Prehematopoietic Stem Cell Transplantation Tear Cytokines as Potential Susceptibility Biomarkers for Ocular Chronic Graft-Versus-Host Disease

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PURPOSE. To determine if cytokine tear levels before hematopoietic stem cell transplantation (HSCT) can help anticipate the occurrence of ocular chronic graft-versus-host disease (cGVHD).

METHODS. In this pilot study, 25 patients undergoing HSCT were followed prospectively for ≤43 months. After ocular examinations, tears were collected before HSCT. Levels of 19 cytokines (epidermal growth factor [EGF], eosinophil 1/CCL11, fractalkine/CX3CL1, IL-1Ra, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8/CXCL8, IL-10, IL-12p70, IL-13, IL-17A, IP-10/CXCL10, IFN-γ, VEGF, TNF-α, and RANTES/CCL5) were measured by multiplex bead assay. A multistate model (MSM) based on four states (HSCT, systemic cGVHD, ocular cGVHD, and death) was developed to identify cytokines associated with each transition probability. Molecules included in the final multivariable model were selected by a supervised principal components analysis. Bootstrap resampling internally validated the final MSM. Model discriminatory ability was determined by time-dependent receiver operating characteristic curves and the corresponding area under the curve (AUC).

RESULTS. The final model, based on fractalkine, IL-1Ra, and IL-6 tear levels, accurately influenced the transition between the four different states. The AUC for this model, based on a new variable built upon the combination of these three molecules, was 67% to 80% throughout follow-up and, thus, had good discriminatory ability.

CONCLUSIONS. In this prospective study, a model based on pre-HSCT tear levels of the inflammatory molecules fractalkine, IL-1Ra, and IL-6 had good prognostic ability for the development of ocular cGVHD after HSCT. These cytokines potentially could act as susceptibility biomarkers for the development of this disease after HSCT.

Keywords: ocular cGVHD, dry eye disease, keratoconjunctivitis sicca, graft-versus-host disease, GVHD, tears, cytokine, biomarker, hematopoietic stem cell transplantation, HSCT

Allogeneic hematopoietic stem cell transplantation (HSCT) is an established and potentially curative form of treatment for malignant and nonmalignant hematologic diseases. However, its use is restricted by the potential development of graft-versus-host disease (GVHD), which represents the most important source of nonrelapse mortality, as well as the main cause of morbidity and loss of quality of life in long-term survivors.1

Chronic GVHD (cGVHD) is a distinct clinical entity from acute GVHD (aGVHD) and not merely a temporal extension of the latter.2 While in the acute form, the dominant changes are necrosis of the target organs (e.g., skin, liver, and mucous membranes, including the conjunctiva), the pathologic hallmarks of cGVHD are autoimmune-like processes induced by dysfunctional immunological recovery leading to chronic inflammation and fibrosis.3 The incidence of cGVHD ranges from 6% to 80% of transplanted patients, depending upon the presence of risk factors and the diagnostic criteria used.1

The eye is one of the most frequently affected target organs in cGVHD, occurring in 60% to 90% of HSCT recipients.5 Ocular cGVHD can lead to serious abnormalities of the ocular surface, and the universal manifestation is dry eye disease (DED) or keratoconjunctivitis sicca (KCS). This pathologic dramatically affects patient quality of life and eventually may lead to permanent visual loss.

At present, we have the possibility of searching not only for clinical factors associated with cGVHD,5,6 but also for molecules that could act as biomarkers. Biomarker categories and definitions have been reported in the Food and Drug Administration–National Institutes of Health (FDA-NIH; Bethes-
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da, MD, USA) BEST Resource,7 where susceptibility biomarkers have been defined as a “biomarker that indicates the potential for developing a disease or medical condition in an individual who does not currently have clinically apparent disease or the medical condition.” There are many biomarkers proposed for cGVHD,6–12 and some for ocular cGVHD.13–15 However, to our knowledge, there are no studies reporting the presence of potential susceptibility biomarkers before HSCT that can help predict ocular involvement after HSCT. Previous research yielded valuable insights in the field, but these findings were limited by their retrospective nature. These studies included patients with an already diagnosed ocular cGVHD and, therefore, the incidence of new cases of ocular cGVHD could not be determined. Furthermore, these studies could not fully elucidate risk factors of the disease, and the results may be influenced by immunosuppressive medication used for prevention and treatment.

To overcome these limitations, we prospectively studied patients with planned HSCT to analyze the incidence and clinical factors associated with development of ocular cGVHD, and determine if tear molecules collected before HSCT could serve as susceptibility biomarkers for the development of post-HSCT ocular cGVHD. The use of pre-HSCT tear cytokine levels avoids the potential influence of any immunosuppressive therapy on the tear molecules. The development of a predictive model of ocular cGVHD based on these molecules could help clinicians in their decision-making process for this severe pathology.

MATERIALS AND METHODS

Patients

Between September 2008 and February 2010, we recruited 36 consecutive patients from the Hematology Department, Barcelona Clinic Hospital (Barcelona, Spain) who were scheduled to undergo allogeneic HSCT for hematologic malignancies. A first visit (baseline or V0) was scheduled before HSCT to evaluate inclusion and exclusion criteria. Inclusion criteria were patients over 18 years of age, who already were scheduled to undergo allogeneic HSCT, who did not have DED (defined below), and in whom a tear sample could be collected. Exclusion criteria were any ocular active disease, current contact lens wear, current use of ophthalmic medications, and any ophthalmic surgery in the previous 6 months. The study was approved by the Institute of Applied Ophthalmology (IOBA) Institutional Review Board and by the University of Valladolid Clinic Hospital and Barcelona Clinic Hospital Ethics Committees. It followed the tenets of the Declaration of Helsinki and the Spanish law of personal data protection. All enrolled patients were informed of the nature of the study and written consent was obtained from each of them.

Study Design and Patient Clinical Evaluation

The total planned follow-up included 10 scheduled visits (V0–V9), starting at V0 or baseline, performed between 7 and 30 days before HSCT, and before any preparatory treatment was administered. Follow-up visits V1 to V9 were held at 1, 3, 6, 9, 12, 18, 24, 30, and 36 months after HSCT. Five patients could not attend the last visit at 36 months due to their health status; therefore, they eventually were evaluated at 43 months after HSCT when their health permitted. Additionally, nonscheduled visits were included if required by patients at any time.

Data collected at V0 included sex and age at the time of transplantation, underlying hematologic disease, type of conditioning regimen, hematologic stem cell source, donor–recipient relation, donor sex, and time between the diagnosis of the background disease and HSCT. Underlying diseases were classified according to the World Health Organization Classification of Tumors of Hematopoietic and Lymphoid Tissues.15 All included patients underwent transplantation procedures according to the standard HSCT protocols used by the Department of Hematology of the Barcelona Clinic Hospital. Conditioning regimens were used according to standard operating procedures,16 including nonablative regimen (fluorarabine plus busulphan or melphalan) or myeloablative regimen (cyclophosphamide plus total body irradiation).

After V0 but before HSCT, GVHD prophylaxis was initiated and consisted of cyclosporine A plus mycophenolate mofetil for cases treated with a nonablative regimen.16 For cases undergoing a myeloablative conditioning regimen, prophylaxis consisted of cyclosporine A plus methotrexate, according to the Seattle scheme.17

Ocular GVHD was diagnosed by the hematology team following the NIH consensus criteria proposed in 2005.18 Ocular cGVHD was diagnosed following the most recent consensus document, which states that the diagnosis “requires at least one diagnostic manifestation of chronic GVHD or at least one distinctive manifestation plus a pertinent biopsy, laboratory, or other tests (e.g., pulmonary function tests, Schirmer’s test), evaluation by a specialist (ophthalmologist, gynecologist), or radiographic imaging showing chronic GVHD in the same or another organ, unless stated otherwise.”19

As distinctive manifestations of chronic GVHD include “new onset of dry, ‘gritty,’” or painful eyes, cicatricial conjunctivitis, KCS, and confluent areas of punctate keratopathy. Other features include photophobia, periorbital hyperpigmentation, and blepharitis,” we decided to establish the diagnosis of “dry eye or KCS” in the context of ocular cGVHD, with abnormal results in at least 3 of 5 DED diagnostic tests: Ocular Surface Disease Index (OSDI) questionnaire score > 12 points, fluorescein tear break-up time (T-BUT) ≤ 7 seconds, corneal fluorescein staining and conjunctival rose Bengal staining > 1 (Oxford scale), and Schirmer test without topical anesthesia ≤ 5 mm in 5 minutes. Lastly, if a patient had a corneal fluorescein staining > 3 in only one eye, DED was diagnosed regardless of the remaining test results. These DED diagnostic criteria have been reported previously and have a demonstrated relevance.15,14,19–21

Patients presenting at V0 with DED signs and symptoms were excluded from the study.

Ocular Clinical Examination and Sample Collection

Ocular clinical evaluations always were performed by the same clinician (MSM). The sequence of tests was the same at each visit: (1) OSDI questionnaire, consisting of 12 questions assessing the presence of DED-related symptoms over the preceding week,22 (2) Tear sample collection was performed at baseline, before HSCT (V0), and before any other test to avoid any ocular vital dye interference. Basal tears (1 µL) were collected from the external canthus of the left eye in all cases, avoiding additional tear reflex as much as possible,23 using a glass capillary tube (Drumond Scientific Co., VW International, Broomall, PA, USA). The sample was placed in a sterile tube containing 9 µL of cold cytokine assay buffer (Millipore Ibérica, Madrid, Spain), and the mixture was frozen immediately and stored at −80°C until assayed. (3) Conjunctival hyperemia, based on the Nathan-Efron 0–4 scale,14 was evaluated under a slit-lamp. (4) Tear stability was evaluated by measuring T-BUT; sodium fluorescein (2%, 5 µL) was applied gently into the outer third of the inferior fornix with a micropipette. The time between the last of three blinks and the appearance of the first
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![Diagram of MSM for the onset of cGVHD and ocular cGVHD](image)

**FIGURE 1.** MSM for examining the onset of cGVHD and ocular cGVHD in patients undergoing HSCT. Each state in this four-state model is represented by a box. Transitions between states are indicated by arrows from one state to another. The number of observed transitions and percentages (in parentheses) are shown.

Cytokine expression data were transformed using the logarithmic base 2 scale.

**Statistical Analysis**

Statistical analyses were performed by a PhD statistician (IF) using the R software (R Foundation for Statistical Computing, Vienna, Austria). Statistical significance was set at 5%.

Quantitative data were expressed as means ± SD and qualitative variables were described in percentages. Median and interquartile range (IQR) were used to summarize distributions of ordinal variables. Univariate tests checked differences in the distribution of each variable across the studied groups. Normality assumptions were checked by the Shapiro-Wilk test. To compare quantitative characteristics, we used the Student’s t-test for two independent samples, or the nonparametric alternative, Mann-Whitney U test, if the normality hypothesis was not valid. To check homogeneity of variances, we used the Brown-Forsythe test. When there was significant heterogeneity of variance, we used the Welch’s t-test. To assess the association between qualitative variables and groups, we used the χ² test or the Fisher’s exact test with small expected frequencies. Kaplan-Meier curves and survival estimations were generated to evaluate the onset of aGVHD, systemic cGVHD, and ocular cGVHD.

**Multistate Model (MSM) Definition**

Using the R package **msstate**, we applied a MSM to evaluate the onset of ocular cGVHD and to identify molecular tear levels that could predict patient risk of ocular cGVHD development. With this model, patients can be classified into several health states at any time during the follow-up period, and transitions between states represent a change in the disease process. Typically, the MSM contained one initial state, one or several endpoints or absorbing states, and different intermediate or transient states. In this study, we defined a four-state model (Fig. 1). The initial state was HSCT, and patient death and ocular cGVHD occurrence were considered as competing endpoints or absorbing states. From the HSCT state, development of systemic cGVHD could be reached as a transient state toward the development of ocular cGVHD. A transition from systemic cGVHD to patient death also was included in the model. We considered that after HSCT, patients may or may not suffer systemic cGVHD, but they also may die due to transplant complications or initial hematologic disease relapse. Although ocular cGVHD can be diagnosed even if no other organs are involved, for the purpose of this study, we assumed that the onset of ocular cGVHD was preceded by systemic cGVHD. Lastly, some patients could have died after systemic cGVHD but without having suffered ocular cGVHD. Figure 1 shows schematically the MSM definition and general construction. The detailed number and description of the patients included are shown in the Results section.

We have assumed that the multistate process is Markovian, meaning that the time course can be described assigning every individual patient to a distinct state at any defined time and that transition intensities depend only on the history of the process through the present state. To estimate transition probabilities, we used the Aalen-Johansen estimator, which provides the distribution of patients’ disease states during follow-up.

**Effect of Individual Baseline Characteristics on Disease Process**

Cytokine/chemokine tear levels before HSCT, and several demographic and clinical characteristics of patients were selected as covariates for the MSM to explain differences
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Table 1. Clinical Data of Patients Undergoing HSCT

<table>
<thead>
<tr>
<th></th>
<th>25</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, mean ± SD, (range in y)</td>
<td>44.1 ± 15.42 (18–63)</td>
</tr>
<tr>
<td>Female/male</td>
<td>10/15</td>
</tr>
<tr>
<td>Underlying disease, n (%)</td>
<td></td>
</tr>
<tr>
<td>Chronic myeloproliferative diseases</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Myelodysplastic/myeloproliferative diseases</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Myelodysplastic syndromes</td>
<td>2 (8)</td>
</tr>
<tr>
<td>Acute myeloid leukemias</td>
<td>6 (24)</td>
</tr>
<tr>
<td>B cell diseases</td>
<td>6 (24)</td>
</tr>
<tr>
<td>T and NK cell diseases</td>
<td>8 (32)</td>
</tr>
<tr>
<td>Hodgkin’s disease</td>
<td>2 (8)</td>
</tr>
<tr>
<td>Plasma cell diseases</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Histiocytic and dendritic cell neoplasms</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Other diagnoses</td>
<td>1 (4)</td>
</tr>
<tr>
<td>Source of hematologic stem cells, n (%)</td>
<td></td>
</tr>
<tr>
<td>Bone marrow</td>
<td>4 (16)</td>
</tr>
<tr>
<td>Peripheral blood stem cell</td>
<td>20 (80)</td>
</tr>
<tr>
<td>Umbilical cord blood</td>
<td>1 (4)</td>
</tr>
<tr>
<td>Donor-recipient relationship, n (%)</td>
<td></td>
</tr>
<tr>
<td>Related</td>
<td>12 (48)</td>
</tr>
<tr>
<td>Nonrelated</td>
<td>13 (52)</td>
</tr>
<tr>
<td>Type of conditioning regimen, n (%)</td>
<td></td>
</tr>
<tr>
<td>Nonablative</td>
<td>15 (60)</td>
</tr>
<tr>
<td>Myeloablative</td>
<td>10 (40)</td>
</tr>
</tbody>
</table>

Results

Clinical Examination

Of the 36 patients initially recruited, 11 were excluded at V0 for the following reasons: rapid evolution of their underlying disease and could not proceed with the scheduled HSCT (n = 3), DED before HSCT (n = 5), and inability to collect tears (n = 3). Therefore, 25 patients (15 males, 10 females; 44.1 ± 15.4 years; range, 18–63 years) were included in V0 and subsequently underwent HSCT (Table 1). A total of 12 patients were lost during the planned follow-up visits. Six died due to complications of the transplant process and four due to progression of the underlying hematologic disease. One patient eventually was followed at a different health center, and one was lost due to unknown causes. Mean follow-up was 19 ± 16.4 months (range, 1–43 months). The number of patients who attended each visit was V0, 25; V1, 16; V2, 11; V3, 9; V4, 6; V5, 4; V6, 3; V7, 3; V8, 4; and V9, 12. Additionally, four patients were seen at nonprogrammed visit times.

Overall, 12 patients (48%) suffered aGVHD from HSCT at a median time, estimated by Kaplan Meier method, of 2.2 months. The targeted organs were skin (4), gastrointestinal (GI) tract (4), mouth (2), and skin and GI tract together (2). A total of 16 patients (64%) developed systemic cGVHD at a median of 15 months, and in these cases the affected organs were skin (5), skin and GI tract (4), mouth (3), GI tract (3), and lungs (1). Among the patients who were diagnosed with systemic cGVHD, 10 (62.5%) developed ocular cGVHD at a median of 33 months after HSCT.

Tear Molecule Detection and Concentration

The percentage of detection and the concentration of the 19 cytokines and chemokines were analyzed in each of the 25 tear samples (Table 2). In more than half of the samples, we used imputation to determine the low concentration values, and these were treated as quantitative variables. Molecules with 20% to 50% detection were treated as qualitative variables that either were or were not detected. Cytokines and chemokines that were not detected in at least 20% of the individuals were not analyzed further.

Multistate Process Estimation

The four-state model, along with the number of patients included in each state and the transitions from one state to another, is shown in Figure 1. The transition probabilities among the four different states were determined by the Aalen-Johansen estimator (Fig. 2). Deaths occurred between 1 and 8 months after HSCT, while systemic cGVHD appeared at 4 months and later, and ocular cGVHD at 9 months and later. At the end of the follow-up period, 43.3 months after HSCT, 40% of patients had died, 10% had systemic cGVHD without ocular complications, and the remaining 50% had ocular cGVHD. Ocular examination results in each event considered are shown in Table 3.

Effect of Individual Baseline Characteristics and MSM Construction

The following individual baseline characteristics had no significant effect on any of the transitions states: age at transplantation, sex, donor’s sex, conditioning regimen,
Among the molecules studied, only fractalkine, IL-1Ra, and IL-6 demonstrated a significant influence in the different state transitions considered (Fig. 3). High levels of fractalkine before transplantation significantly decreased the risk of ocular cGVHD (Fig. 3). For each unit increase of fractalkine, the risk of ocular cGVHD development was reduced 3.3 times.

The detection of IL-6 in tears before transplantation increased by 2-fold the risk of ocular cGVHD. On the other hand, the same increase before transplantation decreased by 2-fold the risk of systemic cGVHD (Table 4). The same increase also was associated with a 3.3-fold reduction in the risk of ocular cGVHD.

Finally, the presence of IL-6 before transplantation significantly increased the risk of death. IL-6 was detected in the tears of only 20% of the patients. Because of the low percentage of detection, it was included in the model as a qualitative variable. The detection of IL-6 in tears before transplantation increased the risk of death 7-fold. Additionally, they are positively self-related, which means that higher levels of fractalkine and IL-1Ra are found in patients in whom IL-6 is detected (Fig. 4).

Using supervised PCA, we built a new variable that combined the presence of IL-6, IL-1Ra, and fractalkine. We designated the new composite variable as “PC1”, and it accounted for 75% of the whole variability of the sample. The correlation of PC1 with the original variables showed that the three molecules were correlated positively with it. Thus, higher values of this variable corresponded to higher levels of fractalkine and IL1Ra and a higher percentage of detection of IL-6.

The composite variable PC1 was significantly associated with the transitions from HSCT to death and from systemic to ocular cGVHD. Thus, a one-unit increase in tear PC1 before transplantation was associated with a 1.6-fold increase in death before the onset of systemic cGVHD (Table 4). The same increase also was associated with a 3.3-fold reduction in the risk of ocular cGVHD.

### Internal Validation of Multivariate Model

For internal validation of the model, we used a bootstrapping technique, repeating the overall modeling process with 1000 bootstrap samples. The three selected molecules, IL-6, IL-1Ra, and fractalkine, appeared in more than 69% of the bootstrap samples. Specifically, the frequency of selection in bootstrap samples was 87.9%, 82.6%, and 69.4% for IL-1Ra, IL-6, and fractalkine, respectively. Additionally, three more molecules appeared in more than 50% of the bootstrap samples: IL-8 (50%), EGF (45%), and IL-13 (35%).

The model discriminatory ability for the development of ocular cGVHD, based on the AUC of the ROC curves, had values between 67% and 80% throughout follow-up (Fig. 5). Thus, the model was highly accurate in predicting the probability of death before the development of systemic cGVHD.

### Estimated Transition Probabilities Using the Aalen-Johansen Estimator

Probabilities are stacked and the distance between two adjacent curves indicates the transition probability of the corresponding state. Deaths occurred between 1 and 8 months after HSCT; systemic cGVHD appeared at 4 months and later, and ocular cGVHD at 9 months and later. At the end of the follow-up period, 43.3 months after HSCT, 40% of patients had died, 10% had systemic cGVHD without ocular complications, and the remaining 50% had ocular cGVHD.
Figure 3. Relative Risk (RR) estimates of each molecule for any of the 4 transitions considered in the model. Points denote the base 2 logarithm of the estimated relative risk [log₂(RR)], and horizontal lines represent the 95% confidence interval (CI) for each molecule in each transition. There is a significantly greater or lower risk if the horizontal line is to the right or to the left, respectively, of the no-effect line (vertical line) and does not cross it. The risk is not significant if the horizontal line crosses the no-effect line. Blanks correspond to nonestimable effects. Fractalkine significantly decreased the transition from systemic to ocular cGVHD. IL-1Ra significantly increased the risk of the HSCT to death transition but decreased the risk of systemic to ocular cGVHD transition. IL-6 significantly increased the risk of transition from HSCT to death.

Table 3. Ocular Examination Results at Each Event of Interest Considered in the MSM

<table>
<thead>
<tr>
<th>Test</th>
<th>Baseline, n = 25</th>
<th>Systemic cGVHD, n = 16</th>
<th>Ocular cGVHD, n = 10</th>
<th>Death, n = 10</th>
</tr>
</thead>
<tbody>
<tr>
<td>OSI questionnaire</td>
<td>0.52 ± 1.13</td>
<td>12.28 ± 23.82</td>
<td>33.33 ± 28.93</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>T-BUT, sec</td>
<td>9.64 ± 3.72</td>
<td>8 ± 4.18</td>
<td>4.9 ± 4.35</td>
<td>8.6 ± 2.88</td>
</tr>
<tr>
<td>Corneal fluorescein staining</td>
<td>1 ± 1</td>
<td>2 ± 2</td>
<td>3 ± 1</td>
<td>0 ± 1</td>
</tr>
<tr>
<td>Rose Bengal conjunctival staining</td>
<td>1 ± 1</td>
<td>2 ± 2</td>
<td>3 ± 1</td>
<td>0 ± 0.75</td>
</tr>
<tr>
<td>Schirmer test without anesthesia, mm</td>
<td>9.64 ± 3.72</td>
<td>12.56 ± 7.92</td>
<td>5.9 ± 5.69</td>
<td>13.2 ± 8.2</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SD in OSI, T-BUT, and Schirmer test. Data are presented as median ± IQR in fluorescein corneal staining and rose Bengal conjunctival staining.

Figure 4. Relationships among fractalkine, IL-1Ra, and IL-6. The three cytokines linked to ocular cGVHD were associated positively with one another, with higher levels of fractalkine and IL-1Ra found in patients in whom IL-6 was detected (D). ND, nondetected.
appearance of ocular cGVHD during the follow-up period of this study.

**DISCUSSION**

One of the greatest barriers to achieving adequate management of patients with ocular cGVHD is the inability to determine their individual risk before the onset of symptoms. Hence, making an adequate treatment plan for each individual’s risk is exceedingly difficult. In our study, our goal was to identify tear cytokine and chemokine levels that indicated the potential for ocular cGVHD development in individuals who have not yet undergone HSCT.

Thus, we recruited a cohort of patients undergoing HSCT and analyzed tear levels of a panel of cytokines and chemokines before transplantation. We observed these patients for a maximum of 43 months to determine who of them developed ocular cGVHD, although there was a considerable loss of patients before the initial visit (11 of 36) that reduced the sample size to 25 patients. The final panel of inflammatory molecules was defined after analysis of the most important candidate molecules from previous studies.13,14

Ocular examination during the follow-up period showed the development of ocular cGVHD in 40% of the patients by worsening of DED symptoms as assessed by the OSDI questionnaire, lower Schirmer test score, decreased T-BUT, and significant worsening of ocular surface integrity as demonstrated by increased fluorescein and rose Bengal staining. In our cohort, only 5 of the 36 patients initially recruited (13.9%) were classified as having DED and subsequently were not included in the study. These results disagreed with those reported recently by Giannaccare et al., who stated that DED already was present in a significant number of patients (42.8%) suffering from hematologic disease before HSCT was performed.38 We considered that patients who suffered DED before HSCT and enrollment could be difficult to analyze and data from such patients could have confounded the interpretation of the data and the conclusions of the work, because elevated tear levels of cytokines may represent preexisting DED. We wanted to be sure that this was not the case; thus, we excluded patients with a previous diagnosis of DED.

In our study 10 of the 25 patients (40%) developed ocular cGVHD after HSCT. This finding agrees with our previous retrospective study in which 38% of transplanted patients suffered ocular cGVHD.39 It also is consistent with the

**Figure 5.** Time-dependent AUC for the transition of systemic to ocular cGVHD for the multivariable model based on fractalkine, IL-1Ra, and IL-6 tear levels. Mean values and the 25th and 75th percentile curves are shown. An AUC value of 0.5 (horizontal line) represents the no-discrimination value.

**Table 4.** Effect of the New Variable Constructed Upon Tear Levels of Fractalkine, IL-1Ra, and IL-6 on Transitions Between Clinical States Considered in the MSM

<table>
<thead>
<tr>
<th>Transition</th>
<th>RR</th>
<th>Lower CI</th>
<th>Upper CI</th>
<th>Log Fold Change</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transplant to systemic cGVHD</td>
<td>0.787*</td>
<td>0.51</td>
<td>1.216</td>
<td>−1.27</td>
<td>0.2804</td>
</tr>
<tr>
<td>Transplant to death</td>
<td>1.631*</td>
<td>1.039*</td>
<td>2.562*</td>
<td>1.63</td>
<td>0.0336</td>
</tr>
<tr>
<td>Systemic cGVHD to ocular cGVHD</td>
<td>0.299*</td>
<td>0.098*</td>
<td>0.913*</td>
<td>−3.54</td>
<td>0.034*</td>
</tr>
<tr>
<td>Systemic cGVHD to death</td>
<td>0.55</td>
<td>0.064</td>
<td>4.695</td>
<td>−1.82</td>
<td>0.5847</td>
</tr>
</tbody>
</table>

* Significant differences (P < 0.05).
potential susceptibility biomarkers for ocular cGVHD. We also considered the other transitions to account for other possible outcomes of these patients, such as death or the intermediate state between HSCT and ocular cGVHD that corresponds to the systemic GVHD. Our intention, considering the broad range of intermediate transition stages and time points, was to avoid underestimation of the probability of ocular complications developing, considering patients who had not suffered the disease, and those in whom it could not develop, because they had not lived long enough.

The rationale for this approach is that it allows greater accuracy in predicting the occurrence of the event of interest (i.e., ocular cGVHD), taking into account a number of intermediate events, such as death and development of systemic cGVHD. To do that, we applied MSMs instead of survival or time-to-event data, as we preferred to evaluate the history of events that were available because we prospectively followed the patients undergoing HSCT. With survival data, censoring usually occurs when the outcome event is not observed during a patient’s follow-up, and so the inevitable result is a biased estimation of probability of the occurrence of the event of interest. Instead, MSMs are very useful for describing event-history data, leading to a better understanding of the changes in disease conditions over time. 50 This approach perfectly suited the purpose of our study and has been used by different research groups to analyze complications of HSCT. For instance, Lee et al. 51 developed a MSM with single, independent, and cross-related outcomes based on four states: transplant, pleural effusion, GVHD, and death, in a fashion similar to that used by us here. Additionally, Eefting et al. constructed a MSM to analyze the impact of donor lymphocyte infusion and GVHD on the probabilities of relapse and nonrelapse mortality over time. 52 As in our case, MSM helped to interpret the impact of postransplantation interventions and clinical events on the determined states and transitions between states.

Among all the 19 cytokine and chemokine molecules studied, fractalkine, IL-1Ra, and IL-6 demonstrated significant influence in the different transitions. Consequently, these three molecules may possess significant biological relevance to GVHD. Elevated expression of fractalkine in tears before transplantation was associated with decreased risk of developing ocular cGVHD. The association between the elevated levels of tear fractalkine and reduced risk of ocular cGVHD is consistent with the known tendency of conjunctival epithelial cells to have reduced fractalkine gene expression in ocular GVHD. This molecule acts as a potent chemotractant for intraepithelial lymphocytes (CX3CR1+ cells). 53, 54 In contrast, fractalkine is increased significantly in tears of DED patients compared to healthy controls, and higher levels are associated with greater clinical severity. 55, 56 Numerous studies have shown that it contributes to the development of several inflammatory diseases, such as primary Sjögren syndrome, rheumatoid arthritis, granulomatosis with polyangiitis, Crohn’s disease, and experimental autoimmune uveitis among others. 54, 56 In this case, findings suggest that this molecule could have an important role in the pathogenesis of GVHD, and a decrease in its secretion and lower levels may be related to an increased survival of these patients, making them prone to suffer ocular complications.

Elevated levels of pre-HSCT tear IL-1Ra were associated with a lower risk of subsequent ocular cGVHD. Additionally, higher levels of this molecule were associated with increased risk of transition from HSCT to death. IL-1Ra is a naturally-occurring cytokine receptor antagonist that serves as a modulator of immune responses, regulating the agonist effects of IL-1 during chronic inflammatory diseases. 7 IL-1Ra additionally has been implicated in the pathogenic mechanisms of DED-associated
ocular surface inflammation, with increased tear levels found in those patients compared to controls.\textsuperscript{25,27} A topical preparation of recombinant IL-1Ra has been used successfully to treat DED patients in a randomized clinical trial.\textsuperscript{28} Moreover, polymorphisms in IL-1 family genes have been associated with variability in the production of the respective cytokines and have been implicated in patient susceptibility to GVHD.\textsuperscript{29} The explanation for this apparent protective role could be related to a regulatory effect during IL-1-related inflammatory cascade.

Finally, detectable levels of IL-6 before transplantation increased the risk of death before development of systemic or ocular cGVHD. IL-6 is a well-known pathophysiologic molecule in GVHD.\textsuperscript{30} Many studies have shown increased levels of IL-6 in patients with severe GVHD.\textsuperscript{60,61} Pre-HSCT tear levels of IL-6 of ocular cGVHD patients were increased compared to healthy controls.\textsuperscript{11} Additionally, Jung et al.\textsuperscript{12} found that IL-6, among other molecules, was elevated in tears collected after the onset of systemic cGVHD compared to transplanted patients in whom it did not develop. Finally, gene expression in the conjunctival epithelium also was upregulated in patients with ocular cGVHD.\textsuperscript{13} Although elevated levels of IL-6 occur in diverse post-HSCT complications, such as infections, mucositis, and venous occlusive disease,\textsuperscript{62} we believe that it could be an excellent prognostic indicator and a pivotal marker of ocular disease severity.

The combination of the three selected prognostic indicators, fractalkine, IL-1Ra, and IL-6, resulted in the best prediction model. Collectively as PC1, they were significantly associated with increased transitions of HSCT to death and systemic to ocular cGVHD. Increases in PC1, either as concentration or rate of detection of component cytokines, also were associated with decreased risk of ocular cGVHD. Internal validation of the model, based on the time-dependent ROC curves in which all were above 0.66, showed it has highly reliable discriminatory power.

At this point, we and others have demonstrated that multiple biomarker-based models, built upon the simultaneous use of a group of biomarkers, may improve diagnostic accuracy and specificity compared to the use of a single-molecule analysis for the diagnosis of ocular cGVHD.\textsuperscript{10,13,14,63} One of the biggest limitations of past studies is the reliance on retrospective analysis of the data. This is a crucial difference in study protocol, as we followed the patients over time, which provided valuable information about the disease course. Inability to evaluate patients at specific time points after transplantation may result in a later diagnosis of ocular cGVHD than if evaluated prospectively at a high frequency. Moreover, as mentioned before, studies that include patients with ocular cGVHD rather than those undergoing HSCT are unable to derive direct incidence data.

The relatively small size of our patient sample was the main limitation of this study. However, the study population was of great quality because it was uniform and very representative of patients undergoing HSCT. To a certain extent, this study was performed as a pilot survey in which a large panel of potentially relevant molecules was analyzed. This was necessary to select the ones of significant interest for future validation studies in a large cohort of patients.

Also regarding the size of our sample, it is known that MSMs may produce poor parameter estimates for small datasets because dividing a process into multiple states may lead to smaller event counts at some of the studied transitions. However, the alternative to this approach is survival analysis, which is much more biased and less adequate methodologically. Small sample sizes always will be a limit as, unfortunately, the severity of GVHD greatly affects patient mortality and morbidity, and subsequent follow-up is hampered. In fact, despite the improved success of HSCT, patient survival and better quality of life after GVHD remain a challenge for medicine today.

Trying to fit a multivariate model with many molecules also is challenging. The number of potential transition predictors may be too large for the small number of individuals in this type of study, and trying to combine them in a multivariable complex model could lead to a serious overfitting problem. In addition, correlations among predictor variables may lead to a multicollinearity problem. However, we were able to exploit the correlation structure between the variables and, thus, reduce the number of variables of the final model.

We would like to emphasize the extraordinary difficulties for the ophthalmologist to follow-up with GVHD patients. Many are extremely ill individuals who usually are followed in oncology or hematology departments. They may have a variety of systemic manifestations resulting in frequent hospitalization or risk of death, requiring variable follow-up regimens. Also, obtaining tear fluid from patients with severe DED is a challenge; thus, limiting the number of samples with a sufficient volume of tear available for molecular analysis. Bead-based arrays using X-MAP technology helps in overcoming this limitation in sample amount.

Lastly, given the severity of these patients, the hematology team had to do several and continuous changes and adjustments to the systemic treatment. Those adjustments have not been reflected in the study because they would have notably increased the complexity of the analysis. We are aware that changes in immunosuppression regimens may have an influence on the development of systemic and ocular cGVHD and should be taken under consideration. In contrast, immunosuppressive regimens used after HSCT have not influenced cytokine tear levels that we measured before transplantation, and this is an important feature of our study.

In conclusion, we believe that the model developed in this pilot study, based on the tear levels of fractalkine, IL-1Ra, and IL-6 before transplantation, demonstrates good prognostic ability, and the chosen molecules could act as potential susceptibility biomarkers of the development of ocular cGVHD. This work establishes important guidelines for future studies focused on the use of predictive models based on molecular biomarkers. If this model is validated, it could be of help in implementing preventive measures in patients undergoing HSCT, and may constitute a useful tool that allows clinicians to identify high-risk patients in an attempt to avoid, or at least minimize, the sight-threatening complications and poor quality of life associated with this severe ocular disease.

**Acknowledgments**

Supported by a national grant from the Ministry of Economy and Competitiveness, Madrid, Spain, SAF-2010-15361 (AES). Supported in part by Instituto de Salud Carlos III, CIBER-BBN, Spain (CB06/01/003 MINECO/FEDER EU), and by an ARVO publication grant. The authors alone are responsible for the content and writing of this paper.

Disclosure: L. Cocho, None; I. Fernández, None; M. Calonge, None; M. Sáinz de la Maza, None; M. Rovira, None; M. E. Stern, Allergan, Inc. (C, E), Novaliq (C), Shire (C); A. Enríquez-de-Salamanca, None; A. Enriquez-de-Salamanca, None

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