

Correlation Between the Inflammatory Marker HLA-DR and Signs and Symptoms in Moderate to Severe Dry Eye Disease

Françoise Brignole-Baudouin,¹⁻³ Luisa Riancho,¹ Dahlia Ismail,⁴ Maëva Deniaud,⁵ Mourad Amrane,⁴ and Christophe Baudouin^{1,2,6}

¹Sorbonne Universités, UPMC Univ Paris 06, Institut de la Vision, Paris, France

²CHNO des Quinze-Vingts, DHU Sight Restore, Paris, France

³Sorbonne Paris Cité, Faculté de Pharmacie de Paris, Université Paris Descartes, Paris, France

⁴Santen SAS, Evry, France

⁵MDstat Consulting, Melun, France

⁶Ambroise Paré Hospital, APHP, DHU Sight Restore, University of Versailles Saint-Quentin en Yvelines, Versailles, France

Correspondence: Françoise Brignole-Baudouin, Institut de la Vision, 17 rue Moreau, Paris, F-75012, France; francoise.brignole@inserm.fr.

Submitted: January 27, 2015
Accepted: February 28, 2017

Citation: Brignole-Baudouin F, Riancho L, Ismail D, Deniaud M, Amrane M, Baudouin C. Correlation between the inflammatory marker HLA-DR and signs and symptoms in moderate to severe dry eye disease. *Invest Ophthalmol Vis Sci.* 2017;58:2438–2448. DOI:10.1167/iovs.15-16555

PURPOSE. To investigate correlations of the inflammatory HLA-DR marker with clinical signs and symptoms commonly used to assess dry eye disease (DED) severity.

METHODS. Baseline data were collected from three clinical studies conducted on moderate to severe DED patients. Characteristics of DED were analyzed and correlations were performed in 311 patients. Data were analyzed after treatment with 1 mg/mL cyclosporine (CsA) and vehicle. We quantified HLA-DR by flow cytometry in impression cytology specimens.

RESULTS. We found HLA-DR significantly increased with diagnosis of Sjögren syndrome ($P < 0.0001$) and meibomian gland disease ($P = 0.0223$). The strongest significant correlation was seen with the corneal fluorescein staining (CFS, $r = 0.30$, $P < 0.0001$). Significant negative relationships were also found with Schirmer's test ($r = -0.20$, $P = 0.0003$) and tear break-up time (TBUT, $r = -0.13$, $P = 0.0226$). Correlations were statistically significant with total Ocular Surface Disease Index and visual analog scale scores ($r = 0.12$, $P = 0.0426$, and $r = 0.14$, $P = 0.0176$, respectively). We found HLA-DR arbitrary units of fluorescence were statistically reduced after CsA treatment compared to vehicle ($P = 0.022$ and $P = 0.021$ in two studies).

CONCLUSIONS. In clinical research on DED, discrepancy is often observed between symptoms and signs. We found HLA-DR correlated significantly with CFS clinical signs and to a lower extent Schirmer's test and weakly with TBUT and symptom reporting questionnaires. HLA-DR was reported to be useful for monitoring anti-inflammatory efficacy treatments in DED, which was confirmed with the reduction of HLA-DR while on CsA treatment. Its expression by conjunctival cells has the potential to serve as a biomarker, bridging signs and symptoms in clinical research in DED, but there is still a need for additional validation studies.

Keywords: dry eye disease, HLA-DR, biomarker

Dry eye disease (DED) is a common ocular syndrome occurring in approximately 5 million Americans aged older than 50 years.¹ It is a complex disease affecting the lacrimal glands and ocular surface.² Inflammation is now recognized to play a key role in developing and maintaining chronic ocular dryness.^{3,4} Dysfunction of tear secretory glands causes changes in tear composition, and hyperosmolarity induces inflammatory cytokine production on the ocular surface.⁵⁻⁷ This cytokine-mediated inflammation can in turn cause loss of tear secretory cells and goblet cells, amplifying tear dysfunction and making DED a self-stimulating vicious circle.⁷⁻⁹ Using immunohistochemistry, lymphocytic infiltration was observed in the conjunctival substantia propria of Sjögren and non-Sjögren DED patients.¹⁰ This was associated with expression and upregulation of inflammatory markers, including the major histocompatibility complex class II antigen HLA-DR and intercellular adhesion molecule ICAM-1 on infiltrating lymphocytes and resident conjunctival epithelial cells. Increased HLA-

DR and ICAM-1 expressions were confirmed on conjunctival superficial epithelial cells in DED patients using impression cytology,¹¹ and was further confirmed by the development of new flow cytometry techniques.¹²⁻¹⁵ Besides the immune cell-mediated inflammatory process occurring in DED, hyperosmolarity stress has also been shown to induce HLA-DR overexpression in human conjunctival epithelial cells in DED patients and in vitro cell culture models.¹⁶ These data highlighted an upregulation of inflammatory HLA-DR marker expression in DED.

In 2007, the DED definition was modified by the Subcommittee of the International Dry Eye Workshop (DEWS) to include tear hyperosmolarity and ocular surface inflammation as contributing factors: "Dry eye is a multifactorial disease of the tears and ocular surface that results in symptoms of discomfort, visual disturbance, and tear film instability with potential damage to the ocular surface. It is accompanied by increased osmolarity of the tear film and inflammation of the



ocular surface.¹⁷ The subcommittee designed a 4-level scheme to assess DED severity, based simultaneously on symptoms with symptom-reporting questionnaires and signs with specific clinical tests.¹⁷ This strategy is also recommended by the Regulatory Authorities in ophthalmic clinical research. Patient-reported symptom questionnaires such as Ocular Surface Disease Index (OSDI) and visual analog scale (VAS) evaluate ocular discomfort, visual disturbance, and the need for tear replacement. Clinical DED signs are assessed with a range of diagnostic tests, including ocular surface staining, lid/meibomian glands assessment, tear flow (Schirmer's test), tear film break-up time (TBUT), and tear osmolarity.¹⁷ Furthermore, impression cytology/flow cytometry technique has emerged as a key and helpful method to investigate, diagnose, and monitor ocular surface inflammation in DED patients and has been successfully used as a research tool in several clinical studies assessing the efficacy of anti-inflammatory agents in DED.^{18,19} One large randomized clinical study assessing the efficacy of cyclosporine A (CsA) in treating DED used flow cytometric analysis to show a decrease in conjunctival epithelial cell HLA-DR marker expression in DED patients treated with 0.05% and 0.1% CsA, but not with vehicle.¹⁸ A significant decrease in interleukin-6 cytokine production and HLA-DR-expressing (i.e., activated) lymphocytes was also observed after treatment with 0.05% CsA.^{19,20} Thus, although the methodology is not yet fully standardized for clinical use, evaluation of HLA-DR expression by impression cytology is an effective and reliable method to assess conjunctival inflammation in DED patients in clinical research.¹⁸⁻²⁰ We used retrospective inclusion data from three major clinical studies involving moderate to severe DED patients to investigate potential HLA-DR marker correlations with ocular tests and symptom-reporting questionnaires commonly used in clinical research on DED.

MATERIALS AND METHODS

This study was a retrospective analysis of baseline characteristic data collected in patients with moderate to severe DED during three large, phase III, randomized clinical studies, known as the SICCANOVE, SANSIKA, and NOSIKA studies. In addition, HLA-DR data at month 1 and 6 were analyzed in the SANSIKA study and at month 6 in the SICCANOVE study, as well as the correlations between HLA-DR and signs and symptoms of DED at month 6.

Clinical Studies

Study design, treatments, duration, primary and secondary objectives, and primary and secondary efficacy and safety criteria are all described in Table 1 and summarized below. Full description of the main inclusion and exclusion criteria is presented in Supplementary Table S1.

SICCANOVE Study. This was a phase III, multicenter, randomized, controlled, double-masked study aimed at demonstrating the superiority and comparing the ocular tolerance and systemic safety of ophthalmic cationic CsA 0.1% emulsion versus vehicle administered once daily in patients with moderate to severe DED after a 6-month treatment period. This study conducted in 61 centers in 6 European countries (France, Germany, Italy, the Czech Republic, Spain, and the United Kingdom) was registered under EudraCT number 2007-000029-23. Patients included had moderate to severe DED at baseline, persisting despite conventional management with all the following: one or more dry eye symptoms with a score equal or higher than 2 (maximum 5); TBUT \leq 8 seconds; corneal fluorescein staining (CFS) score from 2 to 4 on the modified Oxford scale; from 2 mm/5 minutes to 10 mm/5

minutes on the Schirmer's test; and Lissamine green staining score \geq 4 (maximum 9).

SANSIKA Study. This was a 6-month treatment period, multicenter, randomized, double-masked, vehicle-controlled, parallel group phase III study, followed by a 6-month, open label follow-up period. The main efficacy objective was to demonstrate the superiority of 1 mg/mL CsA eye drop emulsion over vehicle in simultaneously improving signs and symptoms in severe DED patients. The safety objectives were to evaluate the ocular tolerability and overall safety of 1 mg/mL CsA administered once daily over 12 months at month 6 and month 12. This study was performed in 50 centers across 9 European countries (France, Germany, Italy, Spain, Belgium, the United Kingdom, Sweden, Austria, and the Czech Republic) and was registered under EudraCT number 2011-000160-97. Included patients had persistent severe DED with all the following: CFS score of 4, from 2 to 10 mm/5 minutes on Schirmer's test, and an OSDI score \geq 23.

NOSIKA Study. This was a 3-month treatment period, multicenter, randomized, double-masked, parallel group, controlled, phase III study, aiming at comparing the ocular efficacy of Cationorm with Vismed in moderate to severe DED patients with keratoconjunctivitis sicca. It also aimed to assess the ocular tolerance and safety of Cationorm. This study, conducted in 19 centers in France, was registered under ANSM number 2011-A00955-36. Included patients used artificial tears for DED treatment for at least 3 months, experienced at least 2 symptoms of ocular discomfort (with a VAS score equal or higher than 23 mm) and had the following signs of DED-related keratitis/keratoconjunctivitis: \leq 4 ocular surface staining score \leq 9 (for a maximum score of 15 on the modified Oxford scale); and a sum of 3 TBUT \leq 30 seconds; or 3 to 9 mm/5 minutes on Schirmer's test.

Institutional review board and/or ethics committee approvals were obtained for each participating center and informed consents were obtained from each patient prior to enrollment in the respective studies. These studies were conducted in accordance with the approved protocols and amendments, all relevant local laws, regulations and guidelines, in accordance with the International Conference on Harmonization, Good Clinical Practice guidelines and with the ethical principles defined by the Declaration of Helsinki.

DED Assessments

For each patient, demographic information (including sex, age, and menopause status); patient's medical histories (e.g., daily artificial tear use, diagnosis of Sjögren disease, meibomian gland disease [MGD] status and age at/time since DED diagnosis); and use of systemic immunosuppressive treatments were collected at baseline. We assessed DED disease severity and characteristics in the eligible eye (Table 1) with 6 commonly used tests to evaluate DED signs and symptoms. Briefly, the CFS was scored using a modified grading Oxford scale (a 7-grade scale, scored 0, 0.5, and 1-5).²¹ The Schirmer's test without anesthesia measured the tear front after 5 minutes on a strip inserted in the lower conjunctival sac over the temporal one-third of the lower eyelid margin.²¹ After instillation of a fluorescein-containing solution, the TBUT was measured by recording the time between the last blink and the formation of lacunae on the ocular tear film.²¹ Tear film osmolarity, an indicator of the composition and stability of the tear film structure, was measured using a commercial osmolarity system (TearLab; TearLab Corp., San Diego, CA, USA)²² and was only performed in study centers equipped with this system. The reported normal range for osmolarity was 275 to 308 mOsm/L, with values above 308 mOsm/L indicating dry eye.²² The Ocular Surface Disease Index is a

TABLE 1. Summary of the Study Designs*

Characteristics of the Study	SICCANOVE Study	SANSIKA Study	NOSIKA Study
Title	A phase III, Multicenter, Randomized, Controlled, Double-Masked Trial of NOVA22007 (Ciclosporin 0.1%) Ophthalmic Cationic Emulsion Versus Vehicle In Patients with Moderate to Severe Dry Eye Syndrome	A Multicenter, Randomized, Double-Masked, 2 Parallel Arm, Vehicle-Controlled, 6-Month Phase III Trial with a 6-Month Open Label Treatment Safety Follow-Up Period to Evaluate the Efficacy and Safety of Cyclokot 1 mg/mL (Ciclosporin/ Cyclosporine) Eye Drops, Emulsion Administered Once Daily in Adult Patients With Severe DED	A Prospective, Multicenter, Randomized, Single-Masked, Parallel Group, Reference Controlled, 3-month Study to Compare the Efficacy, Tolerance and Safety of Cationorm, a Preservative-Free Cationic Ophthalmic Emulsion, with Vismed, a Hyaluronate Sodium Solution in Patients with Moderate to Severe DED with Keratitis or Keratoconjunctivitis
Study design	Phase III, multicenter, randomized, double-masked, parallel group, controlled study	6-month, randomized, multicenter, double-masked, vehicle-controlled, parallel group phase III study with a 6-month open label extension follow-up period	Clinical phase IIIB, prospective, multicenter, randomized, single masked, 2 parallel groups, reference controlled study
Treatment duration	6 months	12 months (6 months randomized, double-masked treatment period and 6 months open label follow-up period)	3 months
Primary objectives	To demonstrate the superiority of NOVA22007 (CsA 0.1%) ophthalmic cationic emulsion, administered once daily versus vehicle in patients with moderate to severe dry eye syndrome after a 6-month treatment period	To demonstrate the superiority of NOVA22007 1 mg/mL (CsA) eye drop emulsion over vehicle in simultaneously improving signs and symptoms in severe DED patients following 6 months of treatment	To assess the ocular efficacy of Cationorm versus Vismed as reference product in patients with moderate to severe DED with keratitis or keratoconjunctivitis after a 1-month treatment period
Secondary objectives	To compare the ocular tolerance and systemic safety of NOVA22007 (CsA 0.1%) ophthalmic cationic emulsion versus vehicle administered once daily in patients with moderate to severe dry eye syndrome after a 6-month treatment period	To evaluate the ocular tolerability and overall ocular safety of NOVA22007 administered once daily in patients with severe DED at 2 time points: at month 6, after the randomized, double-masked treatment period and at month 12, after open label follow-up period	To assess the ocular tolerance and safety of Cationorm
Treatment	<ul style="list-style-type: none"> NOVA22007 0.1% CsA Vehicle 0% CsA 	<ul style="list-style-type: none"> NOVA22007 1 mg/mL Matched vehicle 	<ul style="list-style-type: none"> Cationorm Vismed
Primary efficacy criteria†	<ul style="list-style-type: none"> CFS Patient's global score of all ocular discomfort symptoms using a VAS ranging from 0%–100% 	<ul style="list-style-type: none"> % CFS-OSDI responders (i.e., patients with an improvement of 2 points or more from baseline in CFS and an improvement by 30% or more from baseline in OSDI score) at month 6 	<ul style="list-style-type: none"> OSS score at month 1
Secondary efficacy criteria‡	<ul style="list-style-type: none"> % complete responder for CFS (i.e., patients with a CFS = 0) % VAS responders (i.e., patients with a decrease of 25% versus vehicle on VAS) Lissamine green staining of interpalpebral conjunctiva Patient's individual score for each ocular discomfort symptom using a VAS ranging from 0%–100% Schirmer's test TBUT OSDI questionnaire Investigator's global evaluation Concomitant artificial tear usage 	<ul style="list-style-type: none"> % CFS responders (i.e., patients with 2 points or more improvement of CFS) at month 6 % OSDI responders (i.e., patients with an improvement by 30% or more in OSDI score) at month 6 % VAS responders (i.e., patients with an improvement of 30% or more from baseline in ocular discomfort VAS score) at month 6 % CFS–VAS responders (i.e., CFS AND VAS responders) at month 6 % CFS-OSDI responders (i.e., CFS AND OSDI responders) at months 1 and 3 CFS, OSDI, VAS, Lissamine green total scores at months 1, 3, and 6 	<ul style="list-style-type: none"> OSS score at month 3 CFS clearing (i.e., CFS score = 0) at day 7 and months 1 and 3 Symptoms of ocular discomfort: VAS scores at day 7, and months 1 and 3; Schirmer's test and TBUT at months 1 and 3 Investigator global evaluation at day 7, and months 1 and 3 Tear film osmolarity at day 7, and months 1 and 3 QoL NEI-VFQ questionnaire at month 3 Conjunctival cytology impression

TABLE 1. Continued

Characteristics of the Study	SICCANOVE Study	SANSIKA Study	NOSIKA Study
Safety criteria	<ul style="list-style-type: none"> Local ocular tolerance: ocular symptoms related to study medication instillation and slit lamp examination Safety evaluation: BCDVA, IOP, incidence of ocular and systemic AEs, vital signs, CsA blood level and impression cytology 	<ul style="list-style-type: none"> Schirmer's test score, complete corneal clearing, investigator global evaluation of efficacy, TBUT, and QoL questionnaire (NEI-VFQ/EQ-5D) scores at month 6 Impression cytology for conjunctival cell surface HLA-DR expression, tear film osmolarity at months 1 and 6 Artificial tear use Local ocular tolerance assessed by slit lamp examination Incidence and severity of ocular and systemic AEs BCDVA at months 1, 3 and 6 IOP at months 1 and 6 Vital signs and blood sampling for CsA level at month 6 	<ul style="list-style-type: none"> Ocular and systemic AEs Ocular tolerance: slit lamp examination, BCDVA and IOP

AE, adverse event; BCDVA, best-corrected distance visual acuity; EQ-5D, EuroQoL-5D; IOP, intraocular pressure; NEI-VFQ, National Eye Institute-Visual Function Questionnaire; OSS, ocular surface staining; QoL, quality of life.

* The CSF score was graded according to the modified Oxford scale, the Schirmer's test was performed without anesthesia and the Lissamine green staining was assessed according to the Van Bijsterveld scale. The symptoms of ocular discomfort included itching, eye dryness, sticky feeling, photophobia, pain, burning or stinging, sandy feeling or grittiness or foreign body sensation. The OSS score corresponded to the sum of the nasal and temporal interpalpebral conjunctival and corneal vital staining using the modified Oxford scale.

† Assessed in the eligible eye. In the SICCANOVE study, the eligible eye was defined as the eye with the highest CFS score at baseline. If both eyes had the same score, the right eye was selected. In the SANSIKA study, the eligible eye was defined as the eye with the highest Lissamine green staining score at baseline. If both eyes had the same score, the eye with the worse Schirmer's test score was used. If both eyes had the same Schirmer's test score, then the right eye was selected. In the NOSIKA study, the eligible eye was defined as the eye with the higher OSS score at baseline. If both eyes had the same OSS score, the eye with the worst Schirmer's test score was used. If both eyes had the same Schirmer's test score, then the right eye was selected.

questionnaire with 12 questions commonly recommended by regulatory authorities and used to measure and assess the severity of DED symptoms in DED patients.²³ The visual analog scale ranging from 0% to 100% was used to assess 8 main symptoms of ocular discomfort including burning/stinging, itching, foreign body sensation, blurred vision, eye dryness, photophobia, pain, and sticky feeling.

Flow Cytometry

Conjunctival impression cytology was planned to be performed for 70 patients in selected sites at baseline and the final study visit in the SICCANOVE study (Table 1) for all patients at baseline, months 1, 6, and 12 in the SANSIKA study, and for selected sites at baseline only in the NOSIKA study. All samples were analyzed at the Vision Institute in Paris, France.

Cells collected from the superior and superotemporal bulbar conjunctiva areas using two half-polyether sulfone (PES) membranes (Supor 100, 13-mm diameter, pore 2 μ m; Gelman, Pall Sciences, Saint Germain en Laye, France). They were immediately placed into a tube containing 0.05% paraformaldehyde in PBS and sent with temperature control for flow cytometry analysis. When received, the samples were stored at 4°C in a fridge before analysis, and they were processed as soon as possible. Cells were extracted from the membranes, then subjected to indirect immunolabeling for the HLA-DR antigen and analyzed using a flow cytometer (Cytomics FC500 MCL; Beckman Coulter) according to previously published methods.^{11,13,15} Two parameters, both representative of the HLA-DR expression on the cell surface, could be determined from flow cytometry-derived histograms: the percentage of HLA-DR-positive cells (HLA-DR %) and the quantified fluorescence intensity. The latter was derived from

the HLA-DR mean fluorescence intensities (MFI) converted into arbitrary units of fluorescence (AUF) using fluorescence-calibrated beads (QIFIKIT; Dako Denmark A/S, Glostrup, Denmark); this method, in addition to quantifying the fluorescence, allowed standardization of results during the studies. Percentage of positive cells is a standard parameter in flow cytometry, but in a homogeneously positive cell population, like in DED where a marked inflammation is observed,¹³ it cannot discriminate a population with a high fluorescence expression from another with a lower expression. This means that whenever HLA-DR % reaches a maximum threshold, it may not be the most appropriate parameter to study severe DED patients. Therefore, in our studies, the main focus was on HLA-DR-AUF as the main parameter, as the expression values may not be limited in their maximum levels. In addition, results obtained for HLA-DR % are provided as supplemental data (Supplementary Table S2, S3).

Statistical Analyses

Potential relationships between cytologic HLA-DR parameters (HLA-DR % and HLA-DR-AUF) and demographic/baseline characteristics of moderate to severe DED patients included in the three studies were analyzed using statistical software (SAS, version 9.2; SAS Institute, Inc., Cary, NC, USA). For the qualitative variables including sex, menopausal status, Sjögren status, MGD status, use of immunosuppressive treatments, osmolarity class (i.e., above or below the 308 mOsm/L threshold) and CFS score, descriptive analyses of HLA-DR parameters including median, minimum, and maximum were performed by category, and median test was provided when relevant. For quantitative variables including age, age at/time since DED diagnosis, number of artificial tears used daily, CFS,

TABLE 2. Descriptive Statistics for HLA-DR-AUF Parameter in the Overall Analyzed Population at Baseline

Fluorescence (AUF)	N	Median	Minimum-Maximum	P Value*
Sex				0.1767
Male	51	46,928	6,575-381,294	
Female	260	54,457	1,607-504,052	
Confirmed menopause				0.1730
No	74	45,394	1,607-316,229	
Yes	186	58,152	1,688-504,052	
Presence of Sjögren syndrome				<0.0001
No	215	45,324	1,607-381,294	
Yes	96	71,782	8,667-504,052	
IS ongoing				<0.0001
No	284	48,783	1,607-504,052	
Yes	27	95,377	13,520-412,454	
Meibomian gland disease				0.0223
None	145	55,524	1,607-504,052	
Mild	104	44,853	3,837-477,068	
Moderate	48	65,734	4,225-359,049	
Severe	13	79,265	13,109-201,224	
Osmolarity class				0.5056
<308 mOsm/L	35	66,056	13,937-504,052	
≥308 mOsm/L	47	71,089	11,943-341,638	
CFS score†				<0.0001
1	13	35,738	13,062-157,039	
2	64	32,330	1,688-284,641	
3	39	48,558	4,225-166,773	
4	195	66,371	1,607-504,052	

N, number of patients with available data (maximum of 311 patients); ND, not determined.

* Based on median two-sample test or median one-way analysis.

† The CFS score was based on the modified Oxford scale.

Schirmer's test score, TBUT, osmolarity, OSDI score, and VAS score, scatter plots and Spearman correlation coefficients were used to explore correlation with HLA-DR parameters. In order to exclude a possible confusion effect of age or disease duration at month 6, we also performed a Spearman partial correlation of HLA-DR-AUF with each parameter of interest, adjusted on age or disease duration. A value of $P < 0.05$ was considered to be statistically significant. Nonparametric methods and statistics were preferred according to the skewed distribution of the HLA-DR parameter.

RESULTS

Study Population

A total of 818 patients with moderate to severe DED were included in our retrospective analysis: 488 from the SICCANOVE study, 245 from the first 6-month period of the SANSIKA study, and 85 from the NOSIKA study. Flow cytometry/cytology impression data at baseline were thus available for 311 patients: 87 in the SICCANOVE study (only on selected sites); 183 in the SANSIKA study; and 41 in the NOSIKA study (only on selected sites).

Statistical Results

Population Characteristics. Results of HLA-DR-AUF determined by flow cytometry according to patients' demographic/disease characteristics at baseline are presented in

Table 2. About 70% of the samples were analyzed within 8 days and about 25% within 15 days; the remaining samples were not considered for the analyses since they were received too late.

No significant difference on the HLA-DR-AUF parameter was observed according to sex ($P = 0.1767$) or menopausal status ($P = 0.1730$). Male and female patients had median HLA-DR-AUF values of 46,928 and 54,457, respectively, and females with and without confirmed menopause had median HLA-DR-AUF values of 58,152 and 45,394, respectively (Table 2). Dry eye disease patients with Sjögren syndrome had higher median HLA-DR-AUF values than other DED patients (71,782 versus 45,324, respectively, $P < 0.0001$), indicating that a diagnosis of Sjögren syndrome concomitant to DED was linked to HLA-DR expression (Table 2). An increase in the HLA-DR-AUF median values was observed with increasing MGD severity. Patients with mild, moderate, and severe MGD had respective median HLA-DR-AUF values of 44,853, 65,734, and 79,265, respectively ($P = 0.0223$, Table 2). A higher HLA-DR-AUF median value was observed in patients treated with immunosuppressive agents (IS) compared to patients without IS (95,377 versus 48,783, respectively, $P < 0.0001$, Table 2). Although not significant ($P = 0.5056$), a higher HLA-DR-AUF median value was observed for the osmolarity class ≥ 308 mOsm/L compared to the osmolarity class < 308 mOsm/L (71,089 versus 66,056, respectively; Table 2). A significant increase of the CFS score was observed on the median HLA-DR-AUF values: median HLA-DR-AUF values increased from 32,330 to 66,371 when CFS scores increased from 2 to 4 ($P < 0.0001$; Table 2).

A statistical model was done to clarify if there was a center effect. The value of P was 0.09, meaning that there was maybe a small site effect (not statistically significant). We could consider that this result could not influence the data because the study compared changes from baseline in individual patients.

Correlation Analyses

Spearman correlation coefficients (r) with their respective level of significance (P value) at baseline are presented in Table 3 for the HLA-DR-AUF parameter.

Similar statistically significant correlations of HLA-DR-AUF were observed with age ($r = 0.17$, $P = 0.0023$) and with time since DED diagnosis ($r = 0.16$, $P = 0.0049$). However, there was no significant relationship of HLA-DR-AUF with the mean number of artificial tears used daily ($r = 0.12$, $P = 0.0717$); with MGD status ($r = 0.03$, $P = 0.5985$) or with age at time of diagnosis ($r = 0.10$, $P = 0.0949$; Table 3). A weak but statistically significant correlation was observed with the total OSDI ($r = 0.12$, $P = 0.0426$) and VAS scores ($r = 0.14$, $P = 0.0176$). The strongest significant correlation with $r = 0.30$ ($P < 0.0001$) was observed between HLA-DR-AUF and the CFS score (Fig. 1). We found HLA-DR-AUF significantly correlated negatively with Schirmer's test ($r = -0.20$, $P = 0.0003$; and TBUT [$r = -0.13$, $P = 0.0226$]). However, no significant relationship could be seen between HLA-DR-AUF and the osmolarity ($r = 0.08$, $P = 0.4987$).

Results After CsA Treatment

In the SICCANOVE study, a reduction of HLA-DR-AUF was observed at month 6 in favor of 1 mg/mL CsA emulsion ($P = 0.022$, calculated post hoc; Fig. 2). In the SANSIKA study, a statistically significant reduction in the HLA-DR-AUF was observed at month 1 and month 6 in favor of 1mg/mL CsA emulsion ($P = 0.019$ and $P = 0.021$, respectively; Fig. 3).

TABLE 3. Spearman Correlation for HLA-DR-AUF Parameter in the Overall Analyzed Population at Baseline

Criteria	N	Mean	SD	Median	Min-Max	r	P Value
HLA-DR fluorescence, AUF	311	76,446	77,758	53,331	1,607-504,052		
Age, y	311	59.43	12.94	60.00	20.00-90.00	0.17	0.0023
MGD status score	311	0.81	0.93	1.00	0.00-3.00	0.03	0.5985
Time since DED diagnosis, y	311	8.17	7.39	6.06	0-64.11	0.16	0.0049
Age at DED diagnosis, y	311	51.20	13.85	52.56	7.92-88.10	0.10	0.0949
Mean daily number of artificial tears	311	7.88	8.75	6.00	0-91.33	0.12	0.0717
CFS score*	311	3.07	0.90	3.00	0.5-5.0	0.30	<0.0001
Schirmer's test, mm/5 min	311	4.55	3.06	4.00	0-30.00	-0.20	0.0003
TBUT, s	311	3.95	2.06	3.50	0-14.00	-0.13	0.0226
Osmolarity, mOsm/L	130	311.45	20.27	309.00	275.00-385.00	0.08	0.4987
Total OSDI score	311	48.91	22.50	50.00	0-100	0.12	0.0426
VAS score	311	47.50	20.06	46.63	0-98.00	0.14	0.0176

Correlation was determined between the HLA-DR-AUF parameter and all the following factors: age, mean daily number of artificial tears, CFS score, time since DED diagnosis, total OSDI score, VAS score, Schirmer's test, TBUT, and osmolarity. The corresponding Spearman correlation coefficient (r) and statistical significance (P value) are presented.

* The CFS score was based on the modified Oxford scale.

Correlation Analyses

Spearman correlation coefficients (r) with their respective level of significance (P value) at month 6 are presented in Table 4 for the HLA-DR-AUF parameter. The results of the Spearman partial correlations of HLA-DR-AUF with each parameter were almost not modified after adjusting on age, excluding a confusion effect of age in the significant correlations between HLA-DR-AUF and the signs and symptoms parameters. Results adjusted on disease duration (not shown) gave exactly the same conclusion.

DISCUSSION

The causes of DED are intricate and multiple factors such as age, hormones, immune status, nutrition, viruses/bacteria, and environmental conditions may all play a large and variable part in the alteration of the ocular surface system.^{24,25} Because of this complexity, there is increasing evidence to prove that DED symptomatology can be inconsistent with the clinical signs of the disease (Lemp M, et al. IOVS 2011;52:ARVO E-Abstract 3821).²⁶⁻²⁸ Some discrepancy also comes from the lack of test sensitivity, repeatability, and reproducibility.²¹ A recent study has shown that no correlation above 0.17 was seen between

DED symptoms and commonly used clinical tests, and among patients who showed evidence of DED by consensus of clinical signs, only 57% reported symptoms consistent with a diagnosis of DED.²⁹

We investigated the correlation of HLA-DR-AUF with both symptom-reporting questionnaires and clinical tests commonly used in DED assessment and clinical trials. We found significant correlations of similar strength between HLA-DR-AUF/age and HLA-DR-AUF/time since DED diagnosis (r = 0.17 and 0.16, respectively). These relationships may be explained by the length of time patients suffered from DED, although the absence of non-DED groups did not allow evaluation of the effect of aging on HLA-DR expression in normal eyes. Nevertheless, high HLA-DR expression levels were more likely to be seen in elderly patients who had been diagnosed with DED the longest. Interestingly, in these studies patients treated with systemic immunosuppressive agents presented a higher HLA-DR-AUF median value at baseline despite the use of immunosuppressants. This result is consistent with studies showing that immunosuppressive agents taken systemically are not efficient in the treatment of dry eye to reduce ocular inflammation, even if there is evidence that in patients after bone marrow transplant, ocular inflammation and dry eye increase after reducing systemic immunosuppression.³⁰

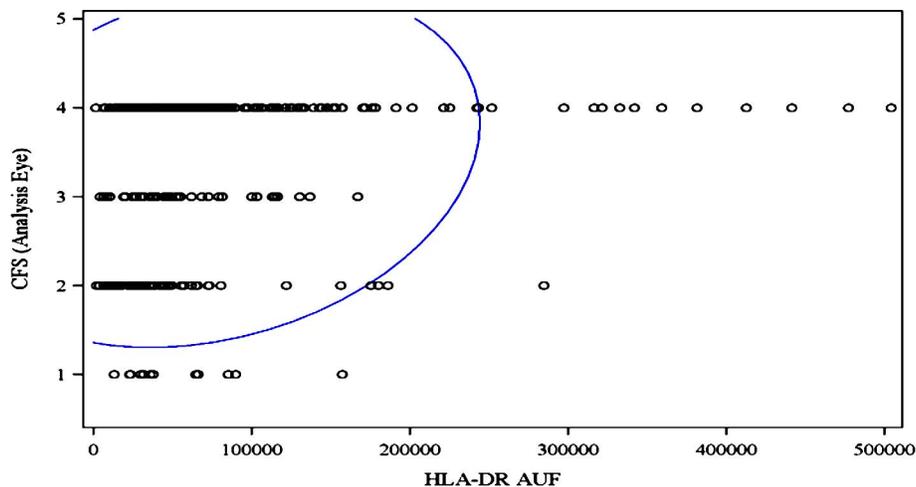


FIGURE 1. Correlation scatter plots of HLA-DR-AUF with CFS score at baseline. The 90% prediction ellipse is shown in blue and the Spearman correlation coefficients of HLA-DR-AUF were r = 0.30 (P < 0.0001) with CFS score.

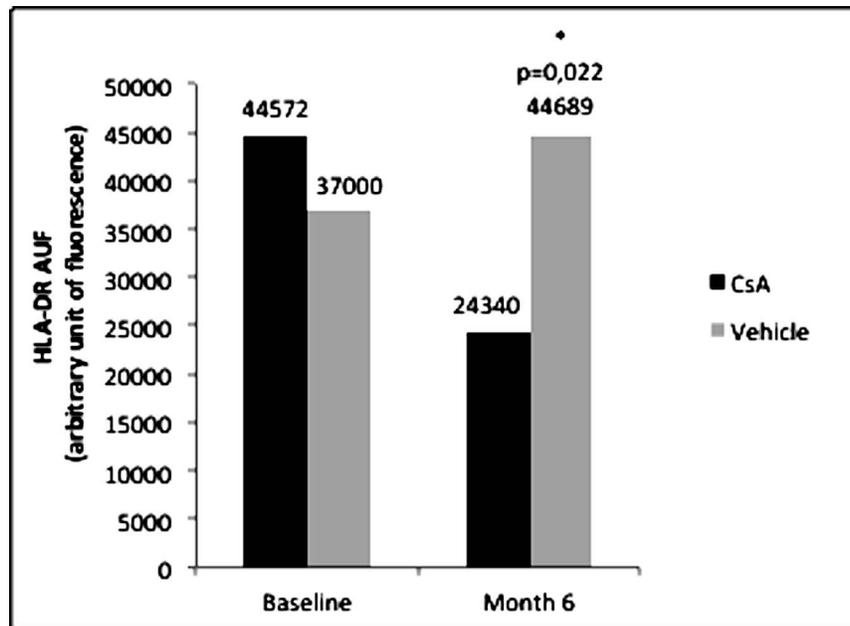


FIGURE 2. SICCANOVE Study (in a subgroup of patients). Evolution of HLA-DR between baseline and month 6. Sample size at baseline and month 6: 41 and 30 in CsA group and 48 and 36 in vehicle group. Value of *P* calculated post hoc. *P* < 5% (test between groups of treatment).

No significant concordance of HLA-DR-AUF was found with the daily use of artificial tears. This may be explained by the fact that worsening DED is associated with development of relative corneal anesthesia,³¹ increased reflex mechanisms,³² and a decrease/loss in corneal sensitivity³³; changes that would lead to stabilization of the need for artificial tears. Interestingly, HLA-DR % was found to significantly correlate with the daily use of artificial tears ($r=0.18$, $P=0.009$, Supplementary Table S3); both HLA-DR % and the daily number of artificial tears are reliable variables for milder DED and tend to saturate in the most severe forms of dry eye. Indeed HLA-DR % in severely affected patients may not reach values over 100%, whereas the level of expression by epithelial cells may continue to progress in severe cases. HLA-DR-AUF has a much wider range of

variation and appears to be a more useful marker than the single percentage of positive cells, which explains why AUF expression was found correlating much more often with clinical criteria than did HLA-DR percentages.

We found HLA-DR-AUF increased with a diagnosis of Sjögren syndrome or mild to severe forms of MGD, concomitant with DED. It also increased in parallel with CFS scores ranging from 2 to 4, which corresponded to the most severe forms of DED. Inconsistencies between individual tests and consensus-based severity were found to be common with mild to moderate DED forms.²⁹

The strongest significant correlation was observed between HLA-DR-AUF (and also %) and the CFS score ($r=0.30$ and 0.26 , respectively): CFS is an indicator of ocular surface damage. We

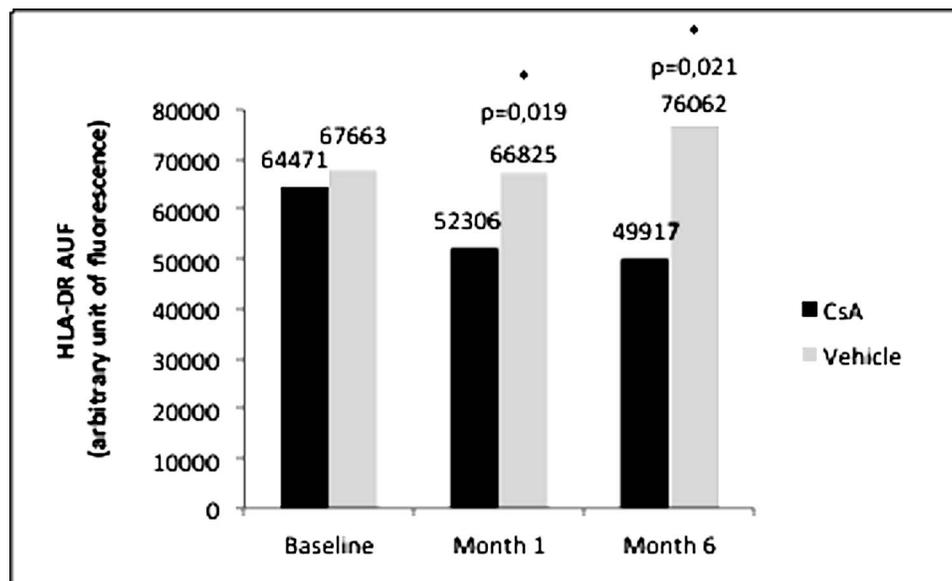


FIGURE 3. SANSIKA Study. Evolution of HLA-DR between baseline and month 6. Sample size at baseline, month 1, and month 6: 119, 76, and 70 in CsA group and 64, 43, and 43 in vehicle group. *P* < 5% (test between groups of treatment).

TABLE 4. Spearman and Spearman Partial Correlation for HLA-DR-AUF Parameter in the SICCANOVE and SANSIKA Studies, at Month 6

At M6	Spearman Correlation With Fluorescence HLA-DR-AUF, <i>r</i> (<i>P</i>)	Spearman Partial Correlation With HLA-DR-AUF Adjusted on Age, <i>r</i> (<i>P</i>)
Age, y		
CsA (<i>n</i> = 97)	0.04 (0.6700)	-
Vehicle (<i>n</i> = 74)	0.24 (0.0385)	
Time since DED diagnosis, y		
CsA (<i>n</i> = 97)	0.02 (0.8200)	0.01 (0.9255)
Vehicle (<i>n</i> = 74)	0.28 (0.0128)	0.23 (0.0554)
CFS score*		
CsA (<i>n</i> = 97)	0.34 (0.0005)	0.35 (0.0005)
Vehicle (<i>n</i> = 74)	0.46 (<0.0001)	0.46 (<0.0001)
Schirmer's test (mm/5 min)		
CsA (<i>n</i> = 97)	-0.31 (0.0020)	-0.31 (0.0023)
Vehicle (<i>n</i> = 74)	-0.23 (0.0422)	-0.24 (0.0376)
TBUT, s		
CsA (<i>n</i> = 97)	-0.25 (0.0138)	-0.26 (0.0114)
Vehicle (<i>n</i> = 74)	0.31 (0.0070)	-0.34 (0.0031)
Total OSDI score		
CsA (<i>n</i> = 97)	0.10 (0.2930)	0.11 (0.2903)
Vehicle (<i>n</i> = 74)	0.22 (0.0579)	0.24 (0.0387)
VAS score		
CsA (<i>n</i> = 97)	0.17 (0.093)	0.18 (0.0831)
Vehicle (<i>n</i> = 74)	0.16 (0.1530)	0.19 (0.1099)

Correlation was determined between the HLA-DR-AUF parameter and all the following factors: age, CFS score, time since DED diagnosis, total OSDI score, VAS score, Schirmer's test, TBUT. The corresponding Spearman correlation coefficient (*r*) and statistical significance (*P* value) are presented.

* The CFS score was based on the modified Oxford scale.

found HLA-DR-AUF correlated negatively with Schirmer's test ($r = -0.20$) and TBUT ($r = -0.13$). These clinical tests both assess tear film and are usually used in conjunction with DED evaluation: the Schirmer's test evaluates the quantity of tears produced while TBUT is a clinical measurement of tear film stability. Therefore, it was not surprising to see that both parameters decreased with an HLA-DR-AUF increase (i.e., with disease severity). The parameters of HLA-DR (AUF and %) also significantly correlated with symptom-reporting questionnaires (OSDI and VAS; $r = 0.12$ and 0.14 , respectively for HLA-DR-AUF and $r = 0.13$ and 0.14 , respectively, for HLA-DR %, Supplementary Table S3). Globally, correlations between HLA-DR-AUF and signs were greater at month 6 than at month 0, and sometimes greater in the vehicle group than the CsA group, supporting a hypothesis on temporal disconnection between the inflammation improvement and the signs improvement under CsA.

Our data thus revealed that the HLA-DR parameter (at least HLA-DR-AUF) correlated significantly with the clinical DED sign test such as CFS and to a lesser extent with Schirmer's test, and weakly with TBUT and also with the symptoms experienced by DED patients. We acknowledge the fact that correlations were not very strong, but they were significant and we could consider them meaningful, as it is well known in DED that very few criteria correlate together.²⁹ One possible explanation for such low correlations could be the time frame difference between biologic and clinical parameters, as we

know that symptoms and signs can be modified rather rapidly under the influence of environment or seasonality.³⁴

Expression of HLA-DR class II antigen is normally restricted to professional antigen presenting cells (APC) in which it presents antigen to CD4-positive T cells promoting lymphocytes activation and infiltration in conjunctival epithelium. Its expression in non-APC has been observed in various pathologic conditions, where enhanced class II expression was related to the degree of inflammation.^{17,35,36} Human leukocyte antigen-DR has also been shown to be upregulated upon interferon gamma with or without the synergistic action of TNF, also in epithelial cells, including conjunctival epithelium.³⁷ Indeed, interferon gamma gene expression was found higher in aqueous-deficient DED and its overexpression correlated with goblet cell loss and severity of the disease.³⁸ In addition, tear interferon gamma was recently found elevated in DED and has been proposed by the authors as a new biomarker for evaporative DED.³⁹

No or weak expression of HLA-DR was observed in normal patient samples; however HLA-DR antigens were greatly expressed in conjunctival epithelial cells of allergic tissues. These results suggest expression of HLA-DR was associated with chronic ocular allergic inflammation potentially linking this to the pathophysiology of the disease.¹⁹

The measurement of this marker could therefore be more largely used to diagnose the increase in inflammation of the ocular surface seen in dry eye and help in classification of DED severity. In clinical studies on anti-inflammatory agents, HLA-DR expression decreased in CsA-treated DED patients.¹⁸⁻²⁰ A recent clinical study showed that supplementation with omega-3 and -6 fatty acids significantly reduced expression of HLA-DR conjunctival inflammatory marker in dry eye patients, as compared to placebo.⁴⁰ Thus, HLA-DR appeared to be a good marker to assess the response to treatment in dry eye and to our knowledge, it is the only one that has been successfully used in several multicenter, international trials. Indeed longitudinal data are required to consider a parameter as a potential biomarker. Taking into account data from previous clinical studies and longitudinal data from SICCANOVE and SANSIKA showing a reduction of the HLA-DR expression after treatment with CsA, the HLA-DR parameter has shown its potential for being an objective and reliable marker associated to ocular surface inflammation in DED.

Identification of HLA-DR may impact DED severity diagnosis in clinical settings and clinical study designs, in particular patient inclusion criteria and primary efficacy endpoints.⁴¹⁻⁴³ Inclusion in ophthalmic clinical studies often relies on assessment of both signs and symptoms and regulatory authorities frequently require an improvement in both. The correlation means that a reduction in HLA-DR expression would be a good indication of improvement in signs and symptoms.

In light of our data and based on the literature, measurement of HLA-DR expression could be considered as an interesting and useful measure of inflammation on the ocular surface and therefore of DED activity.

The exact methodology to analyze the conjunctival HLA-DR varies within the studies. Some used percentage of positive cells, others utilized the fluorescence intensity and recently, Epstein et al.⁴¹ detailed the different steps of this method in standard operating procedures suitable for clinical trials and have suggested to only assessing the highly expressing HLA-DR cells. Nevertheless, the percentages of positive cells are the primary information given by flow cytometry analysis, but it could present variability or discrepancies depending on the placement of background threshold cursor. Interestingly, HLA-DR percentages of posi-

tive cells, even in severe cases, did not reach 100%. This was due to analytic reasons related to the lower levels of expression that did not always discriminate from the isotopic negative control level and overlapped with it, and have to be subtracted from the raw percentage. Conversely, MFI are data directly obtained from the computer without any human intervention. But this parameter can vary depending on the heating time of the laser and in the time of a longitudinal study; moreover, its variations are small in terms of numeric values, varying from a few units relative to the isotopic control or in the time. It is why our group developed a standardized methodology for the cytology impression/flow cytometry technique to analyze HLA-DR expression in multicenter clinical studies using a commercially available fluorescence quantification method based on fluorescence-calibrated beads.^{13,18} Impression cytology/flow cytometry is virtually stress-free and minimally invasive for the patient which justify the interest of HLA-DR quantification as a useful parameter to assess inflammation for clinical research in DED according to the DEWS' recommendations.⁴² Concerning the method validation, conjunctival cells collected on PES membranes are not enough to allow sensitivity, repeatability, and reproducibility for HLA-DR-AUF. We used calibrated beads for this purpose that allow, aside from antigen quantification, a control of the repeatability and the sensitivity of the method as they are submitted to the same technical procedures as the sample. We found that the QIFIKIT beads used in this study expressed antibody binding capacity (ABC units, that we called arbitrary units of fluorescence, AUF, for easier language) at five fluorescence levels from 2.1, 11, 58, 174, to 547 thousands AUF with respective coefficients of variation of 42%, 14%, 3%, 2%, and 0.196%. The higher are the fluorescence levels, the lower and better is the coefficient of variation (CV). These results came from 141 analyses of beads performed at different days.

Epstein et al.⁴¹ described in a reproducibility assay that HLA-DR percentage was not different between two independent assays performed on the same sample. They demonstrated the precision of the mean geographic scores of cell size and structure with no differences between all the sample tested, from normal or DED subjects. They also evaluated the storage conditions in the dark and in a refrigerator and concluded that the samples were still suitable for analysis for 30 days. Despite this work on various standard operating procedures, it is still necessary to add supplementary validation assays. But the limitation to these kinds of evaluations lies in the sample characteristics of samples with a low number of cells. From a practical point of view, the use of an appropriate surrogate matrix has been proposed as exception procedure to the use of a real matrix similar to that of samples in cases of rare or low volume matrices like tears.⁴⁴ Moreover, this method of combining impression cytology and flow cytometry poses some difficulty. Indeed, some samples could not be processed due to various issues, such as:

1. confusion between the PES membrane and its blue paper support that is not suitable for collection. The recent development of a collection device (EYEPRIM; Opia Technologies, Paris, France) could solve this issue;
2. the temperature control during the different steps after the sample collection revealed some samples were frozen;
3. the low number of cells insufficient for the analysis;
4. the fluorescein contamination of some samples influencing the measurement of the fluorescent level of HLA-DR; and

5. the delay between collection and reception, resulting in abnormal flow cytometry images (normally, samples have to be analyzed as soon as received and the sooner after the sample collection, the better).

All these drawbacks should be progressively solved as the techniques would become more standardized and developed in increasing numbers of laboratories.

In clinical studies on dry eye, HLA-DR quantification could be proposed as a surrogate biomarker for patient inclusion and for efficacy evaluation. This method remains quite expensive since it requires many technical steps from cell extraction to immunolabeling and to analysis. It is also currently only performed by some specialized laboratories and has not been developed as an easy and cheap "bedside test."

Moreover, the HLA-DR variable could potentially serve as a biomarker in other ocular diseases, including glaucoma. Several clinical studies have shown a significant increase in expression of immunoinflammatory markers such as HLA-DR, ICAM-1, and chemokine/chemokine receptors by the conjunctival epithelium in glaucoma patients treated over the long term, especially in patients using preserved multitreatment therapies.⁴⁵⁻⁴⁷

Dry eye affects millions of people around the world and with medical advances and changes in lifestyle, this number is likely to increase over the coming decades. Hence, it has become essential to resolve the disparity in signs and symptoms. In particular, clinical studies in dry eye and proinflammatory HLA-DR conjunctival cell expression may represent an interesting objective tool for DED severity evaluation in clinical research and practices, but additional validation studies are still required.

Acknowledgments

The authors thank Magali Le Goff, a professional medical writer from Scinopsis Medical Writing, Fréjus, France.

Disclosure: **F. Brignole-Baudouin**, None; **L. Riancho**, None; **D. Ismail**, Santen SAS (E); **M. Deniaud**, Santen SAS (C); **M. Amrane**, Santen SAS (E); **C. Baudouin**, Alcon (C, F), Allergan (C, F), Thea Laboratories (C, F), Santen SAS (C, F)

References

1. The epidemiology of dry eye disease: Report of the Epidemiology Subcommittee of the International Dry Eye Workshop (2007). *Ocular Surf*. 2007;5:93-107.
2. Stern ME, Beuerman RW, Fox RI, Gao J, Mircheff AK, Pflugfelder SC. The pathology of dry eye: the interaction between the ocular surface and lacrimal glands. *Cornea*. 1998;17:584-589.
3. Dana MR, Hamrah P. Role of immunity and inflammation in corneal and ocular surface disease associated with dry eye. *Adv Exp Med Biol*. 2002;506:729-738.
4. Barabino S, Dana MR. Dry eye syndromes. *Clin Immunol Allergy*. 2007;92:176-184.
5. Luo L, Li DQ, Corrales RM, Pflugfelder SC. Hyperosmolar saline is a proinflammatory stress on the mouse ocular surface. *Eye Contact Lens*. 2005;31:186-193.
6. Luo L, Li DQ, Doshi A, Farley W, Corrales RM, Pflugfelder SC. Experimental dry eye stimulates production of inflammatory cytokines and MMP-9 and activates MAPK signaling pathways on the ocular surface. *Invest Ophthalmol Vis Sci*. 2004;45:4293-4301.
7. Niederkorn JY, Stern ME, Pflugfelder SC, et al. Desiccating stress induces T cell-mediated Sjögren's syndrome-like lacrimal keratoconjunctivitis. *J Immunol*. 2006;176:3950-3957.

8. Baudouin C. A new approach for better comprehension of diseases of the ocular surface. *J Fr Ophtalmol*. 2007;30:239-246.
9. Baudouin C, Aragona P, Messmer EM, et al. Role of hyperosmolarity in the pathogenesis and management of dry eye disease: proceedings of the OCEAN group meeting. *Ocular Surface*. 2013;11:246-258.
10. Stern ME, Gao J, Schwalb TA, et al. Conjunctival T-cell subpopulations in Sjögren's and non-Sjögren's patients with dry eye. *Invest Ophthalmol Vis Sci*. 2002;43:2609-2614.
11. Baudouin C, Haouat N, Brignole F, Bayle J, Gastaud P. Immunopathological findings in conjunctival cells using immunofluorescence staining of impression cytology specimens. *Br J Ophthalmol*. 1992;76:545-549.
12. Tsubota K, Fujihara T, Saito K, Takeuchi T. Conjunctival epithelium expression of HLA-DR in dry eye patients. *Ophthalmologica*. 1999;213:16-19.
13. Brignole F, Pisella PJ, Goldschild M, De Saint Jean M, Goguel A, Baudouin C. Flow cytometric analysis of inflammatory markers in conjunctival epithelial cells of patients with dry eyes. *Invest Ophthalmol Vis Sci*. 2000;41:1356-1363.
14. Barabino S, Montaldo E, Solignani F, Valente C, Mingari MC, Rolando M. Immune response in the conjunctival epithelium of patients with dry eye. *Exp Eye Res*. 2010;91:524-529.
15. Baudouin C, Brignole F, Becquet F, Pisella PJ, Goguel A. Flow cytometry in impression cytology specimens. A new method for evaluation of conjunctival inflammation. *Invest Ophthalmol Vis Sci*. 1997;38:1458-1464.
16. Versura P, Profazio V, Schiavi C, Campos EC. Hyperosmolar stress upregulates HLA-DR expression in human conjunctival epithelium in dry eye patients and in vitro models. *Invest Ophthalmol Vis Sci*. 2011;52:5488-5496.
17. The definition and classification of dry eye disease: report of the Definition and Classification Subcommittee of the International Dry Eye Workshop (2007). *Ocul Surf*. 2007;5:75-92.
18. Brignole F, Pisella PJ, De Saint Jean M, Goldschild M, Goguel A, Baudouin C. Flow cytometric analysis of inflammatory markers in KCS: 6-month treatment with topical cyclosporin A. *Invest Ophthalmol Vis Sci*. 2001;42:90-95.
19. Turner K, Pflugfelder SC, Ji Z, Feuer WJ, Stern M, Reis BL. Interleukin-6 levels in the conjunctival epithelium of patients with dry eye disease treated with cyclosporine ophthalmic emulsion. *Cornea*. 2000;19:492-496.
20. Kunert KS, Tisdale AS, Stern ME, Smith JA, Gipson IK. Analysis of topical cyclosporine treatment of patients with dry eye syndrome: effect on conjunctival lymphocytes. *Arch Ophthalmol*. 2000;118:1489-1496.
21. Methodologies to diagnose and monitor dry eye disease: report of the Diagnostic Methodology Subcommittee of the International Dry Eye Workshop (2007). *Ocul Surf*. 2007;5:108-152.
22. Lemp MA, Bron AJ, Baudouin C, et al. Tear osmolarity in the diagnosis and management of dry eye disease. *Am J Ophthalmol*. 2011;151:792-798.e1.
23. Schiffman RM, Christianson MD, Jacobsen G, Hirsch JD, Reis BL. Reliability and validity of the Ocular Surface Disease Index. *Arch Ophthalmol*. 2000;118:615-621.
24. Sullivan DA, Bloch KJ, Allansmith MR. Hormonal influence on the secretory immune system of the eye: androgen control of secretory component production by the rat exorbital gland. *Immunology*. 1984;52:239-246.
25. Sullivan DA, Wickham LA, Rocha EM, et al. Androgens and dry eye in Sjögren's syndrome. *Ann N Y Acad Sci* 1999;876:312-324.
26. Schein OD, Tielsch JM, Munoz B, Bandeen-Roche K, West S. Relation between signs and symptoms of dry eye in the elderly. A population-based perspective. *Ophthalmology*. 1997;104:1395-1401.
27. Begley CG, Chalmers RL, Abetz L, et al. The relationship between habitual patient-reported symptoms and clinical signs among patients with dry eye of varying severity. *Invest Ophthalmol Vis Sci*. 2003;44:4753-4761.
28. Nichols KK, Nichols JJ, Mitchell GL. The lack of association between signs and symptoms in patients with dry eye disease. *Cornea*. 2004;23:762-770.
29. Sullivan BD, Crews LA, Messmer EM, et al. Correlations between commonly used objective signs and symptoms for the diagnosis of dry eye disease: clinical implications. *Acta Ophthalmol*. 2014;92:161-166.
30. Ramos-Casals M, Tzioufas AG, Stone JH, Siso A, Bosch X. Treatment of primary Sjögren syndrome: a systematic review. *JAMA*. 2010;304:452-460.
31. Viso E, Rodriguez-Ares MT, Gude F. Prevalence of and associated factors for dry eye in a Spanish adult population (the Salnes Eye Study). *Ophthalmic Epidemiol*. 2009;16:15-21.
32. Savini G, Prabhawat P, Kojima T, Grueterich M, Espana E, Goto E. The challenge of dry eye diagnosis. *Clin Ophthalmol*. 2008;2:31-55.
33. Adatia FA, Michaeli-Cohen A, Naor J, Caffery B, Bookman A, Slomovic A. Correlation between corneal sensitivity, subjective dry eye symptoms and corneal staining in Sjögren's syndrome. *Can J Ophthalmol*. 2004;39:767-771.
34. Lopez-Miguel A, Teson M, Martin-Montanez V, et al. Dry eye exacerbation in patients exposed to desiccating stress under controlled environmental conditions. *Am J Ophthalmol*. 2014;157:788-798.
35. Brandtzaeg P, Halstensen TS, Huitfeldt HS, et al. Epithelial expression of HLA, secretory component (poly-Ig receptor), and adhesion molecules in the human alimentary tract. *Ann N Y Acad Sci*. 1992;664:157-179.
36. Hingorani M, Calder V, Jolly G, Buckley RJ, Lightman SL. Eosinophil surface antigen expression and cytokine production vary in different ocular allergic diseases. *J Allergy Clin Immunol*. 1998;102:821-830.
37. Stahl JL, Cook EB, Graziano FM, Barney NP. Differential and cooperative effects of TNF-alpha, IL-1 beta, and IFN-gamma in conjunctival epithelial cell receptor expression and chemokine release. *Invest Ophthalmol Vis Sci*. 2003;44:2010-2015.
38. Pflugfelder SC, De Paiva CS, Moore QL, et al. Aqueous tear deficiency increases conjunctival interferon-gamma expression and goblet cell loss. *Invest Ophthalmol Vis Sci*. 2015;56:7545-7550.
39. Jackson DC, Zeng W, Wong CY, et al. Tear interferon-gamma as a biomarker for evaporative dry eye disease. *Invest Ophthalmol Vis Sci*. 2016;57:4824-4830.
40. Brignole-Baudouin F, Baudouin C, Aragona P, et al. A multicentre, double-masked, randomized, controlled trial assessing the effect of oral supplementation of omega-3 and omega-6 fatty acids on a conjunctival inflammatory marker in dry eye patients. *Acta Ophthalmol*. 2011;89:e591-e597.
41. Epstein SP, Galaria-Rathod N, Wei Y, Maguire MG, Asbell PA. HLA-DR expression as a biomarker of inflammation for multicenter clinical trials of ocular surface disease. *Exp Eye Res*. 2013;111:95-104.
42. Design and conduct of clinical trials: report of the Clinical Trials Subcommittee of the International Dry Eye Workshop. *Ocul Surf*. 2007;5:153-162.
43. Biomarkers Definitions Working Group. Biomarkers and surrogate endpoints: preferred definitions and conceptual framework. *Clin Pharmacol Ther*. 2001;69:89-95.
44. Lee JW, Weiner RS, Sailstad JM, et al. Method validation and measurement of biomarkers in nonclinical and clinical

- samples in drug development: a conference report. *Pharm Res.* 2005;22:499-511.
45. Baudouin C, Pisella PJ, Fillacier K, et al. Ocular surface inflammatory changes induced by topical antiglaucoma drugs: human and animal studies. *Ophthalmology.* 1999;106:556-563.
46. Baudouin C, Liang H, Hamard P, et al. The ocular surface of glaucoma patients treated over the long term expresses inflammatory markers related to both T-helper 1 and T-helper 2 pathways. *Ophthalmology.* 2008;115:109-115.
47. Baudouin C, Hamard P, Liang H, Creuzot-Garcher C, Bensoussan L, Brignole F. Conjunctival epithelial cell expression of interleukins and inflammatory markers in glaucoma patients treated over the long term. *Ophthalmology.* 2004;111:2186-2192.