehta JS. Corneal inlays for presbyopia explanted due to corneal haze. *J Refract Surg*. 2018;34:357–360.
10. Fahd D, de la Cruz J, Jain S, Azar DT. Corneal haze after refractive surgery. In: Alio JL, Azar DT, eds. *Management of Complications in Refractive Surgery*. Cham, Switzerland: Springer Nature; 2018:259–267.
11. Antonios R, Jabbur NS, Ahmed MA, Awwad ST. Refractory interface haze developing after epithelial ingrowth following laser in situ keratomileusis and small aperture corneal inlay implantation. *Am J Ophthalmol Case Rep*. 2018;10:10–12.
12. Mulet ME, Alio JL, Knorz MC. Hydrogel intracorneal inlays for the correction of hyperopia: outcomes and complications after 5 years of follow-up. *Ophthalmology*. 2009;116:1455–1460.
13. Tong L, Zhou L, Beuerman RW, Zhao SZ, Li XR. Association of tear proteins with Meibomian gland disease and dry eye symptoms. *Br J Ophthalmol*. 2011;95:848–852.
14. Zhou L, Beuerman RW, Chan CM, et al. Identification of tear fluid biomarkers in dry eye syndrome using iTRAQ quantitative proteomics. *J Proteome Res*. 2009;8:4889–4905.
15. Matheis N, Okrojek R, Grus FH, Kahaly GJ. Proteomics of tear fluid in thyroid-associated orbitopathy. *Thyroid*. 2012;22:1039–1045.
16. Zhou L, Wei R, Zhao P, Koh SK, Beuerman RW, Ding C. Proteomic analysis revealed the altered tear protein profile in a rabbit model of Sjogren's syndrome-associated dry eye. *Proteomics*. 2013;13:2469–2481.
17. Leonardi A, Palmigiano A, Mazzola EA, et al. Identification of human tear fluid biomarkers in vernal keratoconjunctivitis using iTRAQ quantitative proteomics. *Allergy*. 2014;69:254–260.
18. D'Souza S, Petznick A, Tong L, et al. Comparative analysis of two femtosecond LASIK platforms using iTRAQ quantitative proteomics. *Invest Ophthalmol Vis Sci*. 2014;55:3396–3402.
19. Lim RR, Tan A, Liu YC, et al. ITF2357 transactivates Id3 and regulate TGFbeta/BMP7 signaling pathways to attenuate corneal fibrosis. *Sci Rep*. 2016;6:20841.
20. Majmudar PA, Schallhorn SC, Cason JB, et al. Mitomycin-C in corneal surface excimer laser ablation techniques: a report by the American Academy of Ophthalmology. *Ophthalmology*. 2015;122:1085–1095.
21. Carnahan MC, Goldstein DA. Ocular complications of topical, peri-ocular, and systemic corticosteroids. *Curr Opin Ophthalmol*. 2000;11:478–483.
22. Dos Santos PF, Mansur DS. Beyond ISGlylation: functions of free intracellular and extracellular ISG15. *J Interferon Cytokine Res*. 2017;37:246–253.
23. Ritchie KJ, Zhang DE. ISG15: the immunological kin of ubiquitin. *Semin Cell Dev Biol*. 2004;15:237–246.
24. Taylor JL, D'Cunha J, Tom P, O'Brien WJ, Borden EC. Production of ISG-15, an interferon-inducible protein, in human corneal cells. *J Interferon Cytokine Res*. 1996;16:937–940.
25. Dong C, Gao N, Ross BX, Yu FX. ISG15 in host defense against Candida albicans infection in a mouse model of fungal keratitis. *Invest Ophthalmol Vis Sci*. 2017;58:2948–2958.
26. Wegener KL, Partridge AW, Han J, et al. Structural basis of integrin activation by talin. *Cell*. 2007;128:171–182.
27. Stadtmann A, Zarbock A. The role of kindlin in neutrophil recruitment to inflammatory sites. *Curr Opin Hematol*. 2017;24:38–45.
28. Ishizaki M, Wakamatsu K, Matsunami T, et al. Dynamics of the expression of cytoskeleton components and adherens molecules by fibroblastic cells in alkali-burned and lacerated corneas. *Exp Eye Res*. 1994;59:537–549.
29. Moon KE, Gorski JP, Hugli TE. Complete primary structure of human C4a anaphylatoxin. *J Biol Chem*. 1981;256:8685–8692.
30. Gorski JP, Hugli TE, Muller-Eberhard HJ. C4a: the third anaphylatoxin of the human complement system. *Proc Natl Acad Sci U S A*. 1979;76:5299–5302.
31. Gardner BP, Pleyer U, Mondino BJ, Sumner HL, Frieberg ML, Imperia PS. Complement-derived anaphylatoxins in human donor corneas treated with excimer laser. *Ophthalmic Surg Lasers*. 1995;26:568–571.
32. Rodriguez de Cordoba S, Esparza-Gordillo J, Goicoechea de Jorge E, Lopez-Trascasa M, Sanchez-Corral P. The human complement factor H: functional roles, genetic variations and disease associations. *Mol Immunol*. 2004;41:355–367.
33. Griffiths MR, Neal JW, Fontaine M, Das T, Gasque P. Complement factor H, a marker of self protects against experimental autoimmune encephalomyelitis. *J Immunol*. 2009;182:4368–4377.
34. Kim TH, Lee YH, Kim KH, et al. Role of lung apolipoprotein A-I in idiopathic pulmonary fibrosis: antiinflammatory and antifibrotic effect on experimental lung injury and fibrosis. *Am J Respir Crit Care Med*. 2010;182:633–642.
35. Gangadharan B, Antrobus R, Dwek RA, Zitzmann N. Novel serum biomarker candidates for liver fibrosis in hepatitis C patients. *Clin Chem*. 2007;53:1792–1799.
36. Srinivasan S, Thangavelu M, Zhang L, Green KB, Nichols KK. iTRAQ quantitative proteomics in the analysis of tears in dry

eye patients. *Invest Ophthalmol Vis Sci*. 2012;53:5052–5059.

37. Sen DK, Sarin GS. Immunoglobulin concentrations in human tears in ocular diseases. *Br J Ophthalmol*. 1979;63:297–300.
38. Garrett SC, Varney KM, Weber DJ, Bresnick AR. S100A4, a mediator of metastasis. *J Biol Chem*. 2006;281:677–680.
39. Fei F, Qu J, Li C, Wang X, Li Y, Zhang S. Role of metastasis-induced protein S100A4 in human non-tumor pathophysiologies. *Cell Biosci*. 2017;7:64.
40. Schneider M, Hansen JL, Sheikh SP. S100A4: a common mediator of epithelial-mesenchymal transition, fibrosis and regeneration in diseases? *J Mol Med (Berl)*. 2008;86:507–522.
41. Samolov B, Steen B, Seregard S, van der Ploeg I, Montan P, Kvanta A. Delayed inflammation-associated corneal neovascularization in MMP-2-deficient mice. *Exp Eye Res*. 2005;80:159–166.
42. Ryan DG, Taliana L, Sun L, Wei ZG, Masur SK, Lavker RM. Involvement of S100A4 in stromal fibroblasts of the regenerating cornea. *Invest Ophthalmol Vis Sci*. 2003;44:4255–4262.
43. Nubile M, Lanzini M, Calienno R, et al. S100 A and B expression in normal and inflamed human limbus. *Mol Vis*. 2013;19:146–152.
44. Kenney MC, Chwa M, Alba A, Saghizadeh M, Huang ZS, Brown DJ. Localization of TIMP-1, TIMP-2, TIMP-3, gelatinase A and gelatinase B in pathological human corneas. *Curr Eye Res*. 1998;17:238–246.
45. Kernacki KA, Chunta JL, Barrett RP, Hazlett LD. TIMP-1 role in protection against Pseudomonas aeruginosa-induced corneal destruction. *Exp Eye Res*. 2004;78:1155–1162.
46. Nawaz MS, Giarda E, Bedogni F, et al. CDKL5 and Shootin1 interact and concur in regulating neuronal polarization. *PLoS One*. 2016;11:e0148634.
47. Higashiguchi Y, Katsuta K, Minegishi T, Yonemura S, Urasaki A, Inagaki N. Identification of a shootin1 isoform expressed in peripheral tissues. *Cell Tissue Res*. 2016;366:75–87.
48. Wang Q, Wang X, Le Y, et al. Rnaset2 inhibits melanocyte outgrowth possibly through interacting with shootin1. *J Dermatol Sci*. 2015;80:25–32.
49. Toriyama M, Kozawa S, Sakumura Y, Inagaki N. Conversion of a signal into forces for axon outgrowth through Pak1-mediated shootin1 phosphorylation. *Curr Biol*. 2013;23:529–534.
50. Toriyama M, Shimada T, Kim KB, et al. Shootin1: a protein involved in the organization of an asymmetric signal for neuronal polarization. *J Cell Biol*. 2006;175:147–157.
51. Winiarczyk M, Kaarniranta K, Winiarczyk S, Adaszek L, Winiarczyk D, Mackiewicz J. Tear film proteome in age-related macular degeneration. *Graefes Arch Clin Exp Ophthalmol*. 2018;256:1127–1139.
52. Vanhoof G, De Meester I, Goossens F, Hendriks D, Scharpe S, Yaron A. Kininase activity in human platelets: cleavage of the Arg1-Pro2 bond of bradykinin by aminopeptidase P. *Biochem Pharmacol*. 1992;44:479–487.
53. Rusu I, Yaron A. Aminopeptidase P from human leukocytes. *Eur J Biochem*. 1992;210:93–100.
54. Yoon SH, Bae YS, Mun MS, et al. Developmental retardation, microcephaly, and peptiduria in mice without aminopeptidase P1. *Biochem Biophys Res Commun*. 2012;429:204–209.
55. Chromy BA, Eldridge A, Forsberg JA, et al. Wound outcome in combat injuries is associated with a unique set of protein biomarkers. *J Transl Med*. 2013;11:281.
56. Wright QG. *Role of Microbiota in Strengthening Ocular Mucosal Barrier Function Through Secretory IgA* [master's thesis]. Harvard Extension School, Cambridge, MA: Harvard University; 2017:41.
57. Ljubimov AV, Saghizadeh M. Progress in corneal wound healing. *Prog Retin Eye Res*. 2015;49:17–45.
58. Pisella PJ, Auzerie O, Bokobza Y, Debbasch C, Baudouin C. Evaluation of corneal stromal changes in vivo after laser in situ keratomileusis with confocal microscopy. *Ophthalmology*. 2001;108:1744–1750.
59. Spirn MJ, Dawson DG, Rubinfeld RS, et al. Histopathological analysis of post-laser-assisted in situ keratomileusis corneal ectasia with intrastromal corneal ring segments. *Arch Ophthalmol*. 2005;123:1604–1607.
60. Chen L, Deng H, Cui H, et al. Inflammatory responses and inflammation-associated diseases in organs. *Oncotarget*. 2018;9:7204–7218.
61. Saha S, Harrison SH, Chen JY. Dissecting the human plasma proteome and inflammatory response biomarkers. *Proteomics*. 2009;9:470–484.
62. Brennan K. Risk Management for Corneal Inlays. *Rev Ophthalmol*. 2017. Available at: https://www.reviewofophthalmology.com/article/risk-management-for-corneal-inlays. Accessed February 16, 2019.
63. Azkargorta M, Soria J, Acera A, Iloro I, Elortza F. Human tear proteomics and peptidomics in ophthalmology: toward the translation of proteomic biomarkers into clinical practice. *J Proteomics*. 2017;150:359–367.
64. Wu K, Zhang Y. Clinical application of tear proteomics: present and future prospects. *Proteomics Clin Appl*. 2007;1:972–982.
65. Zhou L, Beuerman RW. Tear analysis in ocular surface diseases. *Prog Retin Eye Res*. 2012;31:527–550.
66. Semba RD, Enghild JJ, Venkatraman V, Dyrlund TF, Van Eyk JE. The Human Eye Proteome Project: perspectives on an emerging proteome. *Proteomics*. 2013;13:2500–2511.
67. Clamp M, Fry B, Kamal M, et al. Distinguishing protein-coding and noncoding genes in the human genome. *Proc Natl Acad Sci U S A*. 2007;104:19428–19433.
68. Roy PJ, Stuart JM, Lund J, Kim SK. Chromosomal clustering of muscle-expressed genes in Caenorhabditis elegans. *Nature*. 2002;418:975–979.
69. Administration USFD. Increased Risk of Corneal Haze Associated With the Raindrop Near Vision Inlay: FDA Safety Communication. 2018. Available at: https://www.fda.gov/MedicalDevices/Safety/AlertsandNotices/ucm623973.htm. February 16, 2019.
70. The Gene Ontology C. Expansion of the Gene Ontology knowledgebase and resources. *Nucleic Acids Res*. 2017;45:D331–D338.