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# Tear proteomic analysis of Sjögren syndrome patients with dry eye syndrome by two-dimensional-nano-liquid chromatography coupled with tandem mass spectrometry

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We examined the tear film proteome of patients with Sjögren's syndrome (SS) and dry eye syndrome (group A), patients with dry eye symptoms (group B) and normal volunteers (group C). Tear samples were pooled from 8 subjects from each group and were subjected to two-dimensional-nano-liquid chromatography coupled with tandem mass spectrometry (2D-nano-LC-MS/MS). The tear breakup time for group A was significantly reduced compared with group B and C ( $P < 0.001$ ). Group A (Schirmer I test,  $2.13 \pm 2.38$  mm/5 min) had markedly lower tear volume than group B ( $5.94 \pm 4.75$  mm/5 min) and C ( $14.44 \pm 6.57$  mm/5 min) ( $P < 0.001$ ). Group A had significantly higher normalized tear protein content ( $1.8291 \pm 0.2241$   $\mu\text{g}/\text{mm}$ ) than group B ( $1.0839 \pm 0.1120$   $\mu\text{g}/\text{mm}$ ) ( $P = 0.001$ ) and C ( $0.2028 \pm 0.0177$   $\mu\text{g}/\text{mm}$ ) ( $P = 0.001$ ). The 2D-nano-LC-MS/MS analysis identified a total of 435 proteins, including 182 (54.8%), 247 (74.4%) and 278 (83.7%) in group A, B, and C, respectively, with 56 (16.7%) proteins including defensin  $\alpha 1$ , clusterin and lactotransferrin unique to group A. In conclusion, dry eye syndrome in SS patients is associated with an altered proteomic profile with dysregulated expression of proteins involved in a variety of important cellular process including inflammation, immunity, and oxidative stress.

Dry eye syndrome is a multifactorial condition characterized by eye irritation symptoms, blurred and fluctuating vision, tear film instability, increased tear osmolarity and ocular surface epithelial dysfunction<sup>1-4</sup>. Sjögren's syndrome (SS) is an autoimmune disorder in which the exocrine glands are the principal target organs, particularly the lachrymal and salivary glands<sup>5,6</sup>, and dry eyes are frequently are the presenting symptoms of SS.

Multiple proteomics-based studies have identified several proteins and peptides as candidate SS biomarkers. A proteomic analysis of the saliva of SS patients exhibited differences in the levels of a number of proteins such as  $\alpha$ -amylase precursor, carbonic anhydrase VI, and  $\beta$ -2 microglobulin compared to healthy controls<sup>7</sup>. Other studies also reveal that lactoferrin is a tear-specific biomarker for SS while anti-transglutaminase, anti-histone, anti-SSA and anti-SSB antibodies are saliva biomarkers of SS<sup>8,9</sup>. The ability to probe the protein content of human tear fluid has enormous potential for deepening our understanding of the pathology of ocular and systemic diseases such as SS and diabetes and enabling novel noninvasive tear-based diagnostic technologies.

Recently, electrospray ionization (ESI) tandem mass spectroscopy (MS/MS) has been used to identify novel protein species in tears<sup>10-13</sup>. The number of proteins found in the tear film continues to grow; however, there is disagreement in the literature regarding the number of proteins in the tear film and the functions of individual proteins. Fung *et al.*<sup>14</sup> reported that approximately 500 proteins were detected and unambiguously identified by liquid chromatography (LC)/MS/MS. Some of these functions are thought to be protective by aiding the ocular surface defense system, or maintain stability of the ocular surface by promoting interaction with other ligands (i.e., lipid binding proteins). The up- or downregulation of these proteins may be indicative of disease mechanisms. In addition, proteomic patterns in tear fluids may reflect histological and functional changes of the lachrymal gland<sup>15</sup>.



The aims of this study were to examine and compare the tear film proteome of SS, dry eye patients and normal volunteers using 2D-LC-nano-MS/MS-based proteomics.

## Methods

**The study population.** Eight subjects with SS and dry eye syndrome (group A) were recruited for the study. SS was diagnosed according to the Fox criteria<sup>16</sup>. Dry eye syndrome was diagnosed by a modified Dry Eye Workshop classification<sup>17</sup> and was considered present if Schirmer I test < 5 mm and tear breakup time (BUT) < 5 seconds, and absent if Schirmer I test > 10 mm and BUT > 10 seconds. Eight patients with dry eye symptoms (group B, Schirmer I test > 10 mm and BUT > 10 sec) but no systemic diseases were also recruited. A subject was excluded if he or she 1) had a history of ocular trauma or abnormality of the nasolacrimal drainage apparatus; 2) used eye drops within one month prior to the study, 3) had systemic immunologic syndromes other than SS; or 4) any other ocular syndromes than dry eye syndrome. Four acinae of the labial gland were taken from the patients and immediately preserved in formalin for pathological examination. In addition, 8 age and sex-matched healthy subjects who received regular physical checkup (group C) in the same interim were included as normal controls.

The study protocol was approved by the Institutional Review Board of Shanghai Tenth People's Hospital and all study participants provided written informed consent. The study was carried out in accordance with the Declaration of Helsinki.

**Western blot assays.** Five mL of blood was collected via the antecubital vein of participants in group A, B, and C for measurement of anti-SSA/SSB antibodies using commercially available kits as instructed by the manufacturer (Emoimraw, Germany). The immunoblotting procedure was carried out as previously described<sup>18</sup>.

**Sample collection and preparation.** Tears were collected by placing a Schirmer strip in the inferior fornix approximately 6 mm from the lateral canthus. The conjunctiva was not anesthetized. The subjects were instructed to keep the eyes closed during the 5-min test. After the wet length was recorded, the strip was placed in a 1-mL amber Eppendorf tube. Gloves were worn by the examiner and by all investigators handling any tear film samples. All the samples were processed in a masked fashion. The samples were placed immediately in ice transport tanks and stored at -80°C until processed.

Proteins were extracted from the Schirmer strip by incubation in 1 mL Tris buffer (pH = 8.3) at 4°C for 10 h. After centrifugation at 12,000 g for 10 min, the supernatants from all patients within each group were pooled and precipitated by acetone as described previously<sup>19</sup>. Precipitated proteins were resuspended in 100 µL Tris buffer and quantitated using the BCA assay kit (Pierce, Rockford, IL). The normalized protein content in the tear samples pooled from all subjects from each group was calculated using the following formula:

Normalized protein content = total protein/filterpaper wet length in Schirmer I test.

Then, 12.5 µg protein from each group was incubated with 25 mM ammonium bicarbonate and 10 mM dithiothreitol (DTT) for 60 min at 56°C followed by incubation with 25 mM iodoacetamide (IAA) for 30 min in the dark at room temperature. Trypsin digestion was done at 37°C for 12 h. The resultant peptides were dried and saved at -80°C.

**Two-dimensional strong cation-exchange/reversed-phase nano-scale liquid chromatography mass spectrometry.** Extracted peptides were desalted using a 1.3 mL C18 solid phase extraction column (Sep-Pak® Cartridge, Waters, Milford, MA), dried using a vacuum centrifuge and resuspended with loading buffer (5 mM ammonium formate containing 5% acetonitrile, pH 3.0), which were then separated and analyzed by 2D-LC-nano-MS/MS. The experiments were performed on a Nano

Aquity UPLC system (Waters) connected to an LTQ Orbitrap XL mass spectrometer (Thermo Electron, Bremen, Germany) equipped with an online nano-electrospray ion source (Michrom Bioresources, Auburn, CA). A 180 µm × 2.4 cm SCX column (Waters), which was packed with a 5-µm PolySULFOETHYL Aspartamide (PolyLC, Columbia, MD), was used for the first dimension for recovery of hydrophobic peptides after a conventional salt step gradient. Then, a RP step gradient from 15% to 50% acetonitrile was applied to the SCX column as previously described<sup>18</sup>. The RP analytical column (20 cm × 75 µm), which was packed with a 1.7-µm Bridged Ethyl Hybrid (BEH) C18 material (Waters), was used for the second dimension separation to elute peptides using a three-step linear gradient, starting from 5% B to 45% B in 40 min (A: water with 0.1% formic acid; B: acetonitrile with 0.1% formic acid), increased to 80% B in 3 min, and then to 5% B in 2 min. The electrospray voltage of 1.1 kV versus the inlet of the mass spectrometer was used.

LTQ Orbitrap XL mass spectrometer was operated in the data-dependent mode to switch automatically between MS and MS/MS acquisition. Survey full-scan MS spectra with two microscans (m/z 300–1800) were acquired in the Orbitrap with a mass resolution of 60,000 at 400 m/z, followed by ten sequential LTQ-MS/MS scans. Dynamic exclusion was used with two repeat counts, 10 s repeat duration, and 60 s exclusion duration. For MS/MS, precursor ions were activated using 35% normalized collision energy at the default activation q of 0.25.

**Data analyses.** All MS/MS spectra were identified by using SEQUEST [v.28 (revision 12), Thermo Electron] against the human International Protein Index (IPI) database (IPI human v3.45 Fasta with 71983 entries). To reduce false positive identification results, a decoy database containing the reverse sequences was appended to the database. The searching parameters were set up as follows: partial trypsin (KR) cleavage with two missed cleavages was included for analysis, the variable modification was oxidation of methionine, peptide mass tolerance was 20 ppm, and fragment ion tolerance was 1 Da. The Trans Proteomic Pipeline software (revision 4.0) (Institute of Systems Biology, Seattle, WA) was then utilized to identify proteins based upon corresponding peptide sequences with ≥95% confidence. The peptide results were filtered by the Peptide Prophet<sup>18</sup> with a P value over 0.95 and a Protein Prophet<sup>19</sup> probability of 0.95 was used for protein identification results. Spectral counts (SC) correlate with protein abundance<sup>20</sup>. The relative abundance of individual proteins was assessed by spectral counting, in which we counted the number of times the unlabeled version of a protein was identified by the fragmentation spectra of its peptides. Additionally, we normalized SC using the following formula:

$$\text{Normalized SC} = \text{SC of specific protein} \times 100 / \text{totalSC of each group}$$

For analysis of the difference of abundance among the proteomes, proteins were divided into three groups according to SC: the high abundance group (SC ≥ 50), the medium abundance group (20 < SC < 49), and the low abundance group (SC < 20). Significant upregulation was defined as a SC ratio ≥ 3 and SC distance ≥ 5; significant downregulation was defined as a SC ratio ≤ 0.33 and SC distance ≤ -5. SC ratio was calculated using the equation:

$$\text{SC ratio} = (\text{tear protein SC of SS patients}) / \text{control SC}$$

and SC distance was calculated using the following formula:

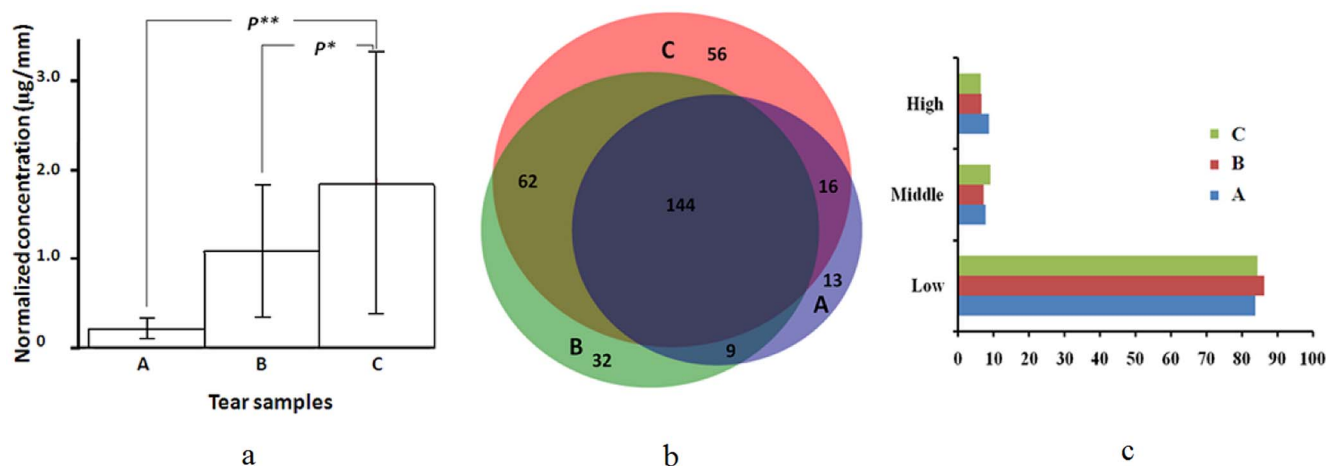
$$\text{SC distance} = (\text{tear protein SC of SS patients}) - (\text{control SC}).$$

**Statistical analysis.** Data were expressed as mean ± standard deviation and analyzed using the SPSS version 16.0 (SPSS, Chicago, IL). Chi-square tests and Student's *t* tests were performed and a *P* value of 0.05 or less was considered significant in our study.

Table 1 | Demographic and baseline characteristics of the study subjects

	Healthy subjects n = 8	Patients with dry eye symptoms n = 8	Patients with SS and dry eye syndrome n = 8
<b>Age, years</b>			
Mean	61.75	56.38	60.13
Standard deviation	6.61	4.63	5.89
<b>Gender</b>			
Male	4	4	4
<b>Tear breakup time, sec</b>	12.38 ± 3.88	3.06 ± 1.76	1.07 ± 1.22
<b>Schirmer I test, mm/5 min</b>	14.44 ± 6.57	5.94 ± 4.75	2.13 ± 1.88
<b>Fluorescent test</b>	0.00 ± 0.00	8.44 ± 6.18	26.49 ± 0.99
<b>Best corrected visual acuity</b>	0.91 ± 0.23	0.91 ± 0.21	0.45 ± 0.35
<b>SSA/SSB antibody (+)</b>	0	0	6
<b>Lip gland biopsy (+)</b>	0	0	8

SS: Sjögren's syndrome.



**Figure 1** | (a) Normalized protein content in tear samples from healthy subjects (A), patients with dry eye symptoms (B) and Sjögren's syndrome (SS) patients with dry eye syndrome (C). Data are expressed as mean  $\pm$  sd of at least three independent experiments.  $P^{**} = 0.035$ , healthy subjects vs. SS patients with dry eye syndrome and  $P^* = 0.213$  patients with dry eye symptoms vs. SS patients with dry eye syndrome. (b) Protein distribution in healthy subjects (A), patients with dry eye symptoms (B) and SS patients with dry eye syndrome (C) identified by 2D-nano-LC-MS/MS. (c) Abundance of tear proteins in healthy subjects (A), patients with dry eye symptoms (B) and SS patients with dry eye syndrome (C) The relative abundance of individual proteins was assessed by spectral counting.

## Results

### Demographic and baseline characteristics of the study subjects.

Demographic and baseline characteristics of the study subjects are summarized in Table 1. The subjects of the three groups were matched in age and sex. Group A patients had a median duration of symptoms for 10.5 (range, 8 to 14) years. The BUT for group A patients was  $1.07 \pm 1.022$  seconds, which was markedly reduced compared with that of group B ( $P < 0.001$ ) and that of group C ( $P < 0.001$ ). The BUT for group B patients was also markedly reduced compared to that of group C ( $P < 0.001$ ). In addition, group A patients had a marked reduction in tear volumes ( $2.13 \pm 2.38$  mm<sup>5</sup>/min) compared to group B and C ( $P < 0.001$  in both). Furthermore, SSA/SSB antibodies were present in 75% (6/8) of the sera samples of group A patients with dry eye syndrome, which was not detected in group B and C.

**Patients with SS and dry eye syndrome have increased normalized protein content in tears.** The total protein contents in tear samples per Schirmer strip were  $395.50 \pm 78.45$ ,  $499.41 \pm 145.59$  and  $423.58 \pm 151.77$  µg in group A, B and C, respectively, with no apparent difference statistically among the three groups ( $P = 0.274$ ). We further determined the normalized protein content in tear samples. The normalized tear protein content in group A was  $1.8291 \pm 0.2241$  µg/mm, which was markedly higher than that of group B

( $1.0839 \pm 0.1120$  µg/mm) ( $P = 0.001$ ) and group C ( $0.2028 \pm 0.0177$  µg/mm) ( $P = 0.001$ ) (Fig. 1a).

**Tear proteomic characteristics of the study subjects.** The 2D-nano-LC-MS/MS analysis identified a total of 435 unique proteins from the tear samples, including 278 (83.7%) proteins in group A, 247 (74.4%) proteins in group B and 182 (54.8%) proteins in group C. One hundred forty-four (43.4%) proteins were shared among group A, B and C. In addition, 62 (18.7%) proteins were present in group A and B, 9 (2.7%) proteins were found in both group B and C, and 16 (4.8%) proteins were shared by group A and C. Fifty-six (16.7%) proteins were unique to group A, 32 (9.6%) proteins to group B and thirteen (3.9%) to group C (Fig. 1b). In addition, the percentage of high and low abundance proteins was comparable among the three groups ( $P > 0.05$ ) (Fig. 1c). Meanwhile, the percentage of medium abundance proteins in group A was markedly higher than that of group C ( $P < 0.05$ ) (Fig. 1c) while the percentage of high abundance proteins was markedly reduced in group A.

**Changes in tear proteins of SS patients with dry eye syndrome.** The 12 most upregulated proteins in tear fluids of group A are shown in Table 2 and the 10 most downregulated proteins in tear proteins of group A are shown in Table 3. Tear proteins unique to group A included proteins in host defense such as defensin  $\alpha 1$  and lactotransferrin, proteins involved in the immune response or

**Table 2** | The 12 most upregulated tear proteins in Sjögren's syndrome patients with dry eye syndrome

Accession No.	Protein Name	Spectral counts
<b>A8K008</b>	CDNA FLJ78387	115
<b>NP_001002858</b>	Annexin A2 isoform 1	94
<b>P02787</b>	Serotransferrin precursor	92
<b>OTTHUMP00000167597</b>	Keratin 4	90
<b>P06702</b>	Protein S100-A9	77
<b>P98088</b>	Mucin-5AC precursor (fragment)	59
<b>P04083</b>	Annexin A1	58
<b>P13645</b>	Keratin, type I cytoskeletal 10	50
<b>P04264</b>	Keratin, type II cytoskeletal 1	47
<b>P05109</b>	Protein S100-A8	41
<b>P01024</b>	Complement C3 precursor (fragment)	41
<b>P60709</b>	Actin, cytoplasmic 1	41


**Table 3 | The 10 most downregulated tear proteins in Sjögren's syndrome patients with dry eye syndrome**

Accession	Protein Name	SC
Q5DSM0	Growth-inhibiting protein 12	516
P31025	Lipocalin-1 precursor	507
P12273	Prolactin-inducible protein precursor	191
Q8N5K4	Igha1 Protein	152
P01833	Polymeric immunoglobulin receptor precursor	85
Q9GZZ8	Extracellular glycoprotein lacritin precursor	80
P61626	Lysozyme C precursor	60
Q16378	Prolinerich protein 4 precursor	56
P01036	Cystatin-S precursor	29
P13647	Keratin, type II cytoskeletal 5	28

inflammatory reaction such as neutrophil elastase 2 and C3, and apoptosis-related proteins like clusterin and annexin (Table 4).

## Discussion

Tear proteomic profile of SS patients with dry eye syndrome may offer important insight into the mechanisms of disease process at the target organs and may lead to identification of biomarkers for the conditions. In the current study, we examined the proteomic properties of tear fluids of SS patients with dry eye syndrome, patients with dry eye symptoms and healthy subjects using nano-flow 2D-LC-ESI-MS/MS. We identified a total of 435 proteins in the tear fluids from all three groups together, suggesting the presence of a wealth of proteins in the tear fluid. The findings indicate that the nano-flow 2D-LC-ESI-MS/MS approach may yield a promising proteomic profile of tear proteins in SS patients with dry eye syndrome from relatively small amounts of samples. We also identified 56 proteins that were uniquely present in the tear fluid of SS patients with dry eye syndrome, which include proteins in the host defense, immune response, inflammation and apoptosis. The findings suggest that the development of dry eye syndrome in SS is a complicated process involving proteins of multiple body systems with participation of novel proteins. These proteins are involved in the inflammatory response

**Table 4 | Changes and functional classification of tear proteins in Sjögren's syndrome patients with dry eye syndrome**

Accession	Protein Name	
<b>Host defense proteins</b>		
P59665	Defensin, $\alpha$ 1	upregulated
P81605	Dermcidin	upregulated
P80511	S100 calcium binding protein A12	upregulated
P31151	S100 calcium binding protein A7	upregulated
Q71D13	Histone 1, H2Bd	upregulated
P20160	Azurocidin 1 (cationic antimicrobial protein 37)	upregulated
Q5DSM0	Lactotransferrin	downregulated
P61626	Lysozyme (renal amyloidosis)	downregulated
<b>Proteins involved in the immune response and inflammatory reaction</b>		
P02765	$\alpha$ -2-Hs-glycoprotein	upregulated
P05155	Serpin peptidase inhibitor, clade G (C1 inhibitor)	upregulated
P08246	Elastase 2, neutrophil	upregulated
P04264	Keratin 1 (epidermolytic hyperkeratosis)	upregulated
P00734	Coagulation factor II (thrombin)	upregulated
P02787	Transferrin	upregulated
P02763	Orosomucoid 1	upregulated
P01024	Complement component 3	upregulated
P19652	Orosomucoid 2	upregulated
POC0L4	Complement component 4A	upregulated
P02652	Apolipoprotein A-II	—
<b>Apoptosis-related proteins</b>		
P55072	Valosin-containing protein	upregulated
O43707	Actinin, $\alpha$ 4	upregulated
P00441	Superoxide dismutase 1, soluble	upregulated
P27797	Calreticulin	upregulated
P10909	Clusterin	upregulated
P08107	Heat shock 70 Kda protein 1A	upregulated
Q96BY2	Modulator Of apoptosis 1	upregulated
P04792	Heat shock 27 Kda protein 1	upregulated
P14174	Macrophage migration inhibitory factor	upregulated
P04083	Annexin A1	upregulated
P30101	Protein disulfide isomerase family A, member 3	upregulated
P00734	Coagulation factor II (thrombin)	upregulated
P23528	Cofilin 1 (non-muscle)	upregulated
P47895	Aldehyde dehydrogenase 1 family, member A3	upregulated
Q15121	Phosphoprotein enriched in astrocytes 15	upregulated
P63104	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein	upregulated
P12814	Actinin, $\alpha$ 1	upregulated
P08758	Annexin A5	downregulated
P31944	Caspase 14, apoptosis-related cysteine peptidase	downregulated
P32119	Peroxiredoxin 2	downregulated
P07858	Cathepsin B	downregulated
P04179	Superoxide dismutase 2, mitochondrial	—
P09211	Glutathione S-transferase PI	—
Q04760	Glyoxalase I	—
Q6NUS1	Programmed cell death 6 interacting protein	—





( $\alpha$ -2-HS-glycoprotein, coagulation factor II, transferrin, and orosomucoid 1 and 2), biosynthesis of IL-8 (apolipoprotein A-II and elastase 2) and activation of the host immune response and the inflammatory response (serpin peptidase inhibitor, clusterin, keratin 1, C3 and 4A). Gene co-expression modules related to primary SS and primary SS/MALT lymphoma were significantly enriched with genes known to be involved in the immune/defense response, apoptosis, cell signaling, gene regulation, and oxidative stress<sup>21</sup>. We further found in this study that tear proteins in SS patients with dry eye syndrome and patients with dry eye symptoms exhibited a marked increase in the contents of proteins involved in the immune response and stress response, development and differentiation. These proteins also interact with one another and they also show increased oxidative activities. The findings indicate that proteins in the tear fluid of SS patients with dry eye syndrome and patients with dry eye symptoms are more likely the result of the host stress response, which is consistent with the findings by Hu *et al.*<sup>21</sup>.

Beta-2-microglobulin, lactoferrin, immunoglobulin (Ig) kappa light chain, polymeric Ig receptor, lysozyme C and cystatin C were found to be involved in all stages of SS<sup>22</sup>. Two presumed proline-rich proteins, amylase and carbonic anhydrase VI, were downregulated in SS patients. In our study, we found that polymeric immunoglobulin receptor precursor, cystatin-S precursor and proline-rich protein 4 precursor were downregulated in the tear fluids of group A patients. Lactotransferrin, which was downregulated in the tear fluids of group A patients in the current study, is a secreted protein and possesses antimicrobial activities against bacteria, fungi and viruses<sup>23</sup>. The protein acts downstream of the inflammatory and immune response cascade and could modulate the host response to bacteria and the immune response. Defensin belongs to the  $\alpha$ -defensin family and is a secreted protein. Defensin 1 $\alpha$ , which was upregulated in the tear fluid of SS patients with dry eye syndrome in this study, is capable of inhibiting the replication of viruses and synthesis of viral proteins<sup>24</sup>. Lysozyme, which has bacteriolytic activities, was downregulated in the tear fluid of SS patients with dry eye syndrome. Our findings that lactotransferrin and lysozymes were downregulated in the tear fluid of group A patients indicate that the ocular surface defense system becomes lessened in group A patients, which may render the patients more susceptible to microbial infections.

Consistent with the findings by Giusfi *et al.*<sup>25</sup>, proteins related to acute and chronic inflammation or involved in oxidative stress injury in this study were upregulated. We found that S100 A12 and S100 A7 were upregulated in the tear fluids of group A patients. S100 proteins are calcium binding proteins. The secretion of S100 A12 is induced by TNF and S100 A12 is highly expressed in the inflammatory response<sup>26</sup>. Other members of the S100 family including S100 A6, S100 A8, S100 A9, and S100 A11 were also upregulated in the tear fluids of group A patients (data not shown), indicating their involvement in the inflammatory response. In addition, proteins associated with apoptosis were markedly upregulated or downregulated. Superoxide dismutase 1 and heat shock proteins were markedly upregulated while peroxiredoxin 2 was downregulated.

Our results lend support to the concept that autoimmunity-mediated dry eye disease has an inflammatory component<sup>27</sup>. Overexpressed proteins were interferon-inducible or were related to lymphocyte filtration and antigen presentation known to be involved in the pathogenesis of primary SS<sup>28</sup>. In the current study, neutrophil elastase 2,  $\alpha$ -2-HS-glycoprotein, C3, Orosomucoid 1 and 2 were significantly upregulated while APO A-II and clusterin showed no apparent changes from normal controls, suggesting the presence of enhanced immune and inflammatory response at the ocular surface. Complement C3 is a pivotal component of the complement system, interacts with C3d and CR2, and plays

a critical role in the activation and proliferation of B cells. In C3 knockout SS mice, SS was diminished or abolished<sup>29</sup>. In addition, apoptosis of ductal cells in the exocrine glands was decreased and caspase-3 levels declined with apparent reduction in the infiltration of leukocytes into the submandibular gland and diminished production of autoantibodies. Cuida *et al.*<sup>30</sup> reported that complement regulatory proteins in the saliva of SS patients including CD59, CD55, CD46 and clusterin could inhibit the activation of complements in tissues. We found that these proteins were reduced in levels or absent in the tear fluids of group A patients, indicating that complements may show unrestrained activities in the tissues. We found here that C3 was upregulated in the tear fluid of group A patients. Given its pivotal role in immune response and inflammation, C3 may be an important molecule for targeting and as a predictor of patient outcomes.

In conclusion, we have demonstrated that dry eye syndrome in SS patients is associated with an altered proteomic profile with dysregulated expression of proteins involved in a variety of important cellular process including inflammation, immunity, and oxidative stress. These findings suggest broad derangement in tear proteins in SS patients with dry eye syndrome. Furthermore, SS and dry eye have similar pathologies such as increased apoptosis, inflammation at the ocular surface, immune response and cytoskeletal remodeling. Further characterization of these proteins could provide potential diagnostic markers and therapeutic targets that may lead to better outcomes for these patients.

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## Author contributions

B.L., W.W. and Y.C. designed the experiments; B.L., M.S., J.L. and G.Y. collected the data; B.L., M.S., W.W. and Y.C. wrote the main manuscript text; all authors reviewed the manuscript.

## Additional information

**Competing financial interests:** The authors declare no competing financial interests.

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