

Changes in Human Tear Proteome Following Topical Treatment of Dry Eye Disease: Cyclosporine A Versus Diquafosol Tetrasodium

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PURPOSE. To compare the changes in human tear proteome and clinical effects following topical cyclosporine A (CsA) 0.05% or diquafosol tetrasodium (DQS) 3% treatment of dry eye disease (DED), and to identify biomarkers for determining disease severity and treatment effectiveness in DED.

METHODS. A total of 18 patients were diagnosed with non-Sjögren DED. Nine patients in each group were treated with topical CsA 0.05% or DQS 3% for 4 weeks. Tear samples were collected after evaluation of tear breakup time, corneal and conjunctival erosion staining, and results of Schirmer's test 1 before and after treatment. Proteomes were characterized using liquid chromatography mass spectrometry, and proteins exhibiting a fold change >1.5 or <0.67 ($P < 0.05$) were considered differentially expressed (DEP).

RESULTS. A total of 794 proteins were identified, with no significant difference observed between pretreatment and posttreatment conditions. Proteomic analysis identified 54 and 106 DEPs between treatment groups (CsA and DQS, respectively), with gene ontology analysis indicating that both treatments enhanced innate and adaptive immune responses and cellular detoxification. Protein-network analysis showed that inflammation associated with the immune response was primarily responsible for the therapeutic process in both groups.

CONCLUSIONS. These results provide insight into the broad scope of changes at the ocular surface in DED and indicated that although both drugs improved the clinical parameters, the activated tear-specific biomarkers differed significantly between treatments. Our findings suggest that the DEPs identified here and those correlated with the clinical parameters might represent candidate biomarkers for DED.

Keywords: cyclosporine A, diquafosol tetrasodium, tear proteome, biomarker, dry eye disease

In-depth analysis of human bodily fluids is among the most promising approaches for determining diagnostic biomarkers and the therapeutic assessment of pharmacological options for human diseases.^{1,2} Recent developments in proteomics and mass spectrometry (MS) analyses have markedly impacted the current understanding of protein pathways, activities, structures, and interactions, even when used to assess small-volume samples, such as those associated with human tears ($<5 \mu\text{L}$).^{3,4}

Tear fluid contains proteins/peptides secreted from the lacrimal glands, meibomian glands, conjunctival goblet cells, corneal epithelial cells, and vascular sources.⁵ Particularly, its high protein concentration and the ease of noninvasive sample collection make it suitable for analyzing the altered status of the

ocular surface (OS) in patients with dry eye disease (DED).^{4,6} DED is a multifactorial disease that causes ocular discomfort and visual disturbance.^{7,8} Currently, tear-film instability and tear hyperosmolarity with subsequent OS inflammation represent the core mechanisms associated with DED.⁹ Treatments for DED have evolved from simply hydrating the OS to modifying the disease process. According to the stepwise therapy suggested by the Dry Eye Workshop II (DEWS II), patients with DED refractory to artificial tears are recommended to use topical anti-inflammatories or secretagogues, such as cyclosporine A (CsA) 0.05% and diquafosol tetrasodium (DQS) 3%, respectively.¹⁰



CsA is an immunomodulatory agent that inhibits interleukin-2 activation of T cells and prevents OS-cell apoptosis, resulting in reduced inflammation and increased tear production.^{11,12} In addition, CsA-loaded nanoemulsions with improved biocompatibility and extended residence time at OS have been designed recently.¹³ DQS is a purinergic P2Y₂ receptor agonist that stimulates water and mucin secretion via calcium-ion flux within OS cells.^{14,15} Although the two drugs exhibit different mechanisms of action, they are equally recommended by DEWS II as topical agents for treating DED patients exhibiting the same severity levels.⁷ Moreover, because both drugs are recommended for treatment of moderate-to-severe DED, the guidelines are difficult to explain to physicians in the absence of evidence showing which drug is more effective according to patient type. In addition, there is a considerable lack of data concerning correlations and interobserver variation among clinical signs and symptoms of DED following proper treatment.^{16,17}

This study elucidated the pretreatment and posttreatment proteomes of human tears following treatment for DED and compared treatment effectiveness at the protein level according to treatment regimen. To the best of our best knowledge, this represents the first study investigating and comparing changes in the tear proteome associated with the use of topical medication.

MATERIALS AND METHODS

Patient Enrollment

This prospective interventional study was part of a multicentered, randomized, evaluator-masked, phase IV clinical trial¹⁸ that was approved by the institutional review board (XC16MIMV0056S). The clinical trial was registered in the Current Research Information System (<http://cris.nih.go.kr>) and World Health Organization International Clinical Trials Registry Platform (<https://www.who.int/ictpr>), and its registration number is KCT0002180. The current study was conducted at Gangnam Severance Hospital (Department of Ophthalmology, Yonsei University College of Medicine, Seoul, Korea). The study adhered to the tenets of the Declaration of Helsinki. After informed consent was obtained, all participants were enrolled following examinations in the more symptomatic eye unless contraindicated.

Adult patients of age ≥ 19 years were enrolled through DED screening as follows: (1) symptomatic dry eye with ocular dryness, and (2) DED-specific fluorescein staining on OS or tear breakup time (TBUT) ≤ 10 seconds. We excluded patients with any ocular history, even in one eye, ocular surgery, ocular injury, ocular infection, eyelid disease, allergy, or autoimmune disease, as well as patients who had used punctal plug, contact lens, or other topical agents within 4 weeks of the screening period. Pregnant or lactating patients were also excluded.

Among enrolled patients after screening, the current study cohort further selected subjects who fulfilled the diagnostic criteria of Asia Dry Eye Society⁸: one or more DED-related symptoms including ocular dryness, tightness, foreign body sensation, irritation, red eye, itching sensation, blurring, or pain; and TBUT of < 5 seconds. For tear proteome analysis, we narrowed the inclusion criteria by further excluding subjects who had used any systemic and topical drugs.

Study Design and Randomization

After screening a total of 154 patients, 153 eligible patients were randomly allocated to receive 0.05% CsA ophthalmic

solution (Cyporin N; Taejoon Pharmaceutical Inc., Seoul, Korea) or 3% DQS solution (Diquas; Santen Pharmaceutical, Osaka, Japan). Patients in the CsA group were instilled with 0.05% CsA twice daily, and patients in DQS group were instilled with 3% DQS six times daily. Among patients enrolled in the clinical trial via our hospital, 32 patients fulfilled the aforementioned narrowed criteria for the present work on proteomic analysis. However, only 18 samples of human tears from one eye of 18 patients had enough volume for proteomic analysis, which consisted of nine samples treated with CsA and nine with DQS. These patients were clinically examined, and their tear samples were collected at 4 weeks after the initiation of treatment. Both efficacy and safety were evaluated at 4 weeks after treatment.

An independent statistical office (Seoul CRO, Co., Ltd., Seoul, Korea) performed permuted stratified block randomization using SAS 9.2 (SAS Institute Inc., Cary, NC, USA). Allocation concealment was maintained over the entire period. For masked condition, all medications were provided to patients after being repackaged in an aluminum pouch and container box with coded product information.

Clinical Examinations and Tear Sample Collection

Each evaluation was performed in the following order to minimize the effect of the previous measurement on other measurements: (1) OS disease index (OSDI) questionnaire was a validated 12-item questionnaire assessing symptoms of ocular irritation consistent with DED, their impact on vision-related function, and environmental triggers. It gave a range of 0 (no symptoms) to 100 (severe symptoms).¹⁹ (2) Corneoconjunctival staining score (range, 0–33), as well as each corneal (range, 0–15), and conjunctival staining scores (range, 0–18) were measured through slit-lamp evaluation with cobalt blue illumination and sodium fluorescein (fluorescein strip; Haag-Streit, Koeniz, Switzerland) using National Eye Institute/industry (NEI) scale.²⁰ (3) TBUT was examined until one or more dry spots appeared in the precorneal tear film from the last blink by slit-lamp microscopy. (4) Schirmer's test 1 (ST1) was performed over 5 minutes without anesthesia (35-mm Whatman filter paper strip; Haag-Streit). (5) A bonded 2 \times 10-mm polyester rod (TRANSORB WICKS; FILTRONA, Richmond, VA, USA) was used to collect tears from lower tear meniscus without any ocular touching or irritation, as previously reported.^{21,22} It was stored at -80°C until the mass spectrophotometric assay was performed.

An interval of at least 15 minutes separated each test. To minimize the extent to which one test influenced the results of the following tests, all tests were performed in the same order with relatively constant temperature ($23\text{--}24^{\circ}\text{C}$) and humidity ($40\text{--}45\%$).

Efficacy, Tolerability, and Safety Assessment

In the original clinical trial before the current study, the primary outcome was defined as the change in staining score on NEI scale after treatment. The secondary outcomes were defined as the change in NEI erosion score, ST1, TBUT, and OSDI. In the present study, we additionally aimed to detect more than 30 differentially expressed proteins (DEPs) with up- and down-regulations for constructing protein network. We also assessed the tolerability, including instillation adherence, using a survey on the sensation of eye drops on instillation scored on a 10-point visual analog scale. Safety variable was the occurrence of adverse events based on physical signs and symptoms, slit-lamp microscopy, visual acuity, IOP, and fundus examination.

TABLE. Clinical Parameters of Patients With DED and Protein Concentrations in Tears

	CsA Treatment (<i>n</i> = 9)	DQS Treatment (<i>n</i> = 9)	<i>P</i> Value
Age, y	46.22 ± 1.44	53.30 ± 15.50	0.41
Sex, <i>n</i> (female)	5	5	
OSDI score (range)	41.42 ± 19.79 (8–94)	40.97 ± 18.93 (8–94)	0.43
TBUT, sec (range)	3.45 ± 0.65 (1.96–4.50)	3.14 ± 0.91 (1.34–4.83)	0.17
Schirmer's test I, mm (range)	7.56 ± 1.78 (3–10)	6.89 ± 1.88 (4–10)	0.21
Corneconjunctival staining score (NEI scale) (range)	10.17 ± 2.70 (5–16)	10.28 ± 4.07 (4–21)	0.94
Corneal staining (range)	4.71 ± 1.37 (3–9)	5.11 ± 1.45 (3–9)	0.27
Conjunctival staining (range)	5.46 ± 2.19 (1–10)	5.17 ± 2.96 (1–12)	0.63
Protein concentrations in tears (μg/μL)			
Pretreatment	15.46 ± 9.51	17.67 ± 12.16	0.27
Posttreatment	13.73 ± 5.44	14.80 ± 7.26	0.28

Data are presented as mean ± SD. *P* values for comparisons of clinical indexes and protein concentrations of tears between groups were determined using the Mann-Whitney *U* test and Student's *t*-test, respectively.

In-solution Digestion

Three pooled tear proteins from nine human subjects for each group, each one of which was approximately 300 μg equally, were digested into peptides by in-solution digestion. With each sample, 8M urea in 100 mM ammonium bicarbonate was mixed to make at least 6M for final concentration, and the mixture was incubated for 20 minutes at room temperature. To denature the proteins, 10 mM dithiothreitol for reduction and 30 mM Iodoacetamide for alkylation were used. Trypsin was added to the samples and incubated at 37°C overnight. The activated trypsin reaction was quenched with 0.4% trifluoroacetate, and peptides were desalted with a C₁₈ Harvard macro spin column. The resultant peptides were dried and stored at –80°C.

Protein Identification Using Liquid Chromatography Tandem MS

Peptides were resuspended in 0.1% formic acid in water and analyzed using the Q Exactive orbitrap hybrid mass spectrometer coupled with the Nanoacquity UPLC (Waters, Manchester, UK). The peptides were eluted through a trap column, ionized through an NSI system coupled with in-house column (100 cm × 75 μm) packed with 2 μm C₁₈ particles at an electric potential of 2.0 kV. The maximal ion injection time for MS/MS was set to 60 ms at a resolution of 17,500. Dynamic exclusion time was set to 30 seconds.

Raw Data Processing

Raw files were searched with the MaxQuant (v. 1.5.1.2)²³ against the Uniprot database. A false discovery rate cutoff of 1% was applied at the peptide spectrum match and protein levels. Protein identification required at least two peptides using the “razor plus unique peptides” setting in MaxQuant.²⁴ Proteins were quantified using the XIC-based label-free quantification (LFQ) algorithm.²⁵ All LFQ intensities were transformed to log₂ values. Proteins that did not display all values in at least one group were filtered out.

Enrichment Analysis Using Gene Ontology and Network Analysis

The gene ontology biological process (GOBP), cellular components, and molecular function (GOMF) terms associated with DEPs were analyzed using the Database for Annotation, Visualization and Integrated Discovery.²⁶ Functional annotation clustering and Kyoto Encyclopedia of Genes and Genomes pathway mapping was also performed. To construct the

network model for DEPs, we collected protein-protein interaction (PPI) information from the STRING 9.1 public database.²⁷ The PPI networks were built with interactome data using Cytoscape.

Statistical Analysis

Statistical analyses on the clinical data were performed using SPSS version 21.0 (IBM Corp., Armonk, NY, USA). The Kolmogorov-Smirnov test was used to confirm normality of the data. To statistically compare data between groups, we used Mann-Whitney *U* test or Wilcoxon signed-rank test for non-normally distributed data. In all statistical tests, a *P* value less than 0.05 was considered statistically significant.

For statistical analysis of proteomics data, Perseus software (v. 1.5.0.31; Max Planck Institute of Biochemistry, Munich, Germany) was used. Proteins with expression greater than ± 1.5-fold change and less than 0.05 *P* value from Student's *t*-test in LFQ intensity were classified to DEPs.

RESULTS

Comparison of the Clinical Effects of CsA and DQS Treatment

Of the 18 DED patients recruited for this study, 18 eyes qualified and completed the study procedures during each visit, with nine eyes treated with CsA eye drops and nine with DQS eye drops. There were no significant differences in drug tolerability, including instillation adherence, between groups (data not shown). No adverse event was observed in this study. We also found no difference in any clinical index according to DED severity between groups before treatment (Table). At 4 weeks after treatment, TBUT, ST1, corneal staining, and conjunctival staining scores (NEI scale), and OSDI showed significant improvements compared with their pretreatment status (Fig. 1), although we observed no significant difference in these parameters between CsA- and DQS-treated groups. These results suggested that both topical drugs exhibited an equivalent therapeutic effect against DED from a clinical standpoint.

Proteomic Profiling of Tears From DED Before and After Treatment

A total of 794 proteins were identified from all tear samples from DED patients, with 654 proteins detected at baseline, and the proportion of common proteins between patients was 93.6% and 85.6% for the CsA and DQS groups before treatment,

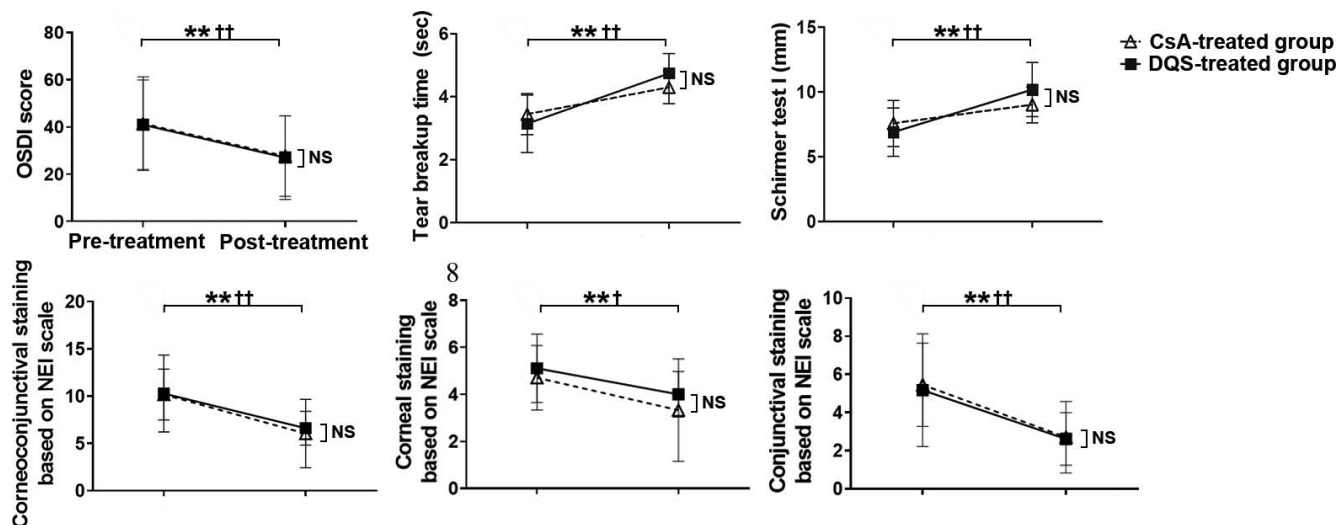


FIGURE 1. Comparison of changes in clinical parameters in patients with DED after treatment. Squares and triangles indicate means, and daggers represent standard deviations in each group. *P* values for comparisons between two groups and between pretreatment and posttreatment samples within each group were determined using Mann-Whitney *U* test and Wilcoxon signed-rank test, respectively. ***P* < 0.01 in CsA-treated group; †*P* < 0.05 and ††*P* < 0.01 in the DQS-treated group. NS, not significant.

respectively ($n = 529$; Fig. 2A). At 4 weeks posttreatment, we detected 601 and 494 proteins in the CsA- and DQS-treated groups, with 54 (10.0%) and 106 (25.6%) DEPs identified between the CsA- and DQS-treated groups (Fig. 2B). Surprisingly, clustering analysis indicated that expressed proteins associated with DQS treatment showed the highest degree of dissimilarity from the other clusters (Fig. 2C), whereas the clustered proteins associated with CsA treatment were distinct from those involved with DQS treatment but showed less difference from clusters associated with pretreatment conditions for both groups.

DEP Identification and Functional Classification Following Treatment

CsA treatment for 4 weeks resulted in significant upregulation (UP-DEP) of 26 proteins and significant downregulation (DN-DEP) of 28 proteins (Supplementary Table S1A), whereas DQS treatment resulted in 51 UP-DEPs and 55 DN-DEPs (Fig. 3A, Supplementary Table S1B).

To determine treatment effects on the tears of DED patients, we analyzed the GOBP associated with DEPs. In the CsA-treated group, UP-DEPs were enriched for cellular processes, including hydrogen peroxide catabolism, vesicle-mediated transport, defense response, immune response, and regulation of cell death (Supplementary Table S2A), and DN-DEPs were associated with defense response to other organisms and the mitogen-activated protein kinase (MAPK) cascade (Supplementary Table 2B). UP-DEPs associated with DQS treatment showed the same enriched cellular processes (Supplementary Table S2C), whereas DN-DEPs were involved in the MAPK cascade, complement activation, and lipid transport (Fig. 3B, Supplementary Table S2D).

We then analyzed the molecular function of DEPs according to GOMF categories, finding that UP-DEPs associated with CsA treatment were predominantly enriched for peroxidase/phosphorylase/peptidase activity, MHC class I binding, and glycosaminoglycan binding (Supplementary Table S3A), whereas DN-DEPs were associated with peptidase-inhibitor activity and calcium-related binding, although these associations were not significant ($P = 0.0793$) (Supplementary Table S3B). UP-DEPs associated with DQS treatment

were enriched for calcium-ion and lipid binding, enzyme activity, and glycoprotein binding (Supplementary Table S3C), whereas DN-DEPs were associated with serine-type endopeptidase activity, cell adhesion, and chaperone binding (Fig. 3C, Supplementary Table S3D).

Differences in PPI Networks According to Treatment

We then constructed PPI network models using DEPs from the CsA- and DQS-treated groups. The results showed that several DEPs formed a network associated with inflammation, including the immune and defense responses. Other networks involved the regulation of cell death and epithelium development, whereas networks associated with DEPs from the DQS-treated group involved lipid metabolism and cell adhesion (Figs. 4A, 4B).

Furthermore, measurement of betweenness centrality²⁸ in the networks for the CsA-treated group revealed the highest level of connectivity for myosin heavy chain 9 (enriched for regulation of proteolysis) with other DEPs, followed by catalase (enriched for hydrogen peroxide catabolism), whereas the networks for the DQS-treated group showed relatively lower but more distributed betweenness, with the associated DEPs mainly involved in the immune response and the response to external stimuli (Supplementary Table S4).

Comparison of Protein Expression Patterns Between CsA and DQS Treatment

We observed similar regulatory patterns following CsA or DQS treatment in 22 proteins. GOBP analysis indicated that similarly downregulated proteins ($n = 13$) following either treatment were enriched for glucose metabolism, cell apoptosis, cell adhesion, and immune-system processes, whereas similarly upregulated proteins ($n = 9$) were related to oxygen transport, defense response, and regulation of cell death (Supplementary Fig. S1A).

Interestingly, 49 proteins showed an inverse pattern of expression following CsA or DQS treatment, with those related to regulation of endopeptidase activity, protein metabolism,

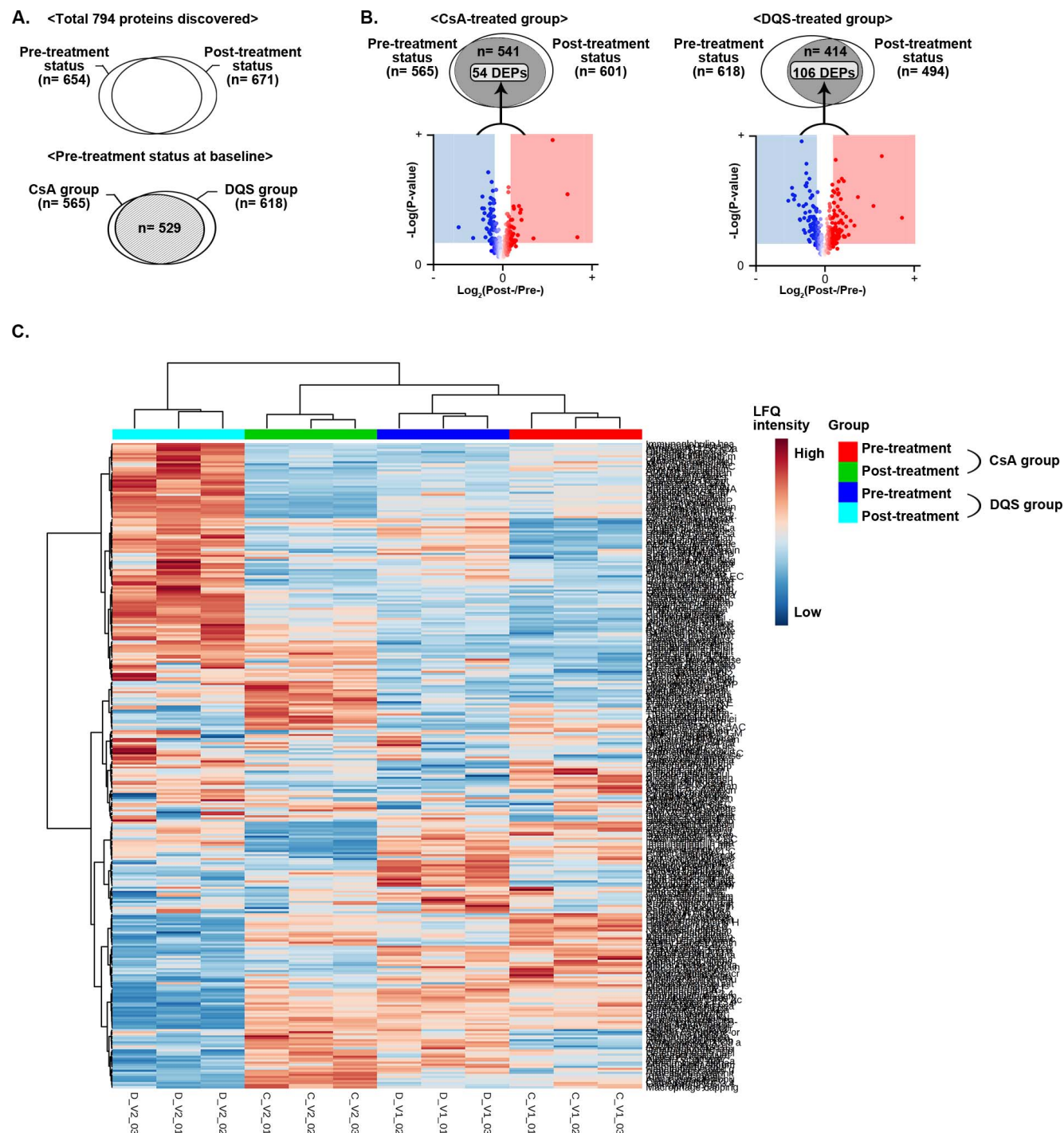


FIGURE 2. Isolation of DEPs in tear samples from patients with DED after treatment. (A) Venn diagram showing total discovered proteins and common proteins in the tears of DED patients before treatment. (B) Detection of DEPs (fold change: >1.5 or <0.67 ; $P < 0.05$) between pretreated and posttreated samples in treatment group. DEPs should be measured at every detection. (C) Heatmap showing the expression of identified proteins and a dendrogram depicting similarities in protein composition among groups. Red and blue colors in the heatmap indicate \log_2 -transformed intensities of LFQ of proteins.

and wound healing upregulated in CsA-treated tears as compared with their downregulation in DQS-treated tears. On the other hand, DQS treatment upregulated proteins involved in regulation of stress response, tissue homeostasis, and defense response as compared with their downregulation in CsA-treated tears. Both drugs similarly upregulated proteins associated with vesicle-mediated transport and immune-system processes (Supplementary Fig. S1B).

DISCUSSION

Although tear osmolality and matrix metalloproteinase (MMP)-9 levels are currently used for point-of-care testing for DED, their efficacy for accurate diagnosis remains controversial.²⁹ Therefore, it is possible that appropriate biomarkers or objective tests for evaluating treatment effectiveness for DED, as well as disease severity, still do not exist. Here, we measured

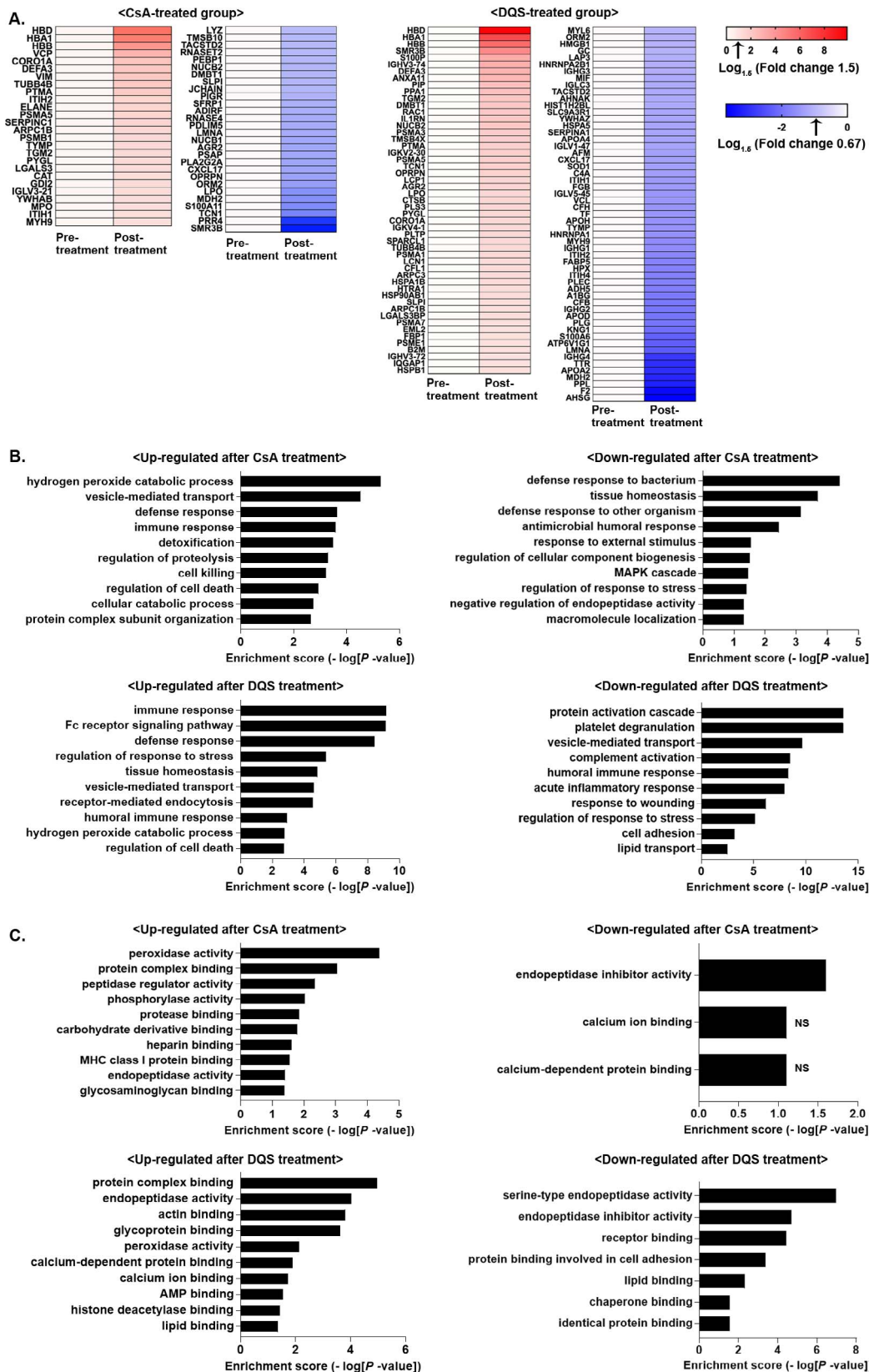


FIGURE 3. Identification and functional classification of DEPs in tears of dry eye patients after treatment. (A) DEPs in tear samples after CsA or DQS treatment as compared with those in pretreated samples (fold change: >1.5 or <0.67 ; $P < 0.05$). Heatmaps show upregulated and downregulated DEPs in both treatment groups according to increasing and decreasing expression ratios. (B) Bar plots showing the top 10 significantly enriched gene ontology (GO) biological processes ($P < 0.05$) for the upregulated and downregulated DEPs in each group. (C) Bar plots showing overrepresented GO molecular functions assigned to upregulated and downregulated DEPs in each group.

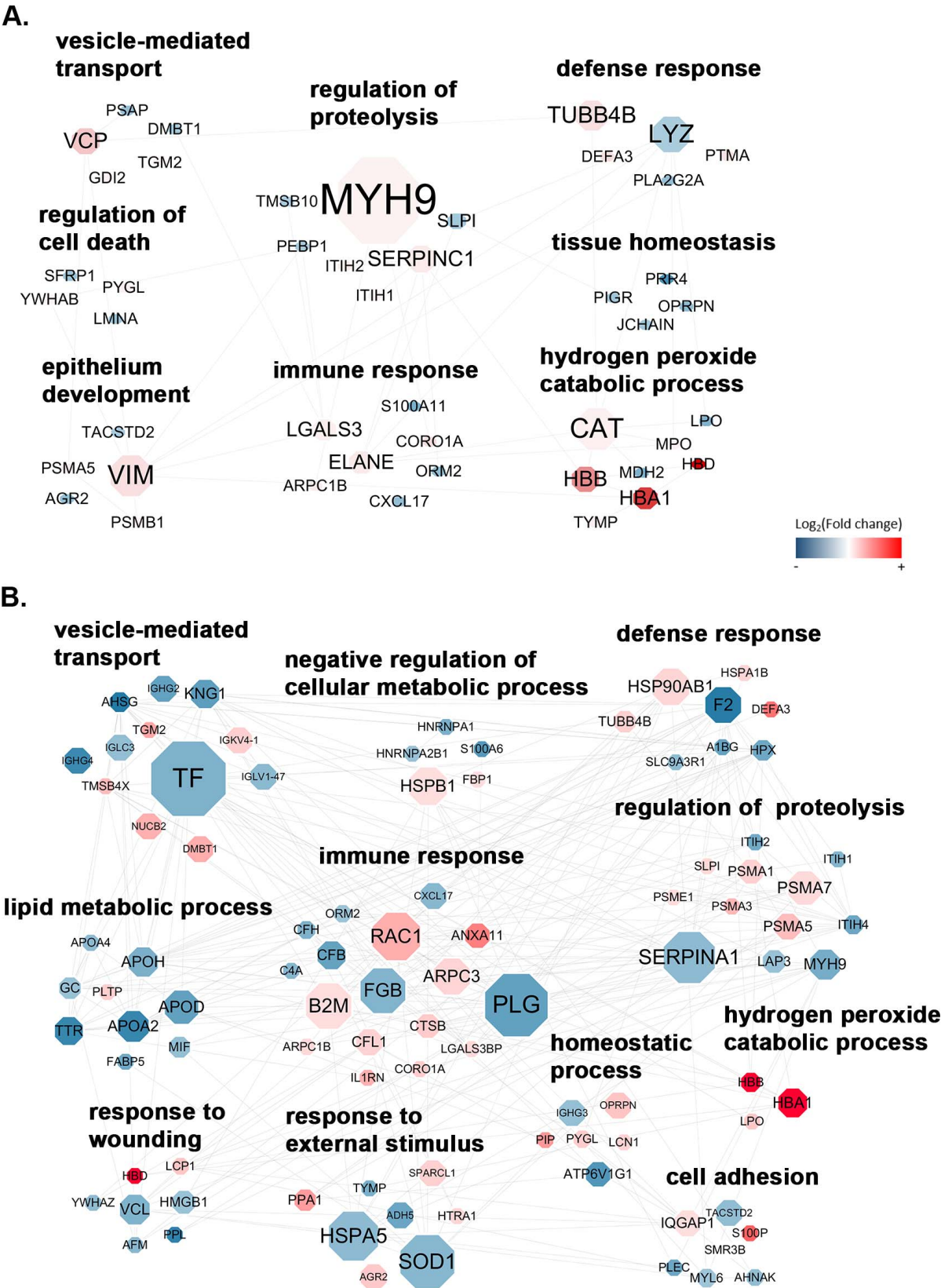


FIGURE 4. PPI networks generated by DEPs identified in tears of dry eye patients after treatment. Larger nodes represent higher connectivity with other DEPs. Connections between nodes (gray lines) designate PPIs. Node colors represent the degree of upregulation (red) or downregulation (blue) for each DEP compared with these levels in pretreated samples. Bold annotations indicate representative biological processes enriched in DEPs according to GO category. (A) Csa-treated group. (B) DQS-treated group.

and compared changes in the tear-fluid proteome following treatment with two different drugs, despite similar clinical outcomes. This finding builds on our previous work evaluating differences in tear proteomes between control and DED patients.²¹ In addition, our findings suggested that proteins associated with the immune system and inflammation were responsible for treatment effectiveness and DED pathophysiology, even after treating with P2Y₂ agonist. Moreover, because the expression profiles of several proteins were significantly altered by treatment and accompanied improved clinical conditions, they might represent candidate biomarkers useful for determining DED severity and treatment effectiveness.

Because DED has multifactorial etiologies and represents heterogeneous entities resulting in various signs and symptoms, we hypothesized that proteomic data would differ between patients. Surprisingly, there was a strong similarity between data from pretreatment tear fluids, despite variability in symptoms and OS-erosion levels. Similarities in the proteome heatmaps (Fig. 2, Supplementary Fig. S1) between pretreatment groups suggest the existence of a common pathophysiologic mechanism involved in DED development, despite the etiologic heterogeneity and symptomatic differences. Previous studies support that tear film instability and inflammation as well as immune response are the core mechanisms associated with DED pathophysiology.^{9,21,30} In the present study, we identified downregulated proteins associated with the innate immune (nuclear factor-kappaB signaling and the Fc-gamma receptor signaling pathway) and inflammation systems (phagocytosis, proteolysis, and the acute-phase response) in relation to clinical improvements, which agreed with previous studies. We found that a 2.1-fold elevation in phospholipase A2 group IIA (PLA2A) relative to the control was subsequently reduced to 0.58- and 0.78-fold elevations by CsA and DQS treatment, respectively. Changes in PLA2A levels were previously identified in tears from patients with DED³¹ and ocular rosacea,³² and other studies reported PLA2 as an inflammatory mediator associated with DED.^{33,34} Although PLA2 expression is elevated in other conditions, such as atopic keratoconjunctivitis³⁵ and contact-lens wearing,^{33,36} and unsuitable as a DED-specific biomarker, its strong correlation with DED-treatment responsiveness suggests it as a candidate for measuring treatment effectiveness and DED severity according to OS status.

In addition, we found dissimilarities in the posttreatment proteomes of patients administered CsA or DQS, despite similar clinical data between groups. We were unable to elucidate a relationship between the clinical parameters and differences in the proteomic; however, these results imply that each drug uses a different pathway to treat DED.

In the CsA-treatment group, levels of valosin-containing protein (VCP) were significantly elevated in tears (by 3.28-fold). VCP is ubiquitously expressed and facilitates protein degradation via the ubiquitin proteasome and autophagy³⁷; however, except for a report identifying its role in retinal disease,³⁸ the specific role of VCP in eyes has not been well documented. By contrast, we observed that VCP levels were downregulated by DQS treatment (by 0.68-fold). In addition, nucleobindin-2 (NUCB2) was downregulated after CsA treatment.³⁹ NUCB2 is distributed in multiple tissues, including adipose tissue, reproductive organs, and tissues associated with immunity and neurons⁴⁰ and affects cellular migration and invasion through the MMP-2 and -9 pathways.⁴¹ A previous study showed that MMP-9-positive patients responded more favorably to CsA than did MMP-9-negative patients.⁴² In the present study, we found that NUCB2 was downregulated by CsA treatment (by 0.65-fold). These results suggest these markers as promising markers of CsA responsiveness against DED.

Furthermore, we found that lipocalin 1 (LCN1) was downregulated following DQS treatment, which agreed with a previous study.⁴³ In addition, a treatment trial involving DED patients reported improved clinical signs in tear film in conjunction with increases in the tear LCN1 levels.⁴⁴ In the present study, we identified upregulation of LCN1 in response to DQS (by 1.68-fold) but not CsA (by 0.92-fold) treatment. Other markers exhibiting drug-specific changes are summarized in Supplementary Tables S1 and S4.

DQS treatment induced a larger number of DEPs in human tears than CsA treatment (106 vs. 54, respectively). However, a larger number of DEPs was not indicative of increased drug efficacy based on the absence of significant differences in clinical improvements observed from both drugs. In particular, CsA treatment resulted in equivalent clinical improvements accompanied by a fewer number of DEPs. Because CsA regulates inflammation, it is possible that it targets specific immunomodulatory markers that result in a larger overall response. In addition, CsA is recommended as an 8-week treatment course for maximum treatment effectiveness,^{10,45} suggesting its possible requirement for a longer treatment period to realize changes at the molecular level relative to DQS. By contrast, DQS directly promotes the secretion of tear components by activating the P2Y₂ receptor, especially in conjunctiva.^{15,46–48} Therefore, DQS efficacy might be associated with the speed with which it achieves maximum effectiveness.

This study had some limitations. First, a small number of subjects took part in the current proteomic study. In fact, the superordinate clinical trial consisted of three arms against DED such as nanoemulsion-CsA 0.05%, emulsion-CsA 0.05% and DQS 3%. However, we analyzed tear samples from patients of nanoemulsion-CsA and DQS group because we intended to compare posttreatment alteration in tear proteome between two different drugs, CsA and DQS. Furthermore, to avoid technical confounder of tear collection, storage, and shipping, we used tear samples from 32 patients enrolled via only our hospital even though 80 patients in each arm were initially registered in the original multicentered trial. However, only 18 samples from 18 patients (nine per each group) had enough volume for proteomic analysis despite our delicate effort to collect nonstimulated tears using polyester fiber rods with an experienced technique. Second, we did not inspect the absence or presence of meibomian gland dysfunction, which is the most common cause of evaporative DED that can lead to a mixed form of DED, consisting of both evaporative and aqueous subtypes. Meibomian gland lipid is an essential component of tear film layer, and its alterations may directly or indirectly affect tear proteome. Moreover, we did not in detail analyze tear proteome according to DED subtypes, such as evaporative, aqueous deficient, or short TBUT-type. However, we tried to focus on the effect of treatment regimens on human tear proteome with general DED. Third, our results may not reflect the full range of effects from their use because the clinical recommendation for maximizing the effectiveness of each drug differed. In the present study, protein levels following DQS treatment were most dissimilar from baseline, whereas those at 4 weeks post-CsA treatment were closer to baseline levels, suggesting that CsA treatment might require a longer period to realize changes relative to DQS.^{7,45} Moreover, in clinical settings, there are several other treatment options, including topical steroids and other types of immunomodulators. In addition, we were unable to suggest specific biomarkers for DED diagnosis and follow-up. Further studies using larger cohorts, which can embrace various subtypes of DED, are required to identify such biomarkers and determine their associated mechanisms in DED.

In summary, we analyzed the tear proteomes of DED patients receiving different treatment regimens, finding that the molecular pathways associated with the clinical outcomes differed significantly between DQS and CsA treatment, despite similar levels of improvement. Proteomic analysis of tears can provide insight into changes at the OS in DED; therefore, further studies using larger sample sizes and in vivo validation of the findings will support the identification of DED-specific biomarkers and the development of therapeutic options for DED management.

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