Tear Film Proteomics Reveal Important Differences Between Patients With and Without Ocular GvHD After Allogeneic Hematopoietic Cell Transplantation

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RESULTS. We identified 282 proteins in tryptic digests of Schirmer strips; 79 proteins were significantly differentially expressed between the two groups, from which 54 were up- and 25 downregulated. The most upregulated proteins were classified as nucleic acid binding and cytoskeletal proteins, while the most extensively downregulated proteins belong to an array of classes including transfer and receptor proteins, enzyme modulators, and hydrolases. In addition to proteins already confirmed as differentially expressed in dry eye disease, we report changes in 36 novel proteins.

CONCLUSIONS. This study reports the proteomic profile of tears in ocular GvHD for the first time and identifies a number of unique differentially expressed proteins. Further studies with a higher number of participants are necessary to confirm these results and to evaluate the reliability of these expression patterns in longitudinal studies.

Keywords: autoimmune disease, cornea, dry eyes, graft versus host disease, keratitis, proteomics, Schirmer’s test, tears

Ocular graft versus host disease (GvHD) is a potentially sight-threatening complication and affects 40% to 60% of patients after allogeneic hematopoietic cell transplantation (HCT). Diagnosis and treatment of GvHD is difficult and, due to its severity and imminent complications, it greatly diminishes the patient’s quality of life (QoL). Currently, no biomarkers for ocular GvHD exist, but to enable an early diagnosis of the disease, such biomarkers are highly desirable.

According to the National Institutes of Health (NIH) consensus criteria definition,2 current diagnosis of ocular GvHD relies on at least one distinctive clinical sign and a pathologic Schirmer test. Distinctive signs include keratoconjunctivitis sicca (KCS), cicatricial conjunctivitis, or fluent areas of punctate keratopathy, mimicking dry eye.2 However, in clinical routine, the low specificity and reliability of these signs reflect the difficulty in diagnosing and monitoring ocular GvHD. Confirming this, the International Dry Eye Workshop also recognized a shortage of objective, sufficient and validated tests for dry eye diagnosis.3 Facing an enormous lack of causal relation between symptoms and objective findings, the diagnosis, determination of treatment and monitoring of response are hindered.

Thus, biomarkers could serve as objective diagnostic, prognostic, and monitoring tools.5 Tear proteomic studies to identify ocular surface disease biomarkers are attractive due to the proximity of tears to the disease site, the relatively noninvasive sampling technique and the possibility to also collect samples from healthy control subjects.4 Tear sampling is possible by various means, including microcapillary tube,5,6 polyurethane sponge,7 and Schirmer strip.8–11 Schirmer test I is a routinely used and well-tolerated clinical diagnostic technique in patients with dry eye syndromes. In brief, the test strip is placed in the lower conjunctival sac and absorption of tears is permitted to occur over a 5-minute period. As such, the extraction of tear proteins from Schirmer strips creates an opportunity for molecular analysis to be carried out without patients having to undergo further clinical procedures.12
Over the last 10 years, extensive data on the tear protein profile has been collected and summarized in several publications, identifying more than 1500 proteins in tears of healthy subjects. Correlation between the tear proteome and ocular or systemic diseases has been examined for glaucoma, thyroid-associated orbitopathy, keratoconus, dry eye disease, multiple sclerosis, cancer, and diabetes. Tear proteome alterations in the context of dry eye were further distinguished between underlying causes like lipid or aqueous deficiency. The latter further subdivided into Sjögren syndrome associated and non-Sjögren associated dry eye. Studies have focused on proteome analysis in Stevens-Johnson syndrome, pterygium, conjunctivochalasia, and allergy, while others investigated dry eye syndrome associated with contact lens wear or occurring after laser-assisted in situ keratomileusis. However, research for tear biomarkers in ocular GvHD has focused mainly on the role of cytokines, but less so on global protein profiling and functional classification of candidate proteins that can be associated with the disease. The purpose of the present study was to analyze proteins and their cellular function in human tears as potential biomarkers forocular GvHD. Therefore, we examined quantity and quality of tear fluid of patients presenting with ocular GvHD after allogeneic HCT and compared these to a group of patients without ocular GvHD after allogeneic HCT.

METHODS

Patients

This prospective, observational study was approved by the local ethical committee (Ethikkommission Nordwest- und Zentralschweiz) and followed the tenets of the Declaration of Helsinki. All patients after allogeneic HCT were referred to our clinic by the Department of Hematology of the University Hospital of Basel. We included 10 consecutive patients with ocular GvHD and 10 consecutive patients without ocular GvHD after allogeneic HCT in our study. Participants were consecutively recruited and examined between March 7 and April 25, 2016, during consultation at the Department of Ophthalmology of the University Hospital Basel. All enrolled patients were informed about the nature of the study and written consent was obtained. Patients received a complete clinical examination including NIH scoring and a Schirmer test. Assessment of symptoms was performed using the standardized Ocular Surface Disease Index (OSDI) questionnaire. Patients were divided into two groups according to their clinical appearance of GvHD: group 1 with ocular GvHD and group 2 with no current symptoms of either ocular or systemic GvHD. According to the NIH consensus criteria, GvHD was divided into four grades of severity for each organ as well as an overall scoring: 0 = none; 1 = mild; 2 = moderate; 3 = severe. Systemic GvHD was classified after physical examination by one physician at the department of hematology, while ocular GvHD was diagnosed by one physician at the department of ophthalmology. In brief, the NIH ocular score is a scale that categorizes ocular GvHD based on the severity of impairment of activities of daily life (ADL), the extent of treatment required and the occurrence of vision loss due to KCS (0 = normal, no symptoms; 1 = mild, no effect on ADL, hydrating drops ≤3 times/day; 2 = moderate, some effect on ADL, hydrating drops >3 times/day or punctal plugs, no vision loss; 3 = severe, significant effect on ADL, loss of vision due to KCS).

Inclusion criteria for group 1 were chronic GvHD with an overall score of 1 to 3 as well as an ocular score of 2 or 3 according to NIH consensus criteria. We did not include patients with an ocular score of 1, as the mild versions of dry eye symptoms could also be attributed to other entities. Inclusion criteria for group 2 were an overall as well as ocular score of 0 at the time of the examination. Exclusion criteria were any kind of ocular infection at the time of the examination, prior keratoplasty or keratoprosthesis, prior refractive surgery, appearance of GvHD within 1 month after the examination, participation in another study involving therapeutic drugs, and <18 years of age or not being able to give informed consent.

Clinical Examination and Sample Collection

Medical history and ophthalmologic examination, ocular NIH scoring, evaluation of the quality of life questionnaire (OSDI score) and Schirmer test I (Schirmer Plus; Gecis, Neungr-Beuvron, France) were performed by one clinician. The OSDI score is a standardized quality of life questionnaire consisting of 12 questions scoring severity of dry eye symptoms. The questionnaire was provided in the patient’s native language. A calculated scale between 0 and 100 was assigned into four grades of severity (0 = normal; 1 = mild; 2 = moderate; 3 = severe) showing dry eye disease with higher scores representing greater disability. The current systemic and topical therapy was registered, including immune-modulating drugs and applied punctal plugs.

Quantitative tear analysis and tear sample collection from each eye using Schirmer test I (5 minutes, without topical anesthesia) was performed prior to any clinical examination. No topical medication was applied at least 15 minutes before the tear collection. To reduce the risk of contamination, medical gloves were worn during strip application. After 5 minutes, the strips were removed, the length of wetting was noted in millimeters and the strips were placed separately in 1.8-mL tubes (CryoPure; Sarstedt, Nümbrecht, Germany). The coded tubes were locked and immediately stored on dry ice for maximum of 8 hours to avoid protein degradation. Samples were stored at −80°C until further processing.

After tear sample collection, all patients received a slit-lamp examination including sodium fluorescein staining (Biofluo Fluorescein Ophthalmic Strips; Omnivision, Neuhausen am Rheinfall, Switzerland) and qualitative tear film analysis by break up time (BUT). Assessment of ocular surface integrity (cornea and the bulbar conjunctiva) was done 2 minutes after application of fluorescein. Corneal and conjunctival staining was scored in six grades of severity (0–V) according to the Oxford scale. Finally, ocular GvHD was scored following the NIH consensus criteria as described above. Regarding the allogeneic HCT, the following information about donor and recipient were recorded: age, sex, disease, time, and graft source (peripheral blood or bone marrow), donor/recipient relationship and HLA-match. In every patient, the overall NIH-score of GvHD as well as the score for each organ (except the ocular score) was determined. Furthermore, the time of first diagnosis was noted.

Analysis of Tear Proteins

Protein Digestion From Schirmer Strips. Schirmer strips were cut into small pieces of approximately 3 × 5 mm and placed into 360 μL, 50 mM Tris-HCl, pH 8.0. All steps were carried out on a thermo shaker (Eppendorf, Schönenbuch, Switzerland) set to 1000 rpm. The proteins were reduced with 10 mM DL-dithiothreitol (DTT, Sigma-Aldrich, Buchs, Switzerland) for 1 hour at 37°C and alkylated with 50 mM iodo-
acetonamide (Sigma-Aldrich Corp.) for 15 min at room temperature. Peptides were eluted from the Schirmer strips by incubation with 1 µg TPKC-treated trypsin (Worthington, Lakewood, NJ, USA) for 2 hours at 37°C. A second 1 µg aliquot of trypsin was added and digestion was continued for 18 hours at 37°C. The pieces from the Schirmer strips were spun at 15,523 g for 1 min and the supernatant was collected and acidified with trifluoroacetic acid (TFA, Applied Biosystems, Rotkreuz, Switzerland) to 1% final concentration.

**Peptide Desalting.** Peptides were desalted on C18 SepPak cartridges (50 mg packing material; Waters, Dättwil, Switzerland). The cartridges were primed with 250 µL methanol and 250 µL 80% acetonitrile/0.1% TFA and equilibrated with 750 µL 0.1% TFA. The digest was applied onto the C18 SepPak cartridge and the flow through was collected. The flow through was reapplied and the cartridge was washed with 1250 µL 0.1% TFA. Bound peptides were eluted with 500 µL 80% acetonitrile/0.1% TFA and collected into an Eppendorf tube. The absorbance of the eluate was measured at 280 nm and peptide concentration was estimated according to Wisniewski et al.34 The digests were aliquoted into 40 µg portions, dried under vacuum and stored at −20°C until assay. Desalted digests from control individuals were pooled, aliquoted and dried using the same method, and served as the internal standard for mass spectrometry.

**Mass Spectrometric Analysis.** The digests from the patient and control strips were analyzed by capillary liquid chromatography tandem mass spectrometry (LC/MS/MS) using a homemade separating column (0.075 mm x 18 cm) packed with Reprosil C18 reverse-phase material (2.4 µm particle size; Dr. Maisch HPLC GmbH, Ammerbuch-Entringen, Germany). The column was connected on line to an Orbitrap FT hybrid instrument (Thermo Fisher Scientific, Reinach, Switzerland). The solvents used for peptide separation were 0.1% acetic acid in water/0.005% TFA (solvent A) and 0.1% acetic acid/0.005% TFA and 80% acetonitrile in water (solvent B). We injected 2 µL of peptide digest with a capillary pump (Proxeon Easy-LC; Thermo Fisher Scientific) set to 0.3 µL/min. A linear gradient from 0% to 40% solvent B in solvent A over 90 minutes was delivered with the nanopump at a flow rate of 300 nL/minute. At the end of the linear gradient, the percentage of solvent B was increased to 75% over 25 minutes. The eluting peptides were ionized at 2.5 kV. The mass spectrometer was operated in data-dependent mode. The precursor scan was done in a mass range of 350–2000 Da. Intense precursors were selected for fragmentation. The MS/MS spectra were then searched against a SwissProt human database updated monthly. The LC/MS/MS data were searched with proteomics software (Proteome Discoverer 1.4; Thermo Fisher Scientific) set to Mascot search engine with 10 ppm precursor ion tolerance, while the fragment ions were set to 0.6 Da tolerance. The following modifications were used during the search: carbamidomethyl-cysteine was set as a fixed and oxidized methionine as a variable modification. The peptide search matches were set to “high confidence,” which corresponds to 1% false discovery rate.

**Relative Protein Quantification.** To analyze equal amounts of protein, a first run was carried out on the mass analyzer instrument (Thermo Fisher Scientific) and the resulting chromatogram was integrated. To compensate for individual differences in peptide amount obtained from the Schirmer strips, the integrated chromatogram from patient and control were adjusted to ensure equal peptide material was injected. All patient and control strips were measured in technical triplicates for quantification by spectral counting.

The search files from the proteomics software (Thermo Fisher Scientific) were loaded into Scaffold (Proteome Software, Portland, OR, USA) and the proteins were quantitated by spectral counting. The peptide false-discovery rate was set to 1%, while the protein threshold was 95%. For protein quantification, the number of usable peptides was set to 3.

**Statistical Analysis.** The mean of the three triplicate measurements from each patient was calculated and this value was used for all subsequent analysis. Missing values (indicating none of that protein found in a patient sample) were assumed to be below quantification (BLQ) and set to half the minimal value available in the dataset. Subsequently, Mann-Whitney U tests were used to compare tear protein concentrations between patients with and without ocular GvHD. Differences of the medians between patients with ocular GvHD and those without were sorted by the absolute value of the log-Median ratio (largest effect). P values were adjusted for multiple comparisons by the false discovery rate (FDR) method. Adjusted P values below 0.05 were considered significant. Calculation of study power was performed using the package “ ssize.fdr.” Assuming a minimal detectable effect size of 2 (effect size = difference/SD) and a sample size of 10 subjects for each group, there was a power of 81% to detect 10% differentially expressed proteins.35 All evaluations were done using R version 3.3.1 (2016) software for statistical computations.

**RESULTS**

**Patient Characteristics**

Patient details are given in Table 1. Group 1 included 10 patients (8 men) with active ocular and systemic GvHD with a mean age of 49 (range: 24–69) years. Group 2 included 10 patients (6 men) without active ocular or systemic GvHD with a mean age of 57 (range: 38–75) years. All patients underwent allogeneic HCT for malignant diseases. At the time of the examination all were in complete remission. Stem cell sources were peripheral blood progenitor cells in all except one patient in group 2 who received bone marrow transplantation. Two patients in group 1 and one patient in group 2 had two allo-HCT. The majority of donors were unrelated in group 1 (8/10 donors) and related in group 2 (8/10 donors) with eight and nine being HLA-matched while the remaining two and one donors had a one locus HLA-mismatch, respectively. In group 1, six donors were male, while in group 2, eight donors were male.

Mean time between first HCT and inclusion into this study was 2384 days (range: 754–4911 days) for the patients in group 1 and 2883 days (range: 96–8082 days) in group 2. Time between the first HCT and diagnosis of any kind of chronic GvHD was as far as data was available 221 days (range: 80–577 days; n = 7) in group 1 and 132.5 days (range: 95–1089 days; n = 4) in group 2. Six patients in group 2 never had acute or chronic GvHD. Three patients had previous oral chronic GvHD (all score 1) that resolved 3, 4, and 5 years before study inclusion. The remaining patient had acute GvHD of the skin grade 2 after HCT with complete resolution 24 years before inclusion into this study. No patient in group 2 ever had ocular GvHD. At the time of examination, no patient in group 2 showed any signs of active ocular or systemic GvHD. The median time between first diagnosis of any signs of chronic
TABLE 1. Demographics of Study Participants

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Group 1 (With Ocular GvHD After HCT), n = 10</th>
<th>Group 2 (Without Active GvHD After HCT), n = 10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>Mean ± SD 49 ± 14</td>
<td>57 ± 11</td>
</tr>
<tr>
<td></td>
<td>Range 24–69</td>
<td>38–73</td>
</tr>
<tr>
<td>Female, n (%)</td>
<td>2 (20)</td>
<td>4 (40)</td>
</tr>
<tr>
<td>Primary diagnosis, n (%)</td>
<td>Myeloma 1 (10)</td>
<td>1 (10)</td>
</tr>
<tr>
<td></td>
<td>ALL 1 (10)</td>
<td>1 (10)</td>
</tr>
<tr>
<td></td>
<td>AML 4 (40)</td>
<td>5 (50)</td>
</tr>
<tr>
<td></td>
<td>NHL 3 (30)</td>
<td>1 (10)</td>
</tr>
<tr>
<td></td>
<td>MDS 1 (10)</td>
<td>2 (20)</td>
</tr>
<tr>
<td>HCT source, n (%)</td>
<td>PB 10 (100)</td>
<td>9 (90)</td>
</tr>
<tr>
<td></td>
<td>BM 1 (10)</td>
<td></td>
</tr>
<tr>
<td>Number of HCT received, n (%)</td>
<td>1 8 (80)</td>
<td>9 (90)</td>
</tr>
<tr>
<td></td>
<td>2 2 (20)</td>
<td>1 (10)</td>
</tr>
<tr>
<td>Unrelated donor, n (%)</td>
<td>8 (80)</td>
<td>2 (20)</td>
</tr>
<tr>
<td>Elapsed time between HCT and ocular exam, d</td>
<td>Mean ± SD 2384 ± 1567</td>
<td>2883 ± 2606</td>
</tr>
<tr>
<td></td>
<td>Range 754–911</td>
<td>96–8082</td>
</tr>
</tbody>
</table>

ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; BM, bone marrow; HCT, hematopoietic cell transplantation; MDS, myelodysplastic syndrome; NHL, non-Hodgkin lymphoma; PB, peripheral blood.

GVHD and current assessment was 2162 days (range: 510–4796 days; n = 7) in group 1.

Therapy & Punctum Plugs

Systemic immunosuppressive therapy was given to eight patients in group 1 and three in group 2. In group 1, six had a calcineurin inhibitor (five receive tacrolimus, one cyclosporin A), four in combination with systemic corticosteroids. One patient each had systemic steroids or mycophenolic acid only and two patients were without systemic immunosuppression. In group 2, three patients were still under systemic cyclosporin A while one used irregular topical tacrolimus for oral aphthosis. All other patients in group 2 didn’t use systemic or topical immunosuppressive agents.

All patients with ocular GVHD (group 1) used topical treatment. Two patients solely applied artificial tear drops. Seven patients used topical corticosteroids with additional hydrating drops, one patient with additional topical cyclosporin A, and one patient with additional cyclosporin A and autologous serum eye drops. One of 10 patients used only cyclosporin A and artificial tears. In contrast only two patients in group 2 used artificial tears while no other topical treatment was necessary.

Punctual plugs were in place in 9 of 10 patients in group 1 and in 2 of 10 patients in group 2. Tables summarizing all systemic and local therapies used in study patients are provided as supporting data (Supplementary Tables S1, S2).

NIH Scoring

Overall GVHD scoring in group 1 was moderate (grade 2) in 5 patients and severe (grade 3) in seven patients, while all patients scored 0 in group 2 (Table 2). In group 1, all patients had more than one organ manifestation of GVHD. Ocular GVHD score was moderate (grade 2) in six patients and severe (grade 3) in four patients of group 1.

OSDI/QoL

Quality of life assessment with the OSDI score showed a mean of 56.3 points (range: 16.7–81.8) in group 1 and 5.5 points (range: 0–15.0) in group 2 (Table 2). Converted into the 4 grades of severity (0–3), group 1 included eight patients with grade 3, one with grade 2, and one with grade 1. Group 2 had no patients with grade 2 or 3, only 2 patients with grade 1 and 8 patients had no dry eye symptoms at all (grade 0).

Break up Time (BUT) & Oxford Score

In group 1 the mean BUT was 2.2 seconds while group 2 had a mean BUT of 4.6. The Oxford score, taken 2 minutes after fluorescein staining, had a mean value of 3.4 (range: 1–5) in group 1 and 0.2 (range: 0–1) in group 2 (Table 2).

Schirmer Test I & Tear Proteins Analysis

The Schirmer Test values of both eyes, according to the NIH criteria, were less than 10 mm in all patients in group 1, with a mean of 1.9 mm (range: 0–10 mm). The Schirmer Test value of both eyes was greater than 10 mm in group 2, with a mean of 23.3 mm (range: 11 to >35 mm; Table 2).

Quantitative protein analysis from Schirmer test strips identified 282 proteins from the UniProtKB data bank. In total, 79 proteins were significantly differentially expressed (up- or downregulated) between the two groups of patients. From the 79 proteins, 54 were upregulated in ocular GVHD patients (group 1). These 54 proteins belong to 17 different protein classes (Fig. 1). Half of them belong to cytoskeletal proteins or nucleic acid binding proteins. Histone H2B (HIST), Ig gamma 1 chain C region (IGHG1), periplakin (PPL), prelamin-A/C (LMNA) and ribosome binding protein 1 (RBP1) were most prominently upregulated (Table 3). We found 25 proteins were significantly downregulated in patients with ocular GVHD, comprising 10 different protein classes (Fig. 2). The functional classification of these proteins was rather broad with a slight predominance for hydrolases, enzyme modulators, transfer/carrier proteins and defense/immunity proteins. Lactotransferrin (LTF), extracellular glycoprotein lacritin (LACRT), proline rich protein (PRP), lipocalin-1 (LCN1) and Cystatin-S (CST4) were the most significantly downregulated proteins (Table 4). The complete list of differentially expressed proteins in GVHD tears can be found in Supplementary Table S3.

Discussion

The objective of this study was to investigate whether proteomic profiling of tear fluid from GVHD patients could...
reveal a putative biomarker signature associated with the disease. Chronic ocular GvHD results from inflammation-related fibrosis in the conjunctiva and lacrimal glands, leading to lacrimal gland insufficiency and a resulting aqueous-deficient dry eye phenotype. However, the exact underlying pathogenic mechanisms leading to ocular surface deterioration in GvHD are unclear. Current diagnosis of ocular GvHD is dependent on quality of life questionnaires and clinical examination, with no established biomarkers available for early detection. Despite this, only a limited number of biomarker studies have been completed to date, with their authors taking a targeted approach to assess changes in inflammatory mediators. To the best of our knowledge, this is the first study to report a discovery-based proteomic analysis of tears collected from GvHD patients.

In this study, we identified and quantified 282 tear proteins. This number is within the range of previously reported proteomes from healthy tears, ranging from 97 unique proteins to over 1500 proteins collected by numerous groups with a variety of different workflows. We found 79 differentially expressed proteins in ocular GvHD tear film versus eyes without GvHD. Compared to patients without ocular GvHD, 54 proteins were significantly increased in the tear film in GvHD. Functional classification of these proteins using PANTHER analysis revealed the most prominently upregulated protein subcategories to be structural proteins, nucleic acid binders and oxidoreductase enzymes (Fig. 1). In comparison to patients without ocular GvHD, 25 proteins were significantly downregulated in the tear film of GvHD patients, distributed over a broad array of functional classes. Among the most prominently downregulated subcategories were enzyme modulators, hydrolases, carrier proteins, receptor binding proteins, and defense and immunity-related proteins (Fig. 2).

This study revealed a broad overlap with previous proteomic studies of various dry eye subtypes. Reductions of the antimicrobial proteins lactotransferrin (LTF) and lysozyme (LYZ), the cysteine protease inhibitors cystatin-S (CST4) and cystatin-SN (CST1), mammaglobin-B (SCGB2A1) and other members of the steroid-binding secretoglobin family (SCGB1D1), and the pro-secretory protein lacritin (LACRT)

**TABLE 2. Ocular Clinical Parameters of Study Participants**

<table>
<thead>
<tr>
<th>Diagnostic Examination</th>
<th>Group 1 (With Ocular GvHD After HCT), n = 10</th>
<th>Group 2 (Without Active GvHD After HCT), n = 10</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>NIH score oGvHD, n (%)</td>
<td>0 (absent) 0</td>
<td>10 (100%)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>1 (mild)</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>2 (moderate)</td>
<td>6 (60%)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>3 (severe)</td>
<td>4 (40%)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>OSDI score</td>
<td>Mean ± SD</td>
<td>56.3 ± 20.6</td>
<td>5.5 ± 4</td>
</tr>
<tr>
<td>Range</td>
<td>16.7 to 20.6</td>
<td>0 to 15</td>
<td></td>
</tr>
<tr>
<td>BUT, s</td>
<td>Mean ± SD</td>
<td>2.2 ± 0.7</td>
<td>4.6 ± 2.5</td>
</tr>
<tr>
<td>Range</td>
<td>1 to 3</td>
<td>2 to &gt;10</td>
<td></td>
</tr>
<tr>
<td>Oxford score</td>
<td>Mean ± SD</td>
<td>3.4 ± 1.2</td>
<td>0.2 ± 0.3</td>
</tr>
<tr>
<td>Range</td>
<td>1 to 5</td>
<td>0 to 1</td>
<td></td>
</tr>
<tr>
<td>Schirmer’s Test I (wetting length, mm)</td>
<td>Mean ± SD</td>
<td>1.95 ± 3</td>
<td>23.3 ± 8</td>
</tr>
<tr>
<td>Range</td>
<td>0 to 10</td>
<td>11 to &gt;35</td>
<td></td>
</tr>
</tbody>
</table>

Comparison of means using the Mann-Whitney nonparametric test.

**FIGURE 1.** Significantly upregulated proteins in ocular GvHD divided into protein class by the PANTHER classification system.
have been previously reported by numerous authors.\textsuperscript{7,8,11,23} One of the top downregulated proteins found in our patients was proline-rich protein 1 (PROL1). The function of this protein is largely unknown, but it has been suggested to be involved in protection of the ocular surface, and its reduced expression has been reported in dry eye and thyroid associated orbitopathy patients.\textsuperscript{9,11,38,39} A comprehensive summary of differential protein expression in dry eye subtypes is provided by Perumal et al.\textsuperscript{9} (Supplementary Table S1). In our MS-based proteomic study, we found expression of 36 proteins that have not been previously reported to be differentially expressed in tear proteomic studies of dry eye related disorders (Supplementary Table S4). Whether this finding can be confirmed in other patients with ocular GvHD, and whether these proteins might be specific for ocular GvHD needs to be further addressed. In the following discussion, we will highlight several significantly differentially expressed proteins found in our GvHD cohort and suggest how they can be related to the underlying pathology of the disease.

In this study, the most highly upregulated proteins in ocular GvHD tears were the histone proteins. This group of DNA-binding proteins is involved in nucleosome assembly. Histones are released from dying cells and are, independently, recognized as important pro-inflammatory mediators (reviewed by Marsman et al.\textsuperscript{40}). Furthermore, histones bound to DNA in the nucleosome, activate TLR-mediated pro-inflammatory signaling.\textsuperscript{41} In response to infection by pathogens, neutrophils release their nuclear contents including DNA, histones, elastases and antimicrobial enzymes to form neutrophil extracellular traps (NETs).\textsuperscript{42} NET formation is an important defense-mechanism for maintenance of ocular surface health. However, in chronic inflammatory conditions NET formation and clearance becomes dysregulated and may contribute further to pathology. Recently, using conjunctival impressions and tear analysis, Jain and colleagues\textsuperscript{43,44} demonstrated an increased abundance of extracellular DNA in tears of severe dry eye patients, including a cohort of GvHD cases. In that study, excess extracellular DNA was correlated with a reduction in endonuclease activity in tears. Subsequent in vitro studies showed that NET formation by neutrophils increased exponentially in response to hyperosmolarity, suggesting a link between hyperosmolar stress and propagation of ocular surface inflammation.\textsuperscript{45} The significant upregulation of histone proteins detected in our study may suggest hyperosmolar stress and increased NET formation at the ocular surface as a key driver of inflammation in ocular GvHD.

Lipocalin-1 (LCN-1; also known as Tear Lipocalin) is a major protein component of normal tears and has numerous suggested functions in the tear film, including lipid binding and scavenging, endonuclease and antimicrobial activities.\textsuperscript{46} LCN-1 is secreted, along with other antimicrobial proteins LYZ and LTF, by the lacrimal glands, and these proteins are
frequently reported as the most downregulated proteins in aqueous-deficient forms of dry eye disease.\textsuperscript{7,11,22,23,47} Our study shows a similar profile of reduced expression for these proteins in GvHD tears, corroborating the clinical evidence of ocular GvHD as an aqueous-deficient phenotype (Table 2). The best-known role of LCN-1 is to function as a lipid-binding protein that helps to stabilize the lipid layer.\textsuperscript{46} with reduced expression of LCN-1 reported in lipid-deficient dry eye tears.\textsuperscript{48,49} Here, we report reduced LCN-1 expression in an aqueous-deficient dry eye phenotype. LCN-1 accounts for 75% of endonuclease activity in tears\textsuperscript{40} and, therefore, has an important role in clearing extracellular DNA from the ocular surface. Reduced endonuclease activity in severe dry eye patients and consequently, reduced clearance of extracellular DNA, has been proposed as a source of chronic inflammation at the ocular surface.\textsuperscript{35,44} Given the observed reduced LCN-1 expression in GvHD tears, in conjunction with the significant increase in abundance of extracellular histones, it is feasible that NET-driven inflammation plays a role in the pathogenesis of ocular GvHD. Interestingly, despite reporting reduced levels of LTF and LYZ in dry eye patients, Perumal et al.\textsuperscript{39} did not report significantly altered levels of LCN-1 by mass spectrometry in any of the dry eye subtypes examined.

The second most highly upregulated protein in GvHD tears detected in the current study was immunoglobulin gamma-1 chain C (IGHG1). Previously unreported by other proteomic studies of dry eye disease, differential expression of IGHG1 in tears has recently been shown by two studies investigating dry eye disease, differential expression of IGHG1 in chain C (IGHG1). Previously unreported by other proteomic try in any of the dry eye subtypes examined.

### Table 4. The Top 10 Downregulated Proteins in Ocular GvHD Tears

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Accession ID</th>
<th>Protein Name</th>
<th>Median Ratio</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>LTF</td>
<td>P02788</td>
<td>Lactotransferrin</td>
<td>0.010</td>
<td>0.0001</td>
</tr>
<tr>
<td>LACRT</td>
<td>Q9GZZ8</td>
<td>Extracellular glycoprotein lacritin</td>
<td>0.014</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>PROL1</td>
<td>Q99935</td>
<td>Proline-rich protein 1</td>
<td>0.019</td>
<td>0.0005</td>
</tr>
<tr>
<td>LCN1</td>
<td>P31025</td>
<td>Lipocalin-1</td>
<td>0.022</td>
<td>0.0003</td>
</tr>
<tr>
<td>CST4</td>
<td>P01036</td>
<td>Cystatin-S</td>
<td>0.026</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>PRR4</td>
<td>Q16578</td>
<td>Proline-rich protein 4</td>
<td>0.033</td>
<td>0.0011</td>
</tr>
<tr>
<td>CST1</td>
<td>P01037</td>
<td>Cystatin-SN</td>
<td>0.036</td>
<td>0.0001</td>
</tr>
<tr>
<td>SCGB1D1</td>
<td>O95698</td>
<td>Secretoglobin family 1D member 1</td>
<td>0.048</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LYZ</td>
<td>P61626</td>
<td>Lysozyme C</td>
<td>0.055</td>
<td>0.0001</td>
</tr>
<tr>
<td>SCGB2A1</td>
<td>P75556</td>
<td>Mammaglobin-B</td>
<td>0.058</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

The expression ratios between the two patient groups are shown for the top 10 most downregulated proteins. The P value is adjusted for multiple comparisons.

was among the significantly upregulated proteins found in the tears of GvHD patients. Elevated conjunctival expression of IVL has been reported in SJS.\textsuperscript{55} We also detected upregulation of the calcium-binding protein S100 A8 (Calgranulin-A; S100A8), another cornified envelope protein.\textsuperscript{56} Upregulation of S100A8 has previously been reported in dry eye\textsuperscript{52} and MGD patients.\textsuperscript{57} Five of the ten GvHD patients studied had some degree of dermato logic manifestion of GvHD and, therefore, the upregulation of PPL, IVL and S100A8 might be a result of dermal GvHD since Schirmer strips always touches patients’ skin. However, the eyelids of our patients were macroscopically not affected by GvHD, hence these changes may also be associated with ocular GvHD. Hyperosmolar stress induces expression of cornification markers in human corneal epithelial cells in vitro,\textsuperscript{58} indicating increased keratinization. Moreover, in mouse desiccating stress models of dry eye disease an increase in epithelial cell turnover is observed.\textsuperscript{59} The upregulation of PPL, IVL and S100A8 in tears may result from increased shedding of dead, cornified epithelial cells and therefore may be an important indicator of the integrity of the cornal epithelium in GvHD patients. Upregulation of cornification markers in GvHD tears suggests pathological keratinization of the corneal epithelium as a significant pathogenic mechanism in loss of tear film stability in GvHD eyes.

Prelamin-A/C (LMNA) was the fourth most highly upregulated protein in GvHD tears compared to patients without GvHD. To date, differential expression of LMNA has not been documented in relation to ocular surface disease. Prelamin-A/C undergoes enzymatic cleavage to yield mature A-type lamins, A and C. The primary function of A-type lamins is to form and stabilize the nuclear membrane.\textsuperscript{60} Overexpression of LMNA has been demonstrated to cause telomere loss and replicative senescence in fibroblasts in vitro\textsuperscript{61} and has been associated with senescence in cultured iPSCs.\textsuperscript{62} Immunosenescence may play a role in the development of GvHD pathology at the ocular surface.

The aim of our study was to determine whether specific changes in the tear proteomic profile could be detected in patients with active ocular GvHD compared to patients without GvHD following allogeneic HCT. In this discussion, we have provided insight into how these differentially expressed proteins could contribute to ocular surface pathology in ocular GvHD. There are a number of limitations associated with this study. Firstly, due to the small number of patients per group, we could only detect strong effect sizes in this study. Despite its small group sizes, this study has identified and confirmed the differential expression of a number of proteins already well known to be altered in dry eye disease. Our study has also added novel proteins that are differentially expressed in GvHD and may have important roles...
in the development of disease pathology. It provides a basis for further studies and longitudinal analyses.

Second, it is possible that systemic immunosuppressive therapies may alter the tear proteome. Patients included in this study were not therapy-naive. In order to maximize “signal-to-noise” ratio, our inclusion criteria for group 1 included only patients with moderate-to-severe disease, the control of which required local and/or systemic immunosuppression. Therefore, it was not possible to study patients with untreated ocular GvHD to correct for potential effects of ongoing systemic or topic immunosuppressive therapy. In light of this, we have reported a list of all systemic and ophthalmological therapies used by study participants as supporting information (Supplementary Tables S1, S2). This issue needs to be addressed further in a prospective study design with multiple assessments over time.

Punctal occlusion for ocular GvHD has been controversial due to the hypothesis that delaying tear outflow may concentrate inflammatory mediators on the ocular surface. We have previously shown punctal occlusion to be safe and effective for reducing objective signs and subjective symptoms of dry eye in ocular GvHD. In the current study, punctal occlusion was used as a local therapy in 90% of Group 1 and 20% of Group 2 patients. In a 3-week duration study, Tong et al. recently demonstrated improvement of symptoms after punctal occlusion in patients with moderate dry eye. Interestingly, follow-up tear proteomic analysis revealed upregulation of lacrimal tear proteins and reduction of inflammatory mediators following punctal occlusion in dry eye patients with low Schirmer test scores at baseline (4.3 ± 1.3 mm). Given that our ocular GvHD patients had very low Schirmer test scores (1.95 ± 3 mm), one might expect a similar beneficial effect of punctal occlusion on the tear proteomic profile. However, our proteomic data showed lacrimal proteins (LTF, LACRT, PROL1, LCN1, CST1, CST4, LYZ) to be among the most significantly downregulated in ocular GvHD patients. This may suggest a more severe impairment of lacrimal gland function in ocular GvHD patients that is not overcome by punctal occlusion.

We identified significantly increased abundance of DNA-binding proteins and cytoskeletal proteins as a hallmark of GvHD. It is possible that presence of these intracellular proteins in tears is solely as a result of cellular necrosis, but these findings may still be relevant for diagnostic testing. Further, it is worth noting that the increased levels of intracellular proteins seen in ocular GvHD tears (Histones, LMNA, PPL, RRBP1) may be in part due to delayed clearance from the ocular surface.

In this study, we report for the first time a discovery-based proteomic analysis of tears from ocular GvHD patients. We have demonstrated significant overlap with previously reported proteomic profiles of dry eye disease patients, with GvHD showing similarity to published profiles of severe dry eye. The sample size of the study reported here was small and, therefore, it has not been possible to conduct validation studies in independent samples. Future longitudinal validation studies and proper correlation with clinical parameters are necessary, and will be carried out to identify potential candidate tear biomarkers for ocular GvHD.

Acknowledgments
The authors thank Graziella Reinhard (study nurse) for her help with the management of our study patients and data handling.

Disclosure: N. Gerber-Hollbach, None; K. Plattner, None; O.E. O’Learly, Roche (F); P. Jenoe, None; S. Moes, None; B. Dreixel, None; A. Schoetzau, None; J.P. Halter, None; D. Goldblum, Roche (F); Santen (C), Haag-Streit (C.R); Thea (R), Bausch & Lomb (R), Johnson & Johnson (R)

References


