Biomarkers in Ocular Chronic Graft Versus Host Disease: Tear Cytokine- and Chemokine-Based Predictive Model

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Purpose. To develop a tear molecule level-based predictive model based on a panel of tear cytokines and their correlation with clinical features in ocular chronic graft versus host disease (cGVHD).

Methods. Twenty-two ocular cGVHD patients and 21 healthy subjects were evaluated in a controlled environmental research laboratory (CERLab). Clinical parameters were recorded, and tears were collected. Levels of 15 molecules (epidermal growth factor [EGF], IL receptor antagonist [IL-1Ra], IL-1β, IL-2, IL-6, IL-8/CXCL8, IL-10, IL-12p70, IL-17A, interferon inducible protein [IP]-10/CXCL10, IFN-γ, VEGF, TNF-α, eotaxin 1, and regulated on activation normal T cell expressed and secreted [RANTES]) were measured by multiplex-bead assay and correlated with clinical parameters. Logistic regression was used to develop a predictive model. Leave-one-out cross-validation was applied. Classification capacity was evaluated in a cohort of individuals with dry eye (DE) of other etiologies different from GVHD.

Results. Epidermal growth factor and IP-10/CXCL10 levels were significantly decreased in ocular cGVHD, positively correlating with tear production and stability and negatively correlating with symptoms, hyperemia, and vital staining. Interleukin-1Ra, IL-8/CXCL8, and IL-10 were significantly increased in ocular cGVHD, and the first two correlated positively with symptoms, hyperemia, and ocular surface integrity while negatively correlating with tear production and stability. Predictive models were generated, and the best panel was based on IL-8/CXCL8 and IP-10/CXCL10 tear levels along with age and sex, with an area under the receiving operating curve of 0.9004, sensitivity of 86.36%, and specificity of 95.24%.

Conclusions. A predictive model based on tear levels of IL-8/CXCL8 and IP-10/CXCL10 resulted in optimal sensitivity and specificity. These results add further knowledge to the search for potential biomarkers in this devastating ocular inflammatory disease.

Keywords: ocular chronic GVHD, dry eye, cytokine, biomarker, tears

Graft versus host disease (GVHD) is a major cause of nonrelapsing mortality and morbidity after allogeneic hematological stem cell transplantation (HSCT). Hematological stem cell transplantation has rapidly evolved in recent years, resulting in an increase in the number of recipients who become long-term survivors. Thus patient quality of life and the possibility of late complications have become increasingly important.[1] Graft versus host disease is an immune-mediated inflammatory disease that causes destruction of host tissues by immunocompetent cells from the donor.[2] There are two forms of GVHD, acute (aGVHD) and chronic (cGVHD), and differentiation between the two forms is currently based on clinical manifestations. Typical ocular complications in aGVHD are pseudomembranous conjunctivitis and acute hemorrhagic conjunctivitis, which appear in approximately 12% to 17% of GVHD patients.[3,4] However, ophthalmic findings are more frequent in chronic GVHD. Approximately 30% to 70% of human leukocyte antigen-matched patients develop cGVHD.[3] Ocular manifestations occur in up to 60% to 90% of patients with cGVHD, primarily affecting structures of the ocular surface.[4]

Ocular cGVHD usually mimics typical dry eye (DE); it frequently severely impairs the patient's quality of life with highly disturbing symptoms and may cause severe vision loss due to corneal involvement.[5] According to the National Institutes of Health (NIH) consensus criteria definition, "diagnosis of chronic GVHD requires at least 1 diagnostic manifestation of cGVHD or at least 1 distinctive manifestation plus a pertinent biopsy, laboratory, or other tests (e.g., Schirmer's test)."[6] However, in 2007 the International Dry Eye WorkShop recognized the lack of adequately validated and objective tests to diagnose DE.[7] The lack of correlation between symptoms and signs in DE (also shown among our own patients)[8] greatly complicates DE diagnosis, and is the main culprit in the frequent failure of clinical trials due to the scarcity of reliable evaluation end points. For this reason, efforts

are being made to find objective biomarkers that could be used as diagnostic, prognostic, and monitoring tools.[9] Inflammatory mediators are being analyzed as potential biomarkers, and to date, an increasing number of cGVHD candidate biomarkers are available for further investigation.[9] Some research groups have reported the possibility that levels of certain cytokines in serum can serve as specific biomarkers of GVHD and thus have the potential to act as accurate diagnostic and prognostic tools.[10,11] Regarding ocular cGVHD, our group has already shown that inflammatory gene expression in conjunctival epithelial cells may act as biomarkers for this disease.[12] However, identifying tear biomarkers in ocular cGVHD is even more desirable, because tears can easily be obtained in a noninvasive manner, and their close anatomical relationship to the disease site makes them highly specific for ocular disease.

With the description of changes in tear cytokine levels, significant progress has been made toward the characterization of underlying inflammatory mechanisms in the ocular surface in DE patients.[13–15] Despite this progress, little is known about the specific pathogenesis of ocular cGVHD. Some studies have already found differences in some tear cytokine levels in cGVHD patients, particularly one study by Riemens et al.,[16] and recently (2015) another one by Jung et al.,[17] which suggested that tear cytokines are useful biomarkers for the diagnosis of cGVHD.

It is clear, however, that any single biomarker lacks the sensitivity and specificity needed for most clinical applications.[18] Thus, multivariate predictive modeling techniques have emerged as useful tools to easily manage not just a single biomarker but a panel of biomarkers, which increases the diagnostic and prognostic power. Such models may be designed to predict a classification variable, for example, susceptibility to ocular cGVHD, based on known continuous parameters, that is, cytokine tear levels. Predictive models have also focused on clinical parameters in an effort to determine which aspects are most predictive of ocular cGVHD diagnosis.[19] Additionally, model validation is necessary as it gives important information about the reliability of the process. Thus, the purpose of this study was to develop and validate a prediction model for the diagnosis of ocular cGVHD based on the levels of a panel of inflammatory mediators in tears and the correlation of those mediators with the clinical ophthalmic phenotype.

Materials and Methods

Patients and Healthy Controls

This study was approved by the Institute of Applied OphthalmoBiology (IOBA) institutional review board and the University of Valladolid Clinical Hospital Ethics Committee and followed the tenets of the Declaration of Helsinki. All enrolled patients and subjects were informed of the nature of this study, and written consent was obtained from each of them.

Ocular cGVHD patients were selected from those referred to IOBA by the Hematology Department of the University of Salamanca Clinical Hospital from among new-onset DE patients, as we excluded patients with previous diagnosis of DE. To ensure as much as possible that the results would not be influenced by the effects of topical medications and/or any other condition, inclusion and exclusion criteria were established to ensure that patients were stable. Consequently, the most severely affected patients were excluded. For the same reason, patients included in the study were asked to discontinue their topical therapies for 7 days before the samples were taken. Only artificial tears and lubricants were allowed. Thus, inclusion criteria for patients were as follows: (1) abnormal results of at least three DE diagnostic tests that included an ocular surface disease index (OSDI) score > 12 points, fluorescein tear breakup time (T-BUT) < 7 seconds, corneal fluorescein staining and conjunctival Lissamine green staining > 1 (Oxford scale), and Schirmer test without topical anesthesia ≤ 5 mm in 5 minutes; (2) feasibility of discontinuing topical anti-inflammatory medications (artificial tears and lubricants were allowed) for 1 week, as judged by the attending ophthalmologist (MC); and (3) patients had to be systemically stable with GVHD under control and no relapse of the patient's primary malignancies and no secondary infections as judged by the referral physician (DC). Exclusion criteria included any ocular active disease other than DE, contact lens wear, any ophthalmic surgery in the past 6 months, any topical medicine other than artificial tears and lubricants, and any systemic medication that was not continuous or constant in treatment and dosage for at least 3 months prior to this study.

Healthy volunteers, similar in age and sex to the study cases, were selected as the control group and were examined to make certain their ocular status was within normal limits. Inclusion criteria were the following: (1) absence of ocular surface-related symptoms (OSDI score ≤ 12 points) and (2) normal limits in at least two of the following four ocular surface tests: fluorescein T-BUT ≥ 7 seconds, corneal fluorescein staining and conjunctival Lissamine green staining (Oxford scale) $\leq$ grade 1, and Schirmer test without anesthesia > 5 mm in 5 minutes. Exclusion criteria for this control group included any present or previous history of ophthalmic or systemic disease, ocular allergy, any ophthalmic surgery, under any medication, pregnancy, or current contact lens wear.

Clinical Examination and Sample Collection

All individuals were evaluated after 20-minute exposure in a controlled environmental chamber (VISIÓN I+D, SL; Valladolid, Spain) located in the Controlled Environmental Research Laboratory (CERLab) at IOBA, University of Valladolid, Valladolid, Spain, with the purpose of normalization and standardization of the conditions of clinical evaluation and collection of samples. The temperature was maintained at 23°C and the relative humidity (RH) at 45%, which corresponds to a comfortable indoor temperature and the average RH in Valladolid.[20–22]

Clinical evaluation was always performed by the same clinicians (LC, VM), each one always evaluating the same tests in the following sequence. First, the OSDI questionnaire, which consists of 12 questions, was administered, and the presence of symptoms over the preceding week was assessed.[23] The OSDI questionnaire was performed for each eye separately, and the eye with the higher score was selected as the most symptomatic and used in clinical evaluation and for tear sampling. A random data table was used to select the study eye in those cases where both eyes were equally symptomatic and for control subjects. Second, tear sample collection was performed before any other tests to avoid any ocular vital dye interference. As previously described by our group,[15] we used a glass capillary tube (Drummond Scientific Co., Broomall, PA, USA) to collect a 1-μL tear sample from the external canthus, avoiding tear reflex as much as possible. The collected sample was then deposited into a sterile tube containing 9 μL cold cytokine assay buffer (Millipore Ibérica, Madrid, Spain) and immediately frozen at −20°C and stored at −80°C until assayed. Tear samples were not pooled. Third, conjunctival hyperemia was evaluated under a slit-lamp and scored on the Nathan-Efron scale (0–4).[24] Fourth, tear stability was evaluated by measuring T-BUT. Five microliters of 2% sodium fluorescein was gently applied 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 dry spot was measured three times, and the mean value was recorded. Fifth, ocular surface integrity was evaluated at the cornea and at the interpalpebral bulbar conjunctiva by vital staining. Corneal integrity was evaluated with a slit-lamp mounted with a cobalt blue filter (Topcon Corp., Tokyo, Japan) and a yellow Wratten no. 12 filter (Eastman Kodak, Rochester, NY, USA). The evaluation was done 2 minutes after instillation of 5 μL 2% sodium fluorescein. For the evaluation of conjunctival integrity, Lissamine green strips (GreenGlo; HUB Pharmaceuticals, LLC, Rancho Cucamonga, CA, USA) were wetted with 25 μL sodium chloride and then gently applied into the inferior fornix. Corneal and conjunctival staining were scored using the Oxford scale (score 0–5).[25] Sixth, tear production was assessed by Schirmer test without topical anesthesia. This test was performed using a Schirmer sterile strip (TearFlo; HUB Pharmaceuticals, LLC, Rancho Cucamonga, CA, USA) placed in the lateral canthus of the inferior lid margin. The length of wetting was measured in millimeters with eyes closed for 5 minutes.

Analysis of Tear Cytokines/Chemokines

The presence and concentration of 15 molecules were determined in tear samples by a multiplex immunobead-based array (Milliplex 15x-MPXHCYTO-60 Human Cytokine/Chemokine Panel; Millipore, Watford, UK), using a Luminex IS-100 (Luminex Corporation, Austin, TX, USA). The following molecules were assayed: epidermal growth factor (EGF), interleukin (IL) 1 receptor antagonist (IL-1Ra), IL-1β, IL-2, IL-6, IL-8/CXCL8, IL-10, IL-12p70, IL-17A, interferon inducible protein (IP)-10/CXCL10, eotaxin 1/CCL11, interferon gamma (IFN-γ), vascular endothelial growth factor (VEGF), tumor necrosis factor alpha (TNF-α), and regulated on activation, normal T cell expressed and secreted (RANTES)/CCL5. Samples were analyzed as previously described,[15] following the manufacturer's protocol.

Statistical Analysis

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

Sample size was statistically determined. Statistical power analysis for logistic regression with continuous predictor and a balanced design determined that a sample of 19 subjects per group would give 80% statistical power to detect odds ratio of 2.5 at a significance level of 0.05.

Quantitative data were expressed as means ± standard deviations (SD), and qualitative variables were described in percentages. Median and interquartile range (IQR) were used to summarize distributions of ordinal variables. Univariate tests to check differences in the distribution of each variable across the studied groups were performed. Normality assumption was 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, the Brown-Forsythe test was used. When there was significant heterogeneity of variance, Welch's *t*-test was employed. To assess the association between qualitative variables and group, the χ^2 test was used or Fisher's exact test with small expected frequencies.

To quantify correlations between cytokine levels and clinical parameters, the Spearman ranked correlation coefficient (ρ) was used. Moreover, a bootstrap confidence interval (CI) for this coefficient was built, using 5000 bootstrap samples.

Tear Cytokine/Chemokine-Based Predictive Model Development and Validation

Cytokines were analyzed as log-transformed variables. Cytokine levels below the limit of detection were imputed using the robust regression on order statistics (robust ROS) method introduced by Helsel and Cohn[26] and implemented in the NADA (nondetects and data analysis) R package.[27] However, molecules that were detected in less than 50% of the samples were not further analyzed.

Logistic regression, adjusted for age and sex, was used to quantify the association between ocular cGVHD group and each cytokine separately. Molecules associated with the outcome at the 10% significance level were identified as candidate biomarkers. Potential biomarkers were evaluated simultaneously to fit a multivariate logistic regression model. The final panel of inflammatory molecules in ocular cGVHD patients was defined as the optimal subset of potential biomarkers. The first step was to identify the few most important candidate biomarkers that help in predicting ocular cGVHD. An exhaustive search was conducted, building logistic regression models with every possible combination of candidate molecules and age and sex as confounding variables. The optimal model was the one with the minimum Akaike information criterion value. The variance inflation factor (VIF) measure was used to check for multicollinearity. A VIF greater than 5 was considered evidence of multicollinearity.

The leave-one-out-cross-validation (LOOCV) procedure was used to estimate the prediction accuracy of the fitted model, and receiver operation characteristic (ROC) curve analysis was used to assess the discriminate ability. The final model was evaluated according to the area under the ROC curve (AUC). In addition, sensitivity and specificity were obtained by setting an optimal threshold using the pROC (display and analyze ROC curves) R package.[28]

The Brier score was used as global measure of precision. It is based upon individual differences between predicted risks in terms of likelihood and observed final outcomes, and ranges from 0 for a perfect degree of agreement to 1 for the worst possible degree of agreement. We used two methods to evaluate the calibration of the model, measured as the degree of agreement between the predicted and observed values. With the calibration in the large (CL) and calibration slope (CS), a perfectly calibrated model will have a CL value of 0 and a CS of 1. For these methods, the scores are not bounded, so the model will be badly calibrated if the values depart from the optimal values. We also used the Hosmer-Lemeshow test, which is significant for badly calibrated models.

In order to evaluate the external capacity of the fitted model to properly classify individuals with DE of different etiologies, an independent dataset of 48 DE patients (14 Sjögren DE and 34 non-Sjögren-associated DE) and 32 healthy controls from previously published studies by our group was used,[20–22] where tear samples were collected in the same standard conditions described above. The percentage of individuals successfully classified was estimated.

Results

Clinical Evaluation

A total of 22 ocular cGVHD patients (15 males, 7 females; 55.6 ± 11.6 years; range, 34–72 years) and 21 healthy

Table 1. Clinical Data of Ocular Chronic Graft Versus Host Disease (cGVHD) Patients

Patient	Previous Diagnosis	Time From HSCT to Ocular cGVHD Diagnosis, mo	Time From GVHD Diagnosis to Development of Ocular cGVHD, mo	Systemic Therapy	Topical Therapy Discontinued 7 d Before Sample Collection
1	Hodgkin disease	36	99	Tacrolimus	Autologous serum 0.4% preserved medroxyprogesterone
2	Non-Hodgkin lymphoma	9	45	None	Autologous serum 0.05% unpreserved cyclosporine A
3	Acute lymphoblastic leukemia	24	63	None	None
4	Acute myeloid leukemia	12	60	None	Autologous serum 0.1% preserved fluorometholone
5	Acute myeloid leukemia	3	126	Cyclosporine A	None
6	Hodgkin disease	18	67	None	Unpreserved 0.05% cyclosporine A
7	Acute lymphoblastic leukemia	12	Unknown	None	None
8	Non-Hodgkin lymphoma	38	49	Tacrolimus	Autologous serum 0.1% preserved fluorometholone
9	Myelodysplastic syndrome	3	81	Tacrolimus	Autologous serum
10	Acute myeloid leukemia	36	Unknown	None	None
11	Chronic myeloid leukemia	12	Unknown	None	0.1% preserved fluorometholone
12	Chronic myeloid leukemia	2	Unknown	None	Autologous serum
13	Acute myeloid leukemia	18	21	Rapamycin Prednisone Imatinib	0.1% preserved fluorometholone
14	Myelodysplastic syndromes	1	Unknown	Rapamycin	Autologous serum
15	Chronic lymphoblastic leukemia	15	Unknown	Rapamycin Tacrolimus Methylprednisolone	0.1% unpreserved dexamethasone
16	Myelodysplastic syndrome	6	26	Prednisone	Autologous serum
17	Chronic myeloid leukemia	27	10	Rapamycin	Autologous serum
18	Multiple myeloma	6	Unknown	Rapamycin Thalidomide Cyclophosphamide	Autologous serum
19	Myelodysplastic syndrome	2	20	Tacrolimus	0.1% preserved fluorometholone Autologous serum 0.05% unpreserved cyclosporine A
20	Myelodysplastic syndrome	6	Unknown	Tacrolimus	Autologous serum 1.5% hydrocortisone ointment
21	Chronic myeloid leukemia	3	Unknown	Prednisone	0.03% tacrolimus ointment Autologous serum
22	Acute myeloid leukemia	10	7	Methylprednisolone	Autologous serum 0.1% unpreserved dexamethasone

HSTC, hematopoietic stem cell transplantation; systemic therapy had to be stable for the 3 preceding months of the study per inclusion/exclusion criteria.

volunteers (12 males, 9 females; 53.1 ± 12.6 years; range, 30–75 years) were recruited. There were no significant differences in sex ($P = 0.665$) or age ($P = 0.4873$) between patients and controls. The clinical histories of the ocular cGVHD patients, including previous diagnosis for HSCT, time from HSCT and systemic cGVHD diagnosis to ocular cGVHD diagnosis, and systemic and topical therapies are shown in Table 1.

Ocular cGVHD patients had significantly more frequent and intense ocular surface symptoms than controls, as reflected by the higher OSDI questionnaire scores (Table 2). Ocular surface examination revealed that patients had moderate conjunctival hyperemia (grade 0 in 1 patient, grade 1 in 10 patients, grade 2 in 6 patients, grade 3 in 4 patients, and grade 4 in 1 patient), although scores were significantly higher compared to control group scores.

Table 2. Ocular Examination Parameters

	Healthy Controls, $n = 21$	Ocular cGVHD Patients, $n = 22$	*P* Value
OSDI questionnaire	4.29 ± 5.11	44.58 ± 21.76	**<0.0001**
Conjunctival hyperemia	0 ± 0	1.5 ± 0.5	**<0.0001**
T-BUT, s	6.79 ± 2.87	2.39 ± 2.30	**<0.0001**
Corneal fluorescein staining	0 ± 0	2 ± 1	**<0.0001**
Lissamine green conjunctival staining	0 ± 0	2 ± 1	**<0.0001**
Schirmer test without anesthesia, mm	13.60 ± 10.25	3.68 ± 2.90	**<0.0001**

Data are presented as mean ± standard deviation in OSDI, T-BUT, and Schirmer test. Data are presented as median ± interquartile range in hyperemia, fluorescein corneal staining, and Lissamine green conjunctival staining. Significant changes ($P < 0.05$) are denoted in bold.

Regarding tear stability and production, ocular cGVHD patients had significantly lower T-BUT and Schirmer test scores than controls. Corneal fluorescein staining and conjunctival Lissamine green staining scores were significantly higher in ocular cGVHD patients (Table 2).

Tear Cytokine/Chemokine Detection, Concentration, and Correlation With Clinical Data

For each of the 15 molecules studied, the percentage of detection in each group and the concentration in each sample were analyzed (Table 3). Epidermal growth factor and IL-17A were detected in significantly fewer ocular cGVHD patients than in healthy controls. For the other 13 molecules, there were no differences between the percent of patients and controls expressing them.

Regarding molecule concentrations, EGF and IP-10/CXCL10 tear levels were significantly decreased, whereas IL-1Ra, IL-8/CXCL8, and IL-10 were significantly increased in ocular cGVHD patients compared to healthy subjects (Table 3; Fig. 1). Interleukin-12, IL-6, IL-8/CXCL8, and RANTES levels were found significantly increased in males compared to females for both ocular cGVHD patients and control subjects (Fig. 2).

Interleukin-1Ra and IL-8/CXCL8 tear levels positively correlated with OSDI questionnaire score, conjunctival hyperemia, fluorescein corneal staining, and Lissamine green conjunctival staining. They were negatively correlated with T-BUT and Schirmer test scores (Fig. 3). Epidermal growth factor and IP-10/CXCL10 levels were positively correlated with T-BUT and Schirmer test scores, and negatively correlated with OSDI, hyperemia, and fluorescein and Lissamine green staining. Other molecules had diverse correlations (Fig. 4): RANTES and TNF-α correlated positively with age; eotaxin correlated positively with age and OSDI questionnaire score; IFN-γ correlated positively with age and hyperemia; and IL-10 correlated positively with OSDI questionnaire score, hyperemia, and corneal fluorescein staining, and negatively with T-BUT.

Multivariate Predictive Modeling

Logistic regression for each of the cytokines, adjusted for age and sex, was fitted. Table 4 shows the estimated odds ratio values (with 95%CI) and AUC based on LOOCV procedure. Elevated levels of EGF and IP-10/CXCL10 were statistically identified as protective factors, while higher levels of IL-8/CXCL8 and IL-1Ra were identified as risk factors for ocular cGVHD development. The elevated level of IL-10 was a borderline risk factor. Classification ability of EGF, IL-1Ra, IL-2, IL-8/CXCL8, IP-10/CXCL10, RANTES, TNF-α, and VEGF was significant, showing AUC values statistically different from 0.5, which corresponds to random chance. An exhaustive search to select the best subset of cytokines for the final multivariate model was performed. The best models by number of molecules included in them, based on the lower value of the Akaike information criterion, are shown in Table 5. Model based on two cytokines, IL-8/CXCL8 and IP-10/CXCL10, along with age and sex, was identified as the optimal model. The estimated odds ratio values were 3.37 (95%CI: 1.24–9.17) and 0.23 (95%CI: 0.07–0.82) for IL-8/CXCL8 and IP-10/CXCL10, respectively.

Table 3. Percentage of Detection and Concentration of the 15 Molecules Analyzed in Tears of Ocular cGVHD Patients and Healthy Controls

	% Detection			Concentration, pg/mL		
Molecule	Healthy Controls	Ocular cGVHD Patients	*P* Value	Healthy Controls	Ocular cGVHD Patients	*P* Value
EGF	100	77.27	**0.0485**	2,154.71 ± 2,385.56	357.83 ± 527.95	**<0.0001**
Eotaxin	47.62	72.73	0.1703	69.95 ± 61.34	83.75 ± 60.95	0.1936
IFN-γ	47.62	77.27	0.09	28.24 ± 26.56	36.86 ± 24.31	0.1203
IL-1β	57.14	63.64	0.9018	15.71 ± 14.51	84.65 ± 299.02	0.3259
IL-1Ra	100	90.91	0.4884	9,384.52 ± 10,474.92	33,330.96 ± 26,982.06	**0.0007**
IL-2	71.43	45.45	0.1566	21.84 ± 17.79	20.16 ± 17.44	0.8093
IL-6	80.95	86.36	0.6981	51.44 ± 48.49	119.5 ± 117.4	0.1169
IL-8/CXCL8	95.24	100	0.4884	385.18 ± 401.72	7,131.18 ± 15,956.77	**0.0003**
IL-10	76.19	86.36	0.4566	28.16 ± 20.74	65.7 ± 82.99	**0.0253**
IL-12 p70	66.67	86.36	0.1623	49.12 ± 55.4	59.32 ± 53.44	0.1331
IL-17A	61.9	22.73	**0.0218**	20.89 ± 17.74	12.16 ± 12.42	0.1913
IP-10/CXCL10	100	90.91	0.4884	60,999.05 ± 56,159.34	10,511.33 ± 20,431.76	**<0.0001**
RANTES	80.95	86.36	0.6981	121.74 ± 108.6	120.55 ± 112.58	0.8126
TNF-α	66.67	72.73	0.92	20.2 ± 17.19	36.43 ± 74.89	0.3203
VEGF	61.9	81.82	0.2648	415.78 ± 347.08	578.01 ± 528.14	0.4575

Significant differences ($P < 0.05$) are denoted in bold.

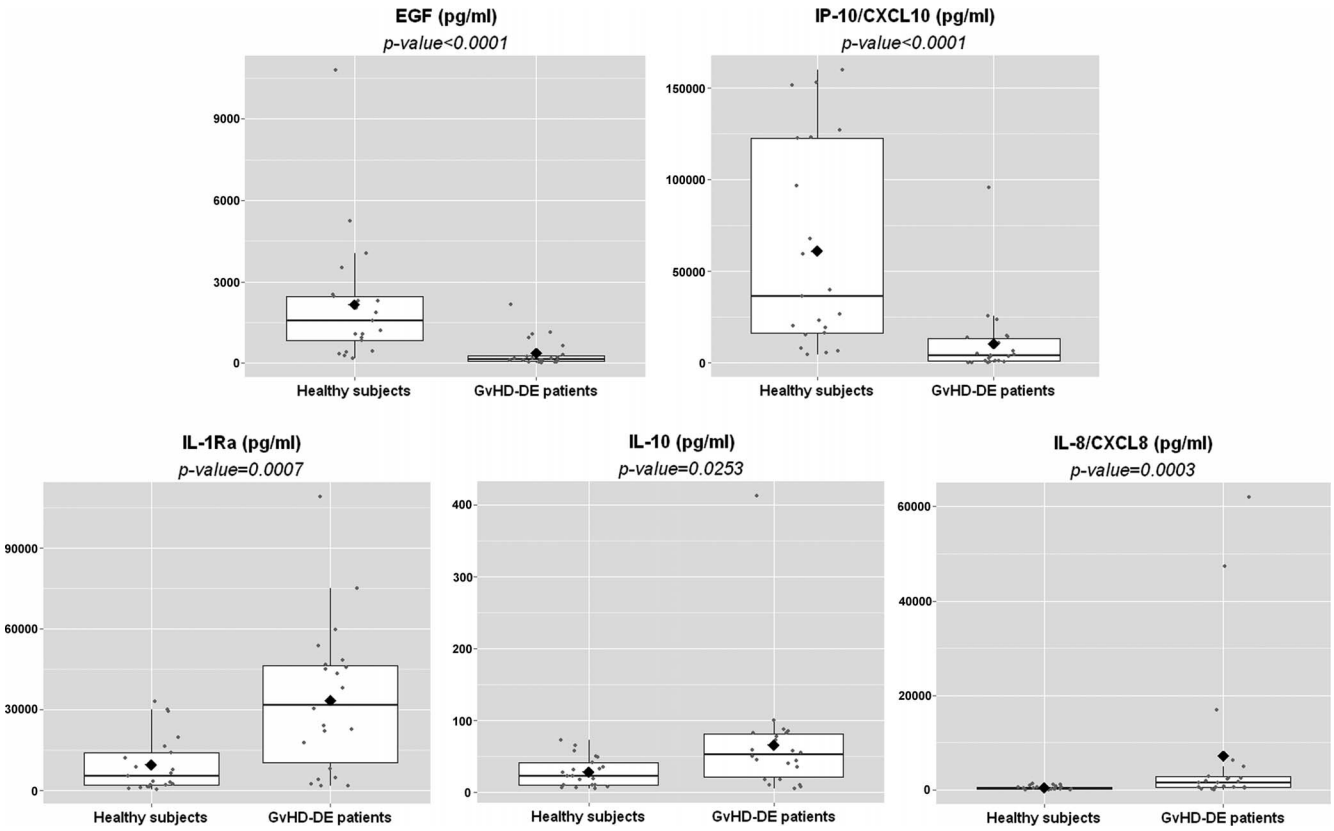

FIGURE 1. Molecules with significant differences in tear levels between ocular chronic graft versus host disease patients and healthy subjects. EGF and IP-10/CXCL10 tear levels were significantly decreased, whereas IL-1Ra, IL-8/CXCL8, and IL-10 levels were significantly increased in ocular cGVHD patients compared to healthy subjects. *Diamonds* represent the mean values. EGF, epidermal growth factor; GVHD, chronic graft versus host disease; IL, interleukin; IP, interferon inducible protein.

Internal Validation

To determine the validity of the IL-8/CXCL8- and IP-10/CXCL10-based model, internal validation by the LOOCV procedure was developed. The Brier score obtained corresponded to an accurate model (Table 6). Furthermore, the CL and CS measures, along with the Hosmer-Lemeshow test, indicated the absence of calibration problems. The model obtained an AUC of 0.9004, with a sensitivity of 86.36% and a specificity of 95.24% (Table 6).

Classification of an External Cohort Without Ocular cGVHD

In order to evaluate the capacity of the fitted model to properly classify individuals without ocular cGVHD, we tested it in cytokine tear levels from a cohort of 48 DE patients (14 severe Sjögren DE and 34 mild/moderate non-Sjögren-associated DE) and 32 healthy controls from previously published studies from our group.[20–22] Results showed that the IL-8/CXCL8- and IP-10/CXCL10-based predictive model (sex and age adjusted) had 100% specificity when classifying both controls and mild/moderate non-Sjögren-associated DE patients; Sjögren-associated DE patients were correctly classified as negative for ocular cGVHD in 78.57% of the cases (Table 7).

DISCUSSION

So far, little is known about the etiopathogenesis of ocular cGVHD, and this limits the emergence of objective and reliable diagnostic tests. In this study we intended to identify molecules that are specifically involved in the pathogenic mechanisms of this disease. To do this, we analyzed tear levels of a panel of inflammatory molecules in ocular cGVHD patients, and we then compared them to those in healthy subjects. Based on this information, we intended to design a tear biomarker-based predictive model that may facilitate the diagnosis of this disease. The final panel of inflammatory molecules included was defined after analysis of the most important candidate biomarkers, and logistic regression models were built with every possible combination of candidate molecules and age and sex as confounding variables.

Evaluation of the enrolled patients revealed that all of them suffered a moderate DE, as shown by the clinical test scores. Ocular cGVHD patients had significantly more DE symptoms, lower Schirmer test scores, and considerably decreased T-BUT values compared to healthy controls. The ocular surface integrity of patients' eyes, as evaluated by fluorescein and Lissamine green, was significantly altered compared to controls. However, the damage was not so severe as to prevent the patients from stopping their anti-inflammatory topical medications for 1 week as required by the inclusion criteria. Sampling patients with more severe disease could potentially have given different results, but then the confounding effects of topical medication upon inflammatory molecules could not have been excluded.

Tear cytokine analysis revealed that levels of EGF and IP-10/CXCL10 were significantly lower in tears of ocular cGVHD patients. Epidermal growth factor is secreted mainly by the lacrimal gland and is one of the most important growth factors present in human tears. Studies from Pflugfelder et al.[13] and Lam et al.[14] found lower levels in patients with Sjögren

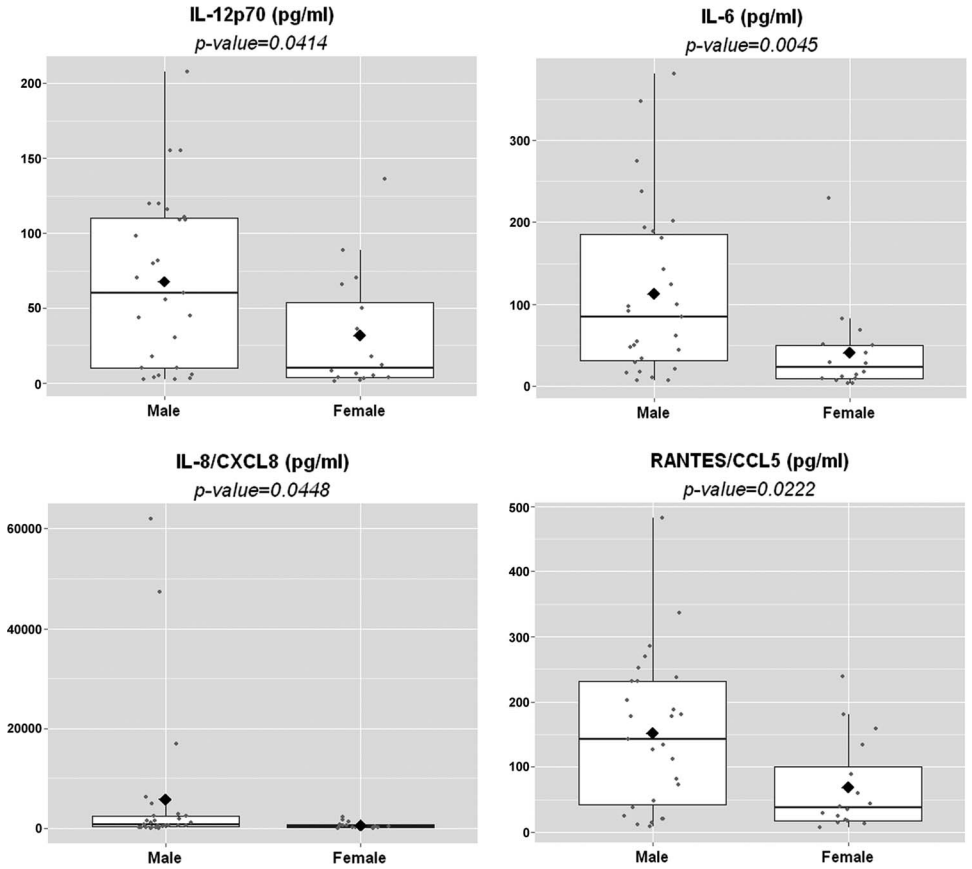

Figure 2. Association between sex and molecule tear levels. IL-12p70, IL-6, IL-8/CXCL8, and RANTES/CCL5 were found significantly increased in males compared to females for both patients and controls together. *Diamonds* represent the mean values. IL, interleukin; RANTES, regulated on activation normal T cell expressed and secreted.

syndrome-associated hyposecretory DE compared to healthy controls. The significant decrease in EGF tear levels found in our ocular cGVHD patients is in agreement with those studies, as cGVHD causes an aqueous-deficient Sjögren-like DE. Additionally, we found that epithelial growth factor receptor (*EGFR*) gene expression in conjunctival epithelial cells of ocular cGVHD patients decreased by 2.6 fold,[12] which confirms the importance of this finding.

We found that IP-10/CXCL10, a chemotactic molecule that attracts T-lymphocytes, natural killer cells, and monocytes,[29] was also decreased in tears of ocular cGVHD patients. Activated T cells lead to the release of the chemokines CXCL9, IP-10/CXCL10, and CXCL11 at tissue sites, and these recruit CXCR3+ T cells, mediating the tissue damage characteristic of GVHD.[30] The importance of the role of IP-10/CXCL10 in GVHD patients has been established by different authors. Using a murine model, Duffner et al.[31] concluded that the migration of donor T cells to GVHD target organs depends on the expression of CXCR3 and contributes significantly to GVHD damage and overall mortality. Meanwhile, Piper et al.[32] found significantly elevated serum levels of IP-10/CXCL10, suggesting that the specific interaction of this molecule with its receptor was critical for the recruitment of T cells to the skin in patients with acute GVHD. Moreover, Westekemper et al.[33] demonstrated by polymerase chain reaction that IP-10/CXCL10 was markedly upregulated in conjunctival biopsies of ocular cGVHD patients compared with healthy controls. The fact that this chemokine was decreased in our patients could be due to selection criteria applied, which allowed only patients whose GVHD was under relatively good control to be included.

On the other hand, we found that the levels of IL-1Ra, IL-8/CXCL8, and IL-10 were increased in tears of the ocular cGVHD group. This is consistent with previous reports of tear levels of these molecules in patients with DE.[13–15,34,35] Interleukin-1Ra is a naturally occurring cytokine receptor antagonist that serves as a modulator of immune response, regulating the agonist effects of IL-1 during chronic inflammatory diseases such as arthritis.[36] 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.[37]

Interleukin-8/CXCL8 is one of the major mediators of the inflammatory response, and patients suffering acute GVHD develop higher levels at times of maximal clinical signs.[38] More importantly, IL-8/CXCL8 is included in two biomarker panels that predicted death, treatment failure, and risk of developing GVHD.[18,39] In agreement with these results, previous results from our group have found that *IL-8/CXCL8* gene expression in conjunctival epithelium from ocular cGVHD patients was almost five times increased compared to healthy controls.[12] More studies are warranted to determine the exact role of IL-8/CXCL8 in ocular cGVHD, as it seems to play an important role in the pathogenesis.

Finally, IL-10 is an anti-inflammatory cytokine that reduces activation of T cells. A lack of this cytokine results in increased allogeneic T-cell responses and strongly aggravates the course of the disease.[40] Several groups have studied the association between increased IL-10 levels and GVHD, and in spite of its anti-inflammatory function, they have reported an association between increased IL-10 levels and GVHD.[41,42] Thus, it has

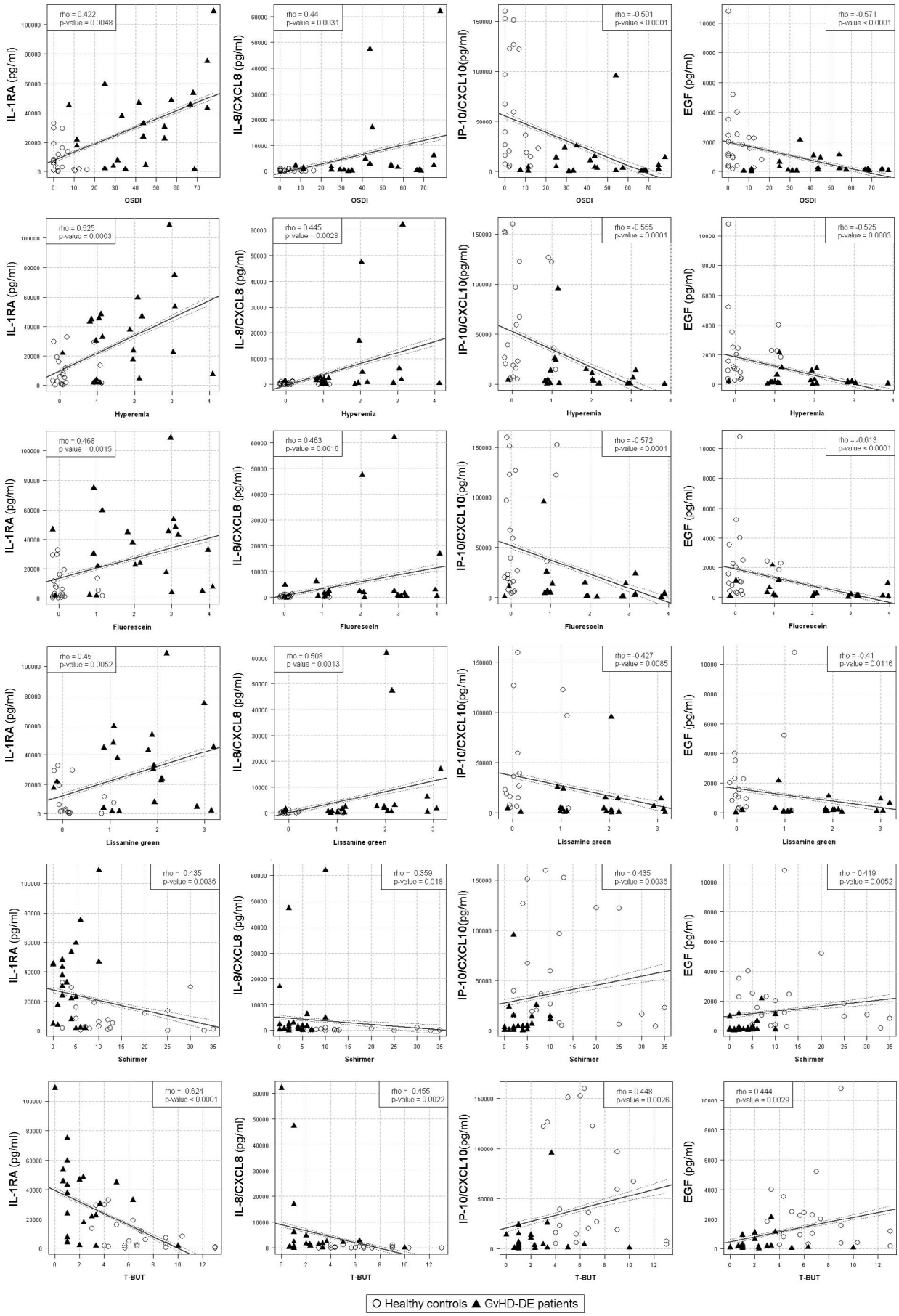

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Figure 3. Correlations between IL-1RA, IL-8/CXCL8, IP-10/CXCL10, and EGF tear levels and clinical parameters. Correlations were determined for the entire study population, both patients and controls together. ▲, ocular cGVHD patients; ○, control group subjects. Values of hyperemia, corneal fluorescein staining, and conjunctival Lissamine green staining have been jittered to reduce overplotting. *Dashed lines* represent the 95% confidence interval. cGVHD, chronic graft versus host disease; EGF, epidermal growth factor; IL, interleukin; IP, interferon inducible protein; ρ, Spearman ranked correlation coefficient.

been suggested that because this molecule is a strong suppressor of T-cell immunity, high levels of IL-10 might lead to functional immunodeficiency contributing to a poor disease prognosis.[10] These results are reinforced by a 7.7-fold increase in *IL-10* conjunctival gene expression in ocular cGVHD patients previously reported by our group.[12] Increased levels of IL-10 are also consistent with those present in tears of patients with Sjögren syndrome-associated DE.[35] Our results are, however, in disagreement with those reported by Riemens et al.,[16] who failed to detect IL-10 in tear levels of ocular cGVHD patients. Interestingly, in the same study, Riemens et al. found increased IFN-γ and IL-6 tear levels, both of which they proposed as key molecules for this disease. However, in our study, tear levels of these molecules in ocular cGVHD patients were not significantly different from those in controls. An explanation for these differences could be the different diagnostic criteria for ocular cGVHD (based on the National Institutes of Health consensus criteria) used in each study, a different tear sample collection method (obtained directly from Schirmer test strips in the study by Riemens et al.[16] instead of by the capillarity collection in ours), and the potential influence of topical medications used by patients in Riemens' study.

Increased IL-10 in tears of GVHD patients has also been recently described by Jung et al.[17] In this study, they investigated levels of IL-2, IL-4, IL-6, IL-10, IL-17A, INF-γ, and TNF-α in tears of patients after HSCT, trying to evaluate whether they are associated with the presence of systemic GVHD, regardless of ocular status. They found that IL-2, IL-10, IL-17A, INF-γ, IL-6, and TNF-α were elevated in patients with cGVHD compared to transplanted patients without cGVHD. While our results are in agreement regarding increased IL-10 in tears of cGVHD patients, we did not find significant increase in IL-2, IL-17A, INF-γ, IL-6, or TNF-α. An explanation for the

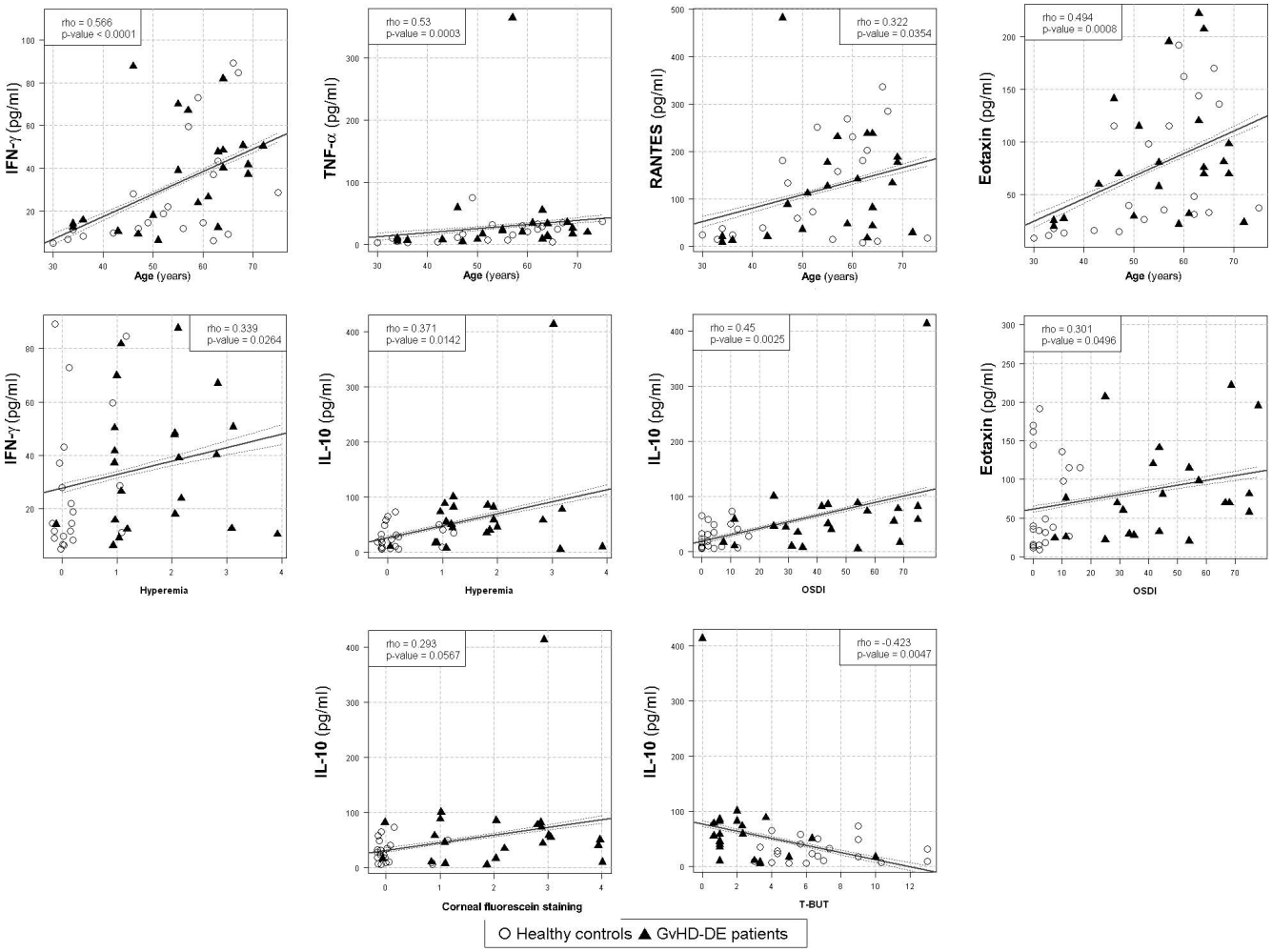

Figure 4. Other correlations between tear molecule levels and clinical parameters. Correlations were determined for the entire study population, both patients and controls together. ▲, ocular cGVHD patients; ○, control group subjects. Values of hyperemia, corneal fluorescein staining, and conjunctival Lissamine green staining have been jittered to reduce overplotting. *Dashed lines* represent the 95% confidence interval. cGVHD, chronic graft versus host disease; EGF, epidermal growth factor; IL, interleukin; IP, interferon inducible protein; ρ, Spearman ranked correlation coefficient.

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Table 4. Estimated Odds Ratio by Logistic Regression Models for Each of the Tear Molecules, Adjusted by Age and Sex

		95% CI for OR				95% CI for AUC	
Molecule	**OR**	**Lower**	**Upper**	***P* Value**	**AUC**	**Lower**	**Upper**
EGF	**0.3881**	**0.2302**	**0.6545**	**0.0004**	**0.8333**	**0.7073**	**0.9594**
Eotaxin	1.3538	0.7494	2.4453	0.3154	0.3961	0.2196	0.5726
IFN-γ	1.5635	0.7963	3.0697	0.1942	0.5455	0.3673	0.7236
IL-1β	1.1648	0.8254	1.6439	0.3854	0.6472	0.4792	0.8152
IL-1Ra	**1.7720**	**1.2091**	**2.5971**	**0.0034**	**0.7078**	**0.5476**	**0.868**
IL-2	0.8687	0.5246	1.4383	0.5842	**0.6861**	**0.5233**	**0.849**
IL-6	1.3057	0.8859	1.9243	0.1777	0.539	0.3596	0.7184
IL-8/CXCL8	**1.7878**	**1.2060**	**2.6501**	**0.0038**	**0.7446**	**0.5936**	**0.8956**
IL-10	1.6817	0.9967	2.8373	*0.0515*	0.5649	0.3847	0.7452
IL-12 p70	1.2188	0.8908	1.6676	0.2160	0.4156	0.2313	0.5999
IL-17A	0.6397	0.3834	1.0674	0.0872	0.4892	0.3083	0.6701
IP-10 /CXCL10	**0.4501**	**0.2766**	**0.7323**	**0.0013**	**0.8182**	**0.69**	**0.9464**
RANTES	0.9491	0.6275	1.4355	0.8044	**0.7424**	**0.5919**	**0.893**
TNF-α	1.1965	0.6958	2.0574	0.5166	**0.6905**	**0.5287**	**0.8523**
VEGF	1.1107	0.7542	1.6358	0.5951	**0.71**	**0.5538**	**0.8661**

EGF and IP-10/CXCL10 were identified as protective factors, while IL-8/CXCL8 and IL-1Ra were identified as risk factors for the development of disease. Significant results are denoted in bold. Borderline *P* values ($0.05 < P < 0.08$) are denoted in italics. AUCs for each of the single molecule models are shown. OR, odds ratio.

discrepancies between the results from the Jung study and ours might be the different sample of patients and controls analyzed, as the authors compared a cohort of transplanted patients with and without cGVHD regardless of their ocular surface status, while we compared patients certainly diagnosed with ocular cGVHD with healthy individuals. Also sample collecting methods were different, as they took tears after buffer washout, whereas we took basal tears by capillarity collection. Finally, tear molecule analysis and cytokine array kits were not exactly the same, as they used a cytometric array with a BD array (Becton, Dickinson and Company, BD Biosciences), while we used a Milliplex cytokine array with Luminex technology. All these methodological differences might be responsible for the differences found between the two studies.

In the present study EGF, IL-1Ra, IL-8/CXCL8, and IP-10/CXCL10 show significant individual diagnostic abilities (IL-10 is borderline), evidenced by their corresponding individual odds ratio and AUC values. Significant diagnostic abilities of IL-10 and IL-17A were also described by Jung et al.[17] However, we did not find significant results for IL-6 and TNF-α as described in their study.[17]

We also correlated cytokine tear levels and clinical tests results. There were significant correlations for EGF, IL-1Ra, IL-8/CXCL8, and IP-10/CXCL10 tear levels with clinical parameters. The majority of the correlations that we found were intuitive, such as that of IL-8/CXCL8 (proinflammatory) tear levels correlating with all of the clinical markers of disease severity. Additionally, EGF showed logical negative correlations with ocular surface damage tests. We found that IP-10/CXCL10 and IL-1Ra, correlated negatively with tests for ocular surface damage (fluorescein staining and Lissamine green) and positively with tear production (Schirmer test) or stability (T-BUT). These results suggest a response by the tissues that counteracts the damage. These findings are in agreement with those described in the literature, as several inflammatory cytokines have been correlated with clinical parameters in DE.[13,35,43] Additionally, our results agree with those found by Jung et al.[17] regarding correlation between IL-10 and OSDI score, hyperemia, corneal staining, and T-BUT; in contrast, we did not find significant correlations between IL-2, IL-6, IL-17A, IFN-γ, and TNF-α with clinical parameters as described by those authors.[17] This might be due to the fact that our correlation studies include a healthy nontransplanted group, while Jung et al.[17] studied only transplanted patients.

The aim of our study was to develop a tear cytokine-based predictive model for ocular cGVHD patients. Predictive models are developed to give health care providers objective estimates of risks of having a particular disease to assist them in the decision-making process.[44] A variety of such approaches have recently been used in an attempt to diagnose ocular cGVHD, including clinical parameters-based predictive models, such as

Table 5. Better Multivariate Logistic Regression Models of Ocular cGVHD by Size (Number of Variables)

	Age	**Sex**	**EGF**	**IL-10**	**IL-17A**	**IL-1Ra**	**IL-8/CXCL8**	**IP-10/CXCL10**	**AIC**
M0	✓	✓							64.64
M1	✓	✓	✓						49.90
M2	✓	✓					✓	✓	26.32
M3	✓	✓	✓				✓	✓	26.72
M4	✓	✓			✓	✓	✓	✓	26.50
M5	✓	✓		✓	✓	✓	✓	✓	28.27
M6	✓	✓	✓	✓	✓	✓	✓	✓	*

Variables included in the multivariate model are those that showed a *P* value less than 0.1 individually: EGF, IP-10/CXCL10, IL-17A, IL-8/CXCL8, and IL-10 IL-1Ra and confounding variables as age and sex. M0, M1, M2, M3, M4, M5, and M6 are models based on 0, 1, 2, 3, 4, 5, and 6 variables, respectively. The better model by size is that with the lower AIC; thus the M2 model (two cytokine levels IL-8/CXCL8 and IP-10/CXCL10) was the best. AIC: Akaike information criterion.

* The complete model does not converge; it is not a valid model.

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Table 6. Internal Validation of IL-8/CXCL8 and IP-10/CXCL10 Ocular cGVHD Predictive Model

Accuracy	Calibration			Discrimination		
Brier Score (95%CI)	Calibration in the Large (95%CI)	Calibration Slope (95%CI)	Hosmer-Lemeshow *P* Value	AUC (95%CI)	Sensitivity % (95%CI)	Specificity % (95%CI)
0.1018 (0.035, 0.1853)	−0.2918 (−1.2163, 0.6326)	0.7463 (0.4194, 1.0731)	0.3866	0.9004 (0.7936, 1)	86.36 (72.02, 100)	95.24 (86.13, 100)

The Brier score is a measure of accuracy. The calibration in the large, calibration slope and Hosmer-Lemeshow test are calibration measures. The AUC, sensitivity, and specificity are discrimination measures for the predictive models.

the National Institutes of Health Eye Score and Schirmer's test,[19] and a multiple conjunctival gene-based predictive model.[12]

Multiple biomarker–based models, built upon the simultaneous use of a group of biomarkers, may improve performance compared to the use of a single-molecule analysis for diagnosis issues. Some investigators have attempted to use proteomic approaches to identify candidate biomarkers in GVHD practice, with promising results.[18,45,46] Regarding ocular cGVHD, as mentioned above, our group has already proposed a biomarker-predictive model based on conjunctival epithelial *EGFR*, *IL-6*, *IL-9*, and *NAMPT* gene expression that showed very good sensitivity and specificity.[12] Although tear cytokines have been proposed by Jung et al.[17] as cGVHD biomarkers based on individual AUC and odds ratio values, to our current knowledge, no multiple biomarker–based predictive models have been described utilizing tears of ocular cGVHD patients. Thus, we intended to apply this technology to the accurate diagnosis of ocular complications of cGVHD; and the predictive model that resulted, based on the tear levels of IL-8/CXCL8 and IP-10/CXCL10, showed very good sensitivity (86.36%) and specificity (95.24%). As expected, discriminant capacity of this multiple biomarker model was higher than those models obtained by any single cytokine alone.

Technical resources available at the time of sample collection and analysis, compared to the previous method using impression cytology, make these results more valuable.[12] Both gene expression and tear cytokine level predictive models have shown to have good sensitivity and specificity, although the latter does not reach the results previously obtained (sensitivity of 100% and specificity of 92.9%).[12] A tear-based model has the advantage that tears are more accessible and that the handling is easier than with impression cytology, and tears can be obtained without the need for anesthesia, which would make it easier to implement in daily clinical practice.[47] Moreover, the advent of point-of-care diagnostic tests for measurement of molecule levels in tears provides a new opportunity to diagnose and monitor ocular cGVHD patients in everyday clinics. The current tear cytokine/chemokine model also provides complementary information about the processes studied. However, limitation in the amount of tear sample that can be obtained from these patients (sometimes even 1 μL is difficult to obtain) could be a downside of this predictive model compared to the gene based.

We did perform an internal validation of our predictive model, but it is known that this is not enough to demonstrate acceptable outcomes of a model on the initial sample only, being absolutely necessary to confirm that the model predicts well in a different subset of individuals.[44] In order to further generalize the discriminatory capability of our model we used an external cohort of patients with Sjögren DE, non-Sjögren DE, and healthy controls from previous studies of our group.[20–22] Classification results obtained by our two-molecule-based model seem very promising, as it identified most of these patients as negative for ocular cGVHD. However, the external validation cannot be considered complete yet, and it would be worth checking classifier performance using a new cGVHD sample with and without ocular involvement.

We want to emphasize that although these cytokines and chemokines are expected to be elevated in other inflammatory diseases of the ocular surface, and not to be exclusively increased in tears of ocular cGVHD patients, based on the very good preliminary classification results, we are convinced that the changes observed reflect the underlying origin of the DE disease.

There are a number of limitations in the present study. First of all, obtaining tear fluid from these severe DE patients who have very little in the way of tears is a challenge. Fortunately, bead-based arrays utilizing X-MAP technology overcome this limitation in sample amount. Also, the low number of ocular cGVHD patients recruited may be a limitation; but while currently there are relatively few patients who manifest the disease, the number is increasing. Additionally, the small number of patients recruited is the result of the inclusion criteria, which restricted the participation of severe and uncontrolled cases that were unable to discontinue their topical medication. Nevertheless, although the sample in this study was not big, it was large enough to establish levels of cytokines/chemokines in tears and serve as the basis for further studies. We are aware that the predictive model has to be further validated in a bigger cohort of patients with ocular cGVHD in order to confirm its sensitivity and that the results obtained so far are totally relevant.

Finally, one could think that systemic anti-inflammatory therapy used on these patients may influence cytokine levels in the tear film. We believe that this is possible, but unfortunately, the severity of the underlying disease made it impossible to

Table 7. Classification Performance of the Fitted Model in a Cohort of Sjögren and Non-Sjögren-Associated DE Patients and Healthy Controls

			Percentage of Well-Classified Individuals	
	Well Classified, *n*	Wrongly Classified, *n*	%	95%CI
Healthy controls	32	0	100	86.66, 100
Mild/moderate non-SS DE	34	0	100	87.36, 100
Severe SS DE	11	3	78.57	48.82, 94.29
Total	77	3	96.25	88.68, 99.03

SS, Sjögren syndrome; DE, Dry eye.

remove these medications and analyze tear cytokine and chemokine levels in untreated patients.

In conclusion, this study shows that ocular cGVHD patients presented different patterns of inflammatory molecules in tears compared to normal controls and other kinds of DE. This information adds further knowledge to the understanding of the molecular mechanisms underlying this disease. Additionally, the predictive model based on IL-8/CXCL8 and IP-10/CXCL10 tear levels showed good sensitivity and specificity results. Due to the accessibility of tear film, it may become a useful tool in daily clinical practice, as biomarkers are now considered a cornerstone for the diagnosis of several pathologies. Future directions include evaluation of these biomarkers with a larger number of samples collected in prospective studies that will facilitate the successful design of subsequent clinical trials.

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