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Corneoscleral Limbus in Glaucoma Patients: In Vivo Confocal Microscopy and Immunocytological Study

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PURPOSE. To investigate morphologic changes of the corneoscleral limbus in glaucoma patients using laser scanning confocal microscopy (LSCM) and impression cytology (IC).

METHODS. Eighty patients with glaucoma and 20 with dry eye were enrolled; 20 healthy subjects served as controls. Patients underwent the Ocular Surface Disease Index (OSDI) questionnaire, tear film break-up time, corneal staining, Schirmer test I, and LSCM of the limbus. Laser scanning confocal microscopy evaluated the limbal transition epithelium (LTE) regularity, dendritic cell (DC) density, and palisades of Vogt (POV). Impression cytology was performed and samples stained with HLA-DR and IL6.

RESULTS. Glaucomatous patients were divided into three groups: Group 1 (40 eyes): one drug; Group 2 (20): two drugs; and Group 3 (20): three or more drugs. Limbal transition epithelium regularity was worse, and DC density higher in Groups 2, 3, and dry eyes compared with Group 1 and controls ($P < 0.01$). Preserved drugs worsened LTE regularity and induced higher DC density compared with preservative-free (PF) drugs ($P = 0.041$; $P = 0.004$). Despite typical POV architecture was preserved, signs of inflammation were found in glaucoma groups. HLA-DR and IL-6 positivity were higher in Groups 2, 3, and dry eye compared with controls ($P < 0.001$), and in preserved versus PF drugs ($P < 0.05$; $P < 0.001$). Dendritic cell density and LTE regularity correlated with HLA-DR, IL-6, and OSDI score in glaucoma groups and dry eyes ($P < 0.001$).

CONCLUSIONS. Laser scanning confocal microscopy and IC documented antiglaucoma therapy induced morphologic alterations of limbus, which may play a role in the glaucoma-related ocular surface disease. Further studies are required to determine if limbal changes affect stem cell viability.

Keywords: corneoscleral limbus, primary open angle glaucoma, glaucoma therapy, ocular surface, in vivo laser scanning confocal microscopy, impression cytology

The corneoscleral limbus (CL), which represents one of the components of the ocular surface morphofunctional unit, is the most crucial structure involved in the maintenance of the corneal epithelial integrity because hosts the niches for corneal epithelial stem cells.[1] Several conditions may affect the CL integrity such as ocular surface inflammatory diseases, chemical burns, aniridia, contact lens wear, infections, surgical procedures, and the use of topical medications.[2]

In patients with glaucoma, long-term medical therapy leads to critical modifications of ocular surface structures and adnexa, including the conjunctiva, conjunctiva-associated lymphoid tissue, cornea, Meibomian glands, eyelids, and periocular skin.[3–6] To date, the impact of antiglaucoma medications on CL was only hypothesized by Schwartz and Holland, who first introduced the concept of the iatrogenic limbal stem cell deficiency (LSCD).[7] The authors observed the occurrence of LSCD in glaucomatous patients treated with pilocarpine and beta blockers who previously underwent surgeries involving the CL. Given that antiglaucoma medications are known to alter corneal and conjunctival epithelia and their deep layers,[4–6,8] it is likely that their chronic use is specifically toxic also to the superficial and deep structures of the limbal region.

Currently, laser scanning confocal microscopy (LSCM) can noninvasively assess the ocular surface permitting an in vivo cytology. Laser scanning confocal microscopy was used to describe limbal features in healthy subjects,[9] in contact lens wearers,[10] in dry eye,[11] during superior limbic keratoconjunctivitis,[12] and to document ocular surface changes in patients diagnosed with different types of LSCD.[13]

In glaucoma, LSCM was widely used to describe ocular surface tissue alterations induced by medical therapy,[5,6,8,14,15] the bleb functionality after filtration surgery,[16] and transscleral aqueous humor outflow modifications induced by medical and surgical approaches.[17,18] This imaging technique was also used to evaluate modifications induced by different IOP-lowering

formulations on CL in rabbit models.[19,20] To date, no previous study has been specifically focused on studying the CL status in patients with glaucoma.

We conducted the present study to describe the microscopic features of CL in medically controlled glaucomatous patients by using LSCM and immunecytology, in order to elucidate modifications induced by therapy.

MATERIALS AND METHODS

Patient's Enrollment

This was a case control observational study that adhered to the tenets of the Declaration of Helsinki. Our institutional review board (Department of Medicine and Ageing Science, G. d'Annunzio University of Chieti-Pescara, Chieti, Italy) approved the project. We examined 80 consecutive Caucasian patients (80 eyes) with medically controlled POAG, who came to our attention at the Glaucoma Centre. Twenty age- and sex-matched patients (20 eyes) with primary or secondary Sjogren syndrome (SS)-related dry eye (diagnosed according to criteria reported by the International Dry Eye Workshop)[21] referring to our Ocular Surface Diseases Centre were also enrolled. Twenty age- and sex-matched healthy Caucasian subjects (20 eyes) referring to the General Clinic were used as controls. Written informed consent was obtained from all subjects prior to enrolment, after explanation of the nature and possible consequences of the study.

Glaucomatous patients had to respect the following inclusion criteria: corrected visual acuity greater than or equal to 8/10, refractive error less than or equal to 3 diopters (D; spherical-equivalent), mean IOP at the time of diagnosis greater than or equal to 22 mm Hg and medically controlled at enrolment (IOP < 18 mm Hg: mean of three measurements taken at 9 AM, 12 PM, and 4 PM), central corneal thickness (CCT; Ultrasound pachimetry, Altair; Optikon 2000, Rome, Italy) ranging from 530 to 570 μm, visual field (VF) test (30-2 test, full-threshold, Humphrey field analyzer II 750; Carl Zeiss Meditec, Inc., Dublin, CA, USA) showing at least three contiguous points on the total deviation probability plot at the less than 2% level, Glaucoma Hemifield Test "outside normal limits," and ophthalmoscopic signs of glaucomatous optic consistent with the VF alterations. Topical therapy had to be the same in both eyes without variation during the 12 months immediately prior to enrolment.

Exclusion criteria were the followings: history of systemic or ocular inflammatory diseases, and systemic or topical therapies in the last 12 months that could have modified the CL status, previous ocular surgery, laser therapy, ocular trauma, chemical burn, end-stage glaucoma, pregnancy, and contact lens wear. History of corneal dystrophy, inflammatory diseases of the ocular surface, and dry eye prior to glaucoma diagnosis and initiating treatment were also considered exclusion criteria.

Patients with dry eye and healthy controls had to show a best-corrected visual acuity greater than or equal to 8/10, a refractive error less than or equal to 3 D (spherical equivalent), mean IOP lower than 18 mm Hg, CCT ranging from 530 to 570 μm, absence of glaucomatous optic neuropathy, and a normal VF examination. At the moment of enrolment patients with dry eye were treated with lubricants five to seven times a day (preservative-free [PF] hypotonic solution of sodium hyaluronate) and did not receive topical steroids during the last 3 months. Exclusion criteria for dry eye patients were the followings: lymphoma, AIDS, sarcoidosis, diabetes mellitus; corneal dystrophy and nondry eye–related ocular surface inflammatory diseases; systemic or topical therapy with

medications having potential corneal toxicity; diagnosis of glaucoma; topical therapy with steroids or NSAIDs; contact lens use; and previous ocular surgery.

None of the healthy subjects had either a history of topical or systemic therapy nor were they affected with any ocular or systemic diseases in the last 12 months. Pregnant women and contact lens wearers were also excluded. Both eyes were evaluated in the study, but one eye per subject was randomly chosen (using a computer generated random number list) for the statistical analysis.

Patient Clinic Assessment

Each subject underwent a careful slit-lamp examination of both the anterior and posterior segment of the eye.

Ocular Surface Disease Index (OSDI) Questionnaire and Tear Film Function Tests

After the enrolment and biomicroscopic examination, all subjects were asked to complete the OSDI questionnaire and underwent tear film function tests to assess the clinical status of the ocular surface. The tear film break-up time (BUT), Schirmer test I (STI; 30 minutes after BUT measurements), and corneal staining were performed after the completion of the OSDI questionnaire. The BUT was recorded as the average of three consecutive measurements; STI result was expressed as the length of the strip that was wet after 5 minutes; corneal staining was evaluated with sodium fluorescein 1% (using the van Bijsterveld method).

LSCM of the CL

The day after the tear film function assessment, LSCM was performed using a digital confocal laser-scanning microscope (HRT III Rostock Cornea Module, diode-laser 670 nm; Heidelberg Engineering GmbH, Dossenheim, Germany), to study the superficial and deep structures of the CL.

The confocal laser-scanning device was equipped with a water immersion objective (633/N.A. 0.95 W; Zeiss, Jena, Germany) and permitted an automatic z-scan determination of depth of focus within the CL. Thus, high-contrast digital images of limbal layers with a field of view of 400×400 μm were acquired. The theoretical confocal section thickness is approximately 10 μm. This is the slice thickness (voxel), which is imaged by the confocal microscope to form a 2-dimensional, pixel-based digital image. The lateral and transverse resolution is 4 μm.

Laser scanning confocal microscopy was carried out under topical anesthesia with 0.4% oxybuprocaine. Proper alignment and positioning of the head was maintained with the help of a dedicated movable-target red fixation light for the fellow eye. A digital camera mounted on a side arm provided a lateral view of the eye and objective lens to monitor the position of the objective lens on the surface of the eye. A drop of 0.2% polyacrylic gel served as coupling medium between the poly (methyl methacrylate) contact cap of the objective lens and cornea. The examination was performed in 8 clock-hour positions of the limbal area (12, 3, 6, 9, corresponding to superior, nasal, inferior, or temporal limbus; and the four intermediate positions between the above points), using a previously described method.[13]

Sequential images derived from automatic scans and manual frame acquisition throughout the limbal area in the scheduled sectors. The automatic brightness mode was selected during examination.

The presence of palisades of Vogt (POV) and a progressive morphologic transition of epithelial cells from the conjunctival
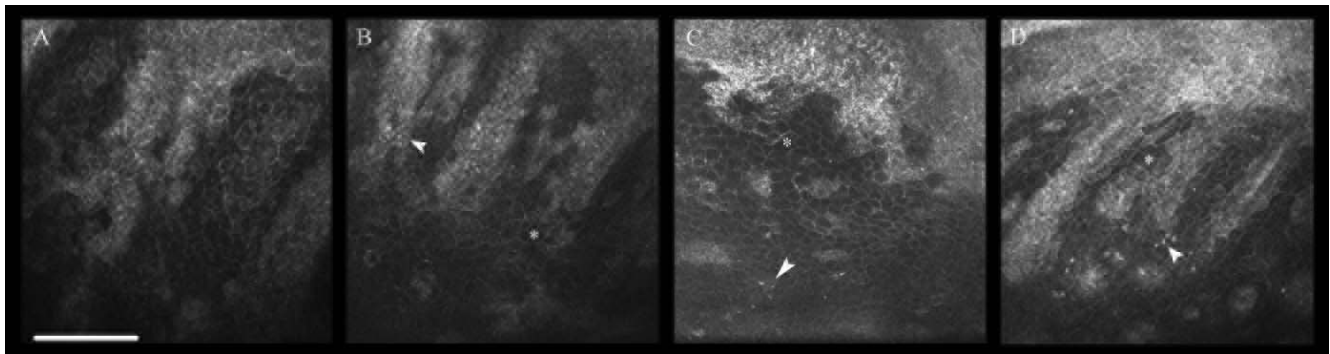
FIGURE 1. Grading of the LTE regularity at LSCM. (**A**) Grade 0: normal LTE features (26-μm depth) characterized by homogeneity of cell shape and size, and normal epithelial reflectivity (58.34). (**B**) Grade 1: good degree of regularity with LTE features (29 μm) quite similar to grade 0, except for mild signs of inhomogeneity of cellular size and shape (*asterisk*), and epithelial hyper-reflectivity (71.35). Punctate reflective elements (*arrowhead*) represent signs of inflammation. (**C**) Grade 2: moderate degree of regularity (30 μm), with more evident sings of epithelial hyperreflectivity (89.00), presence of punctate reflective elements (*arrowhead*), inhomogeneity of cellular size and shape (*asterisk*). (**D**) Grade 3: poor degree of regularity (28 μm), characterized by very evident inhomogeneity in cellular size and shape (*asterisk*), marked epithelial hyperreflectivity (99.75), irregular and less distinguishable border demarcation, and several punctate reflective elements, sometimes clustered (*arrowhead*). *Scale bar*: represents 100 μm.

to the corneal phenotype in the peripheral cornea adjacent to the limbus, were considered indicators of normal CL anatomy.[9] Palisades of Vogt and limbal transition epithelium (LTE) were considered present when revealed in three or four limbal quadrants, partially present if revealed in only one or two sectors, and absent if not detected at all. Normal features of POV are characterized by hyperreflective linear radial stromal ridges alternating with columns of basal epithelial cells, originating from the subconjunctival deep stroma.

The normal LTE consists of conjunctival-type bright epithelium with indistinct cell boundaries, presenting a gradual transition toward the corneal epithelium. The demarcation between the two types of epithelia is generally irregular, with small islands of corneal epithelial cells dispersed within the sheet of conjunctival epithelium. An arbitrary grading scale of 0 to 3 was adopted to quantify the regularity of the LTE considering the following parameters: overall epithelial reflectivity, homogeneity of cell shape and size, cell border demarcation, and evidence of punctate reflective elements (as presumed sign of inflammation). The overall epithelial reflectivity was calculated by determining the average gray value of the image using Image J software (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA). This value corresponded to the sum of gray values of all pixels in the entire image divided by the number of pixels. An average gray value less than 60.00 indicated a normal reflectivity, from 60.01 to 75.00 mild reflectivity, 75.01 to 90.00 moderate, and greater than 90.01 high reflectivity. Therefore, grade 0 corresponded to the normal aspect, whereas grade 1, 2, and 3 to good, moderate, and poor degree of regularity, respectively (Fig. 1).

Punctate reflective elements seen in confocal microscopy are isolated or confluent noncellular hyperreflective spots, sized approximately from 2 to 6 μm. These elements most probably correspond to deposition of phlogistic material, such as mucoproteins or immune complexes, and have been documented in different ocular surface tissues during inflammatory diseases.[6,10,14,22]

Limbal dendritic cells (DC) were also examined to determine their density, at the basal layer and basal membrane of the limbal epithelium. Dendritic cells can be seen with a different morphology depending on their maturity. Mature DC have a slender nucleated cell body from which extends a maze of long membrane processes resembling dendrites of nerve cells; immature DC have a large cell body with fewer and shorter processes, if any. Dendritic cell density (given as cells/

mm²) was calculated, using the analysis software of the instrument, by averaging numbers of cells from five images in each position (randomly selected among the recorded images), counted manually within a region of interest of standardized dimensions (250 × 250 μm).

Eighty images of the CL were acquired (20 for each quadrant) for each eye; 40 randomly selected high-quality images (10 for each quadrant) were analyzed. Typical confocal session lasted 5 minutes and none of the subjects experienced any visual symptoms or ocular surface epithelial complications as a result of the examination. A single operator (VF) performed confocal examinations and selected the images, which were evaluated by a second operator (LB). Both operators were masked for the subject history and for grouping.

Impression Cytology (IC) of the CL

The IC was scheduled to evaluate the expression of HLA-DR and IL-6 at the transition epithelium of the limbus. These markers, which can be normally expressed in the normal conjunctiva,[23] are known to be over-expressed in medically treated glaucomatous patients, and are considered two of the most prominent inflammatory markers of the ocular surface.[24,25] Impression cytology sampling of the LTE was performed from 36 to 48 hours after confocal microscopy to avoid misinterpretation due to the mechanical pressure during execution of LSCM. Each patient received two consecutive cytological biopsies at the superior-nasal (from 12 to 3 clock-hour positions) and superior-temporal (from 12 to 9 clock-hour positions) portions of the LTE, with two different membranes. Given that these sectors offered a better exposure of areas of interest, the sampling was easier and thus less bothersome for the patients. In order to be sure that limbus (and not cornea or conjunctiva) was precisely impressed during procedure, membranes were cut in an arched shape (approximately 9-mm length, 2-mm large) reproducing and matching the limbal curvature. Samples with cells covering more than 80% of the membrane area, or samples covering between 50% and 80% where cells were confluent and present in a defined area of the membrane (not scattered), were considered suitable for diagnostic purposes.

For IC the Millicell-CM 0.4-mm membrane (Millipore, Bedford, Massachusetts, USA) was used and cells were fixed with cytology fixative (Bio-fix; Bio Optica, Milan, Italy). For HLA-DR and IL-6 immunofluorescence staining, the Millicell

TABLE 1. Demographics and Clinical Data of Healthy Controls, Dry Eye, and Glaucoma Groups

	Age, y ± SD	Sex, M/F	IOP, mm Hg ± SD	MD, dB ± SD	Mean Time on Therapy,§ mo ± SD
Healthy controls	55.38 ± 4.64	9/11	16.35 ± 3.43	1.32 ± 0.46	NA
Dry eye	56.63 ± 3.66	10/10	15.82 ± 2.65	1.65 ± 0.35	68.6 ± 3.1
Group 1	56.80 ± 4.52	20/20	15.63 ± 3.70	−2.42 ± 0.56*	70.2 ± 4.5
Group 2	57.28 ± 3.53	10/10	13.58 ± 2.75	−4.45 ± 1.03†	66.1 ± 3.3
Group 3	58.45 ± 4.61	11/9	14.25 ± 3.75	−6.45 ± 1.76‡	69.5 ± 4.2

M, male; F, female; MD, mean defect; NA, not applicable.
* $P < 0.05$ vs. healthy controls, dry eye, and Groups 2 and 3.
† $P < 0.05$ vs. healthy controls, dry eye, and Groups 1 and 3.
‡ $P < 0.05$ vs. healthy controls, dry eye, and Groups 1 and 2.
§ The time on therapy matched with the time of diagnosis.

membranes were hydrated with distilled water and placed in 80% alcohol for 2 minutes. The membranes were washed in distilled water and PBS was added for 2 minutes, followed by two washes with Wash Buffer (Dako, Glostrup, Denmark) of 2 minutes each. Subsequently, filters were incubated with ribonuclease A (Sigma-Aldrich Corp., St. Louis, MO, USA) diluted 1:300 in PBS for 25 minutes at room temperature. Specimens were washed, and protein block (Dako) was added for 10 minutes at room temperature. Finally HLA-DR antibody 1:50 or IL-6 1:200 (Abcam, Cambridge, UK), both diluted in antibody diluent (Dako), were incubated overnight at 4°C. Samples were washed and anti-mouse IgG1 Alexa fluor 488 (Invitrogen, San Giuliano Milanese, Italy) for HLA-DR or anti-rabbit Alexa Fluor 488 (Invitrogen) for IL-6 diluted 1:200, and propidium iodide at 1:150 (both in antibody diluent; Dako), were added and incubated for 1 hour at room temperature. Membranes were mounted with a drop of fluorescent mounting medium (Dako) and cells visualized with a Zeiss confocal laser-scanning microscope (510; Carl Zeiss Micro-Imaging, GmbH, Vertrieb, Germany). Five different fields for each sample were evaluated, positive (red nucleus and green cytoplasm) and negative (red nucleus) cells were counted and the positivity percentage was calculated. Two independent observers (CC, MF) masked to the details of the staining technique, performed all evaluations of impression cytology specimens. Digital images of representative areas were taken.

The presence, features, and the degree of regularity of the LTE, the presence and features of POV, and the density of limbal epithelium DC, were the primary outcomes; the secondary outcomes were the positivity expression of HLA-DR and IL-6.

Statistical Analysis

The sample size calculation indicated that at least 120 patients were necessary to have an α of 0.05 and a β of 0.80. Analysis was performed using SPSS Advanced Statistical 13.0 Software (Chicago, IL, USA). Student's *t*- and χ^2 tests were used to evaluate age and sex differences, respectively, between healthy, glaucomatous, and dry-eye patients. Mann-Whitney *U* test was used to determine differences among groups of patients. Spearman's correlation analysis was used to investigate the relations between LSCM parameters and OSDI score, BUT, ST, and corneal staining and among LSCM parameters. An average for each patient was used for the statistical analysis. *P* values less than 0.05 were considered statistically significant.

RESULTS

The demographic and clinical data of the enrolled subjects are shown in Table 1. According to the number of antiglaucoma medications they were taking, glaucomatous patients were divided into three groups: Group 1 (40 eyes), one drug; Group 2 (20 eyes), two drugs; Group 3 (20 eyes), three or more drugs. Patient treatment is shown in Table 2. All patients in Group 1 did not modify therapy from treatment onset. More than 90% of dry-eye patients presented a severity level 2, according to criteria reported by the International Dry Eye Workshop.[21]

Ocular Surface Disease Index (OSDI) Questionnaire and Tear Film Function Tests

Ocular Surface Disease Index questionnaire score, STI, BUT, and corneal staining were significantly different between glaucoma groups and controls, and between overall Group 1 compared with Groups 2 and 3 ($P < 0.05$). Ocular Surface Disease Index score was significantly different between preserved and PF drugs ($P < 0.05$). Dry eyes showed all tests significantly different with respect to controls and PF drugs (Group 1; $P < 0.05$), but not different compared with Groups 2 and 3 (Table 3).

TABLE 2. Therapy of Glaucomatous Patients

Group	Therapy	*N*
Group 1	Single	40
	β-blockers	20
	Timolol 0.5% (BAK 0.01)	10
	PF-timolol 0.5%	10
	PGAs	20
	Latanoprost 0.005% (BAK 0.02)	10
	PF-tafluprost 0.015%	10
Group 2	Double	20
	Latanoprost 0.005%-timolol 0.5% fixed combination (BAK 0.04)	7
	Bimatoprost 0.03% (BAK 0.005) and BAK-preserved timolol 0.5%, unfixed combination	5
	Brimonidine 0.2% (BAK 0.05) and BAK-preserved timolol 0.5%, unfixed combination	4
	Dorzolamide-timolol 0.5% fixed combination (BAK 0.0075)	4
Group 3	Triple or more	20
	BAK preserved bimatoprost 0.03%, brimonidine and timolol 0.5% fixed combination (BAK 0.05)	10
	BAK preserved latanoprost 0.005%, and dorzolamide timolol 0.5% fixed combination	7
	BAK-preserved bimatoprost 0.03%, BAK-preserved brimonidine 0.2%, and dorzolamide-timolol 0.5% fixed combination	3

PGAs, prostaglandin analogs; BAK, % (mg/mL).

TABLE 3. Clinical Characteristics of Patients and Controls

	OSDIs	BUT	STI	Corneal Staining
Controls	8.4 ± 3.2*	14.2 ± 2.3*	17.9 ± 3.8*	0.4 ± 1.2*
Dry eye	48.9 ± 4.2†	4.2 ± 1.5†	5.8 ± 1.2†	2.1 ± 1.9†
Group 1	13.9 ± 2.7‖	10.6 ± 1.7‖	10.2 ± 2.1‖	1.6 ± 1.1‖
PF drugs	9.4 ± 2.0‡	11.1 ± 1.9	10.3 ± 2.2	1.6 ± 1.3
Preserved drugs	18.4 ± 2.2§	10.1 ± 1.8	10.2 ± 2.3	1.8 ± 1.2
Group 2	46.6 ± 5.7	3.9 ± 1.2	6.7 ± 2.3	2.4 ± 2.1
Group 3	47.4 ± 6.2	3.8 ± 1.2	6.2 ± 2.1	2.2 ± 2.3

* $P < 0.05$ vs. overall Group 1 (except for OSDIs), Groups 2, 3, and dry eye.
† $P < 0.05$ vs. overall Group 1 and healthy controls.
‡ $P < 0.05$ vs. preserved drugs.
§ $P < 0.05$ vs. controls, dry eye, overall Group 1, Groups 2 and 3.
‖ $P < 0.05$ vs. Groups 2 and 3.

Confocal Microscopy of the CL

Laser scanning confocal microscopy was able to show the distinct morphology of the limbal structures in all examined subjects; reliable images of the analyzed quadrants were obtained in all cases. For all considered parameters there were no significant differences between the explored quadrants.

Limbal Transition Epithelium

Limbal transition epithelium was recognized in all subjects and preserved the typical architecture (from 15–30 μm of depth). This structure was observed at least in three quadrants out of four in all cases. In Group 1, LTE did not show morphologic differences compared with controls and between drug classes, even though eyes treated with BAK-preserved formulations presented some punctate reflective elements compared with eyes treated with PF drugs. Groups 2 and 3, and dry eyes showed epithelial cell hyperreflectivity with more numerous punctate reflective elements, when compared with Group 1 and healthy controls. These features were quite similar between Groups 2 and 3. In dry eyes morphologic features did not differ with respect to Groups 2 and 3, except for a lower evidence of punctate reflective elements. The grade of regularity of the LTE was significantly worse in Groups 2, 3, and in dry eyes compared with overall Group 1 and control ($P < 0.05$; Table 4). In Group 1, overall patients treated with preserved drugs showed a worse grade of LTE regularity with respect to overall patients treated with PF drugs ($P < 0.05$; Table 4). When analyzing the different classes of medications, preserved PGA, and preserved β-blockers presented a worse LTE regularity compared with PF formulations ($P < 0.05$; Table 5). The planar reconstructions of the conjunctival-limbus-cornea transition of the surface epithelia are showed in Figure 2.

Limbal DC

Dendritic cells were found in all enrolled subjects and for the most part appeared as bright linear elements with dendritic processes (mature cells) located at the level of the basal layer and basal membrane of the limbal epithelium (from 30 and 50 μm of depth). Their size was up to 30, 50, and 40 μm in controls, dry eyes, and overall glaucoma patients, respectively. In glaucoma groups DC appeared more hyperreflective, somewhere presented a clustering tendency, and were found in 80%, 90%, and 95% in Groups 1, 2, and 3, respectively. Dendritic cell densities were significantly higher in glaucoma groups and in dry eyes compared with controls ($P < 0.001$); overall Group 1 also significantly differed from Groups 2 and 3 ($P < 0.05$; Table 4). Overall patients taking preserved medications presented higher DC density compared with overall patients treated with PF medications and controls ($P < 0.05$; Table 4). In Group 1, patients taking preserved PGA or ß-blockers presented higher DC density compared with patients treated with PF formulations and controls ($P = 0.004$); moreover, PF PGA showed a higher DC density compared with PF β-blockers ($P < 0.05$; Table 5).

In both glaucomatous and dry-eye patients, scattered punctate reflective elements were found interspersed between DC and epithelial cells (Fig. 3).

Palisades of Vogt

Palisades of Vogt preserved the typical architecture and were recognized in all enrolled subjects, and were most prominent in the superior and inferior sectors. These structures were observed at least in three quadrants out of four in all cases. Groups 2, 3, and dry-eye patients presented a mild hyper-reflectivity of stromal ridges and higher evidence of punctate reflecting elements (within stromal ridges or columns of epithelium) compared with controls and overall Group 1. Dry eyes showed more punctate reflecting elements with respect to glaucomatous patients (Fig. 3). No evident morphologic

TABLE 4. Confocal and IC Parameters in Healthy Controls, Dry Eye, and Glaucoma Groups

	LSCM Parameters	IC Parameters		
	LTE	DC Density, Cells/mm²	HLA-DR, %	IL-6, %
Controls	0.48 ± 0.53*	21.94 ± 7.07†	1.95 ± 1.46‡	2.00 ± 1.71‡
Dry eye	2.20 ± 0.67	66.72 ± 17.32	37.10 ± 13.56	47.65 ± 7.70
Group 1	1.67 ± 0.83§	68.22 ± 21.67‖	24.25 ± 7.13	35.15 ± 13.70
Preserved drugs	1.95 ± 0.59#	77.77 ± 20.55§	26.40 ± 7.64¶	39.40 ± 13.96**
PF drugs	1.40 ± 0.96	58.68 ± 18.71	21.85 ± 6.26‡	26.45 ± 5.36‡
Group 2	2.45 ± 0.59	97.49 ± 24.99	35.05 ± 8.14	49.40 ± 8.55
Group 3	2.85 ± 0.47	104.84 ± 20.77	42.00 ± 5.83	55.00 ± 8.03

* $P < 0.001$ vs. Groups 2, 3, and dry eye; $P < 0.05$ vs. Group 1.
† $P < 0.001$ vs. Groups 1–3 and dry eye.
‡ $P < 0.001$ vs. Group 1 (overall), 2, 3, and dry eye.
§ $P < 0.05$ vs. PF drugs and controls.
‖ $P < 0.05$ vs. Groups 2 and 3.
¶ $P < 0.05$ PF drugs.
# $P < 0.05$ vs. PF drugs.
** $P < 0.001$ PF drugs.

TABLE 5. Confocal and IC Parameters in Group 1

| | LSCM Parameters | IC Parameters | | |
| | LTE | DC Density, Cells/mm² | HLA-DR, % | IL-6, % |
Drug Class				
Controls	0.48 ± 0.53*	21.94 ± 7.07†	1.95 ± 1.46‡	2.00 ± 1.71‡
Dry eye	2.20 ± 0.67	66.72 ± 17.32	37.10 ± 13.56	47.65 ± 7.70
Preserved ß-blockers	2.10 ± 0.22§	68.24 ± 61.82‖	21.70 ± 2.98#,**	27.40 ± 7.47¶,**
PF ß-blockers	1.80 ± 0.83	26.55 ± 8.39¶	12.60 ± 3.74¶	21.90 ± 3.66¶
Preserved PGA	1.60 ± 0.54§	89.58 ± 14.67‖	26.80 ± 9.33#	46.40 ± 16.58¶
PF PGA	1.21 ± 1.30	53.86 ± 15.31	16.80 ± 6.21	41.00 ± 11.62

* $P < 0.01$ vs. all drug classes and dry eye.
†, ‡ $P < 0.05$ vs. all drug classes and dry eye.
§ $P < 0.05$ vs. PF ß-blockers and PF PGA.
‖ $P = 0.004$ vs. PF drugs and controls.
¶ $P < 0.001$ vs. PF drugs.
# $P < 0.05$ vs. PF drugs.
** $P < 0.05$ vs. preserved PGA.
‡‡ $P < 0.05$ vs. PF PGA.

differences were found between drug classes in Group 1, and between overall Group 1 and controls.

Impression Cytology of the LTE

HLA-DR– and IL-6–positive cells were found in traces in controls, whereas were clearly observed in glaucomatous and dry eye patients (Table 4; Fig. 4). In overall Group 1, Groups 2, 3, and dry eyes the cell positivity percentages of both markers were significantly higher compared with controls ($P < 0.001$; Table 4). Significant differences were also found between overall preserved and overall PF-drugs for both HLA-DR and IL-6 ($P < 0.05$ and $P < 0.001$, respectively); no significant differences were found between Groups 2, 3, and dry eyes, for both markers (Table 4). In Group 1, significant differences were found for both markers between PF PGA and PF ß-blockers, and between preserved PGA and preserved ß-blockers (Table 5; Fig. 4).

Spearman's correlation analysis indicated that limbal DC density and LTE regularity significantly correlated with HLA-DR, IL-6, and OSDI score in glaucoma groups and dry eye ($P < 0.001$).

DISCUSSION

Most of the literature demonstrated that antiglaucoma medications negatively affect the ocular surface.[4,24–26] While the conjunctiva, cornea, and adnexa have been extensively studied,[3–6,8,14] dedicated analyses of the corneoscleral limbus are lacking.

Our study showed that the limbus changes morphology in response to the antiglaucoma therapy. These changes were for the most part inflammatory, especially in patients treated with preserved medications or controlled with multitherapy. Thus, inflammation (alone or combined with allergy or toxicity) can be hypothesized as the main mechanism involved in the iatrogenic limbal alterations, as proposed for other ocular surface structures.[5]

To date, limited information of the CL status was reported in researches that investigated the effects of antiglaucoma medications on conjunctiva and cornea. Baudouin et al.[4] observed inflammatory cell infiltration in conjunctival specimens taken close to the limbus, reporting a greater number of cells in patients receiving preserved timolol compared with patients receiving PF-timolol. Liang et al.[20] observed inflammatory cells with LSCM, in the limbal area of rabbit eyes
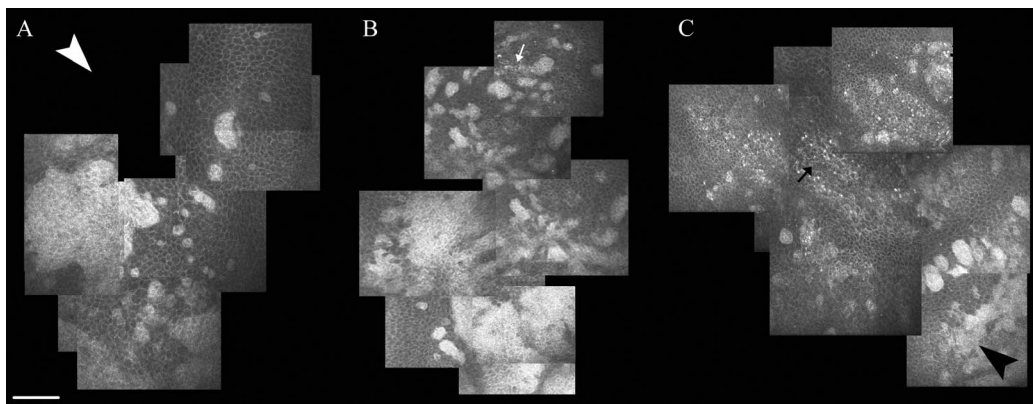

FIGURE 2. Planar reconstruction of the LTE. (**A**) Limbal transmission epithelium in a healthy eye: conjunctival-type bright epithelium with indistinct cell boundaries, presenting a gradual transition toward the corneal epithelium. The demarcation between the two types of epithelia is irregular, with small islands of corneal epithelium dispersed within the sheets of conjunctival epithelium. (**B**) Limbal transmission epithelium in dry eye: the normal architecture of the LTE is preserved. The epithelium of the small corneal epithelial islands appears hyperreflective; scattered punctate reflective elements (*white arrow*) are recognizable around the corneal epithelial islands. (**C**) Limbal transmission epithelium of Group 3: the normal architecture of the LTE is preserved, even though the epithelium shows an increased reflectivity and several punctate reflective elements (*black arrow*), especially in the conjunctival side of the limbus. *White* and *black arrowhead* indicates the conjunctival and corneal side of the limbus, respectively. *Scale bar*: represents 100 μm.
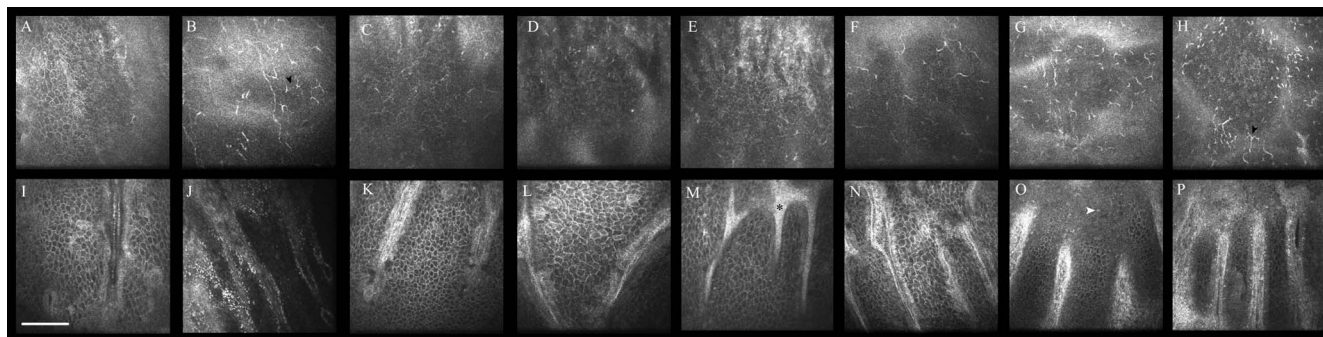
FIGURE 3. Dendritic cells distribution and features of POV at LSCM. (**A, B**) Dendritic cells distribution in healthy and dry eyes, respectively. (**C–F**) Dendritic cells distribution in Group 1 (PF-β-blocker; PF-PGA; preserved β-blocker; preserved PGA). (**G, H**) Dendritic cells distribution in Groups 2 and 3, respectively. DC show a dendritic shape (*black arrowheads*), and are significantly more represented in dry eye, and Groups 2 and 3; in Group 1 DCs are slightly, but significantly more represented in patients taking preserved medications. Punctate reflective elements appear interspersed between DCs, increasing in glaucoma groups (*arrowheads*). palisades of Vogt (taken at superior quadrants) showed linear radial stromal ridges (*asterisk*) more hyperreflective in Groups 2, 3, and dry eye; also punctate reflective elements were more commonly observed in Groups 2, 3, and dry eye (*arrowheads*; [**I**] healthy controls; [**J**] dry eye; [**K–N**] Group 1: PF-β-blocker; PF-PGA; preserved β-blocker; preserved PGA; [**O, P**] Groups 2 and 3, respectively). *Scale bar*: represents 100 μm.

receiving BAK preserved latanoprost or BAK 0.02%. Similar modifications were not observed in eyes receiving PF-tafluprost. These findings were also in line with a study of Pauly et al.,[19] who clearly observed limbal inflammatory cells after exposure to BAK preserved latanoprost or BAK 0.02%, but sporadically after exposure to PF-latanoprost, in an acute rabbit toxicologic model. Zhivov et al.[27] reported an increase of Langerhans cells in the corneal side of the limbus in healthy humans volunteers after 12-week application of 0.01% BAC solution. These studies highlighted the potential major role of BAK in inducing the limbal inflammation.

Our results were consistent with these findings because we documented a higher limbal inflammation in patients treated with BAK-preserved medications compared with patients treated with PF formulations, both with LSCM and IC. In detail, BAK stimulated the local immune system by activating and increasing the number of resident DC; moreover, the presence of punctate reflecting elements within the LTE, the basal epithelial membrane and the deeper POV represents an additional sign of inflammation. These punctate reflecting elements were considered as confocal signs of inflammation, previously reported within the acinar wall and the interstice of meibomian glands during inflammatory conditions, such as the meibomian gland disease and glaucoma therapy.[14,22] In our study, POV were recognized in all healthy subjects. This result was in agreement with previous evidence,[13] but in disagreement with others,[28] supporting an interindividual variability of

such structures.[29] In patients with glaucoma, despite the presence and global architecture of POV was not affected by therapy (probably because of the deep location of palisades), the presence of punctate reflecting elements at this level indicated a deep inflammation induced by drugs. These deep inflammatory modifications, moreover, were in line with inflammatory signs observed within the superficial limbal layers. However, one cannot exclude that after decades of therapy morphologic alterations of POV can also be observed.

These findings suggest the potential role of antiglaucoma therapy in disturbing the equilibrium of the environment where limbal stem cells reside. Evidence that the inflammatory aspects of POV were similar between glaucoma and dry eye, with the latter being recognized as a potential cause of limbal stem cell deficiency,[30] seems to further support this hypothesis. Francoz et al.[31] found that glaucoma medications and dry eye significantly lowered the limbal epithelial thickness, as seen with spectral-domain optical coherence tomography (SD-OCT). They proposed that limbal epithelial thickness might be intended as a surrogate of the limbal stem cells status since it appears to directly correlate and represent the stem cell density.[32] To further support this hypothesis, Lin et al.[33] reported that the instillation of BAK 0.5% four times per day for 4 weeks in a mouse model induced the typical manifestations of LSCD, including corneal neovascularization, severe inflammation in the stroma, and diffuse epithelial defect.
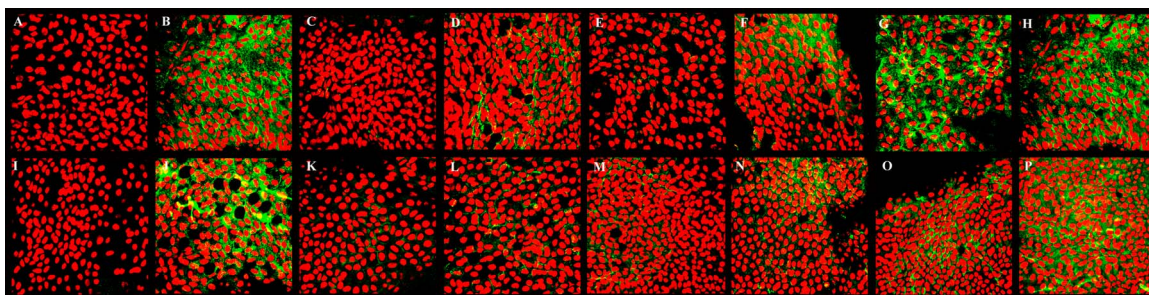

FIGURE 4. HLA-DR and IL-6 immune staining of the LTE. *Top line*: IL-6 expression positivity; *bottom line*: HLA-DR expression positivity. (**A, I**) healthy eyes; (**B, J**) dry eye; (**C–F**) and (**K–N**) Group 1: PF-β-blocker; PF-PGA; preserved β-blocker; preserved PGA; (**G, H**) and (**O, P**) Groups 2 and 3, respectively. The cytoplasmic positivity (*green*) of both markers are significantly more expressed in Group 1 taking preserved drugs, Groups 2, 3, and dry eye compared with Group 1 taking unpreserved drugs and controls. Nuclei of cells were stained with propidium iodide (*red*). Magnification ×630.

The mechanisms leading to POV alterations in glaucoma were not previously considered and, therefore, are unknown; nevertheless, inflammatory processes occurring within limbal epithelium and diffusing for contiguity in depth finally reaching POV, may be hypothesized.

In accordance with reports focused on other ocular surface structures, patients receiving PF drugs better preserved limbal morphology at LSCM, confirming the better ocular surface tolerability of medications without preservative.[3,6,8,14,15] However, DC density and immunoinflammatory marker positivity were higher in patients receiving PF medications compared with controls. The control subjects presented values in accordance with those previously reported.[34,35] This suggested the presence of a mild to moderate limbal inflammation also when using PF formulations, which was in accordance with previous observations.[24] In addition, our results also suggested that inflammation might be the first step in the cascade of limbal alterations, since the inflammatory parameters, such as DC density, HLA-DR and IL-6 positivity, were the main aspects that differentiated Group 1 from controls. In Group 1, we found a higher inflammatory activity of PGA on limbus with respect to β-blockers at both LSCM and IC. These results reflect the inflammatory nature of PG, and appear in line with modifications occurring to other structures such as Meibomian glands, which presented a pattern of interstitial inflammation slightly more evident in eyes treated with PF-PGA compared with eyes treated PF-β-blockers.[14]

Overall, the inflammatory changes were more pronounced in patients in multitherapy, and were quite similar to those observed in patients with dry eye. In detail, density of DC did not differ between multitreated glaucoma and dry-eye patients, suggesting that antiglaucoma drugs induced an activation of the local immune system similar to that observed in dry eyes. This was also confirmed by the immune-inflammatory marker positivity and scores of clinical parameters, which did not differ between multitreated glaucomatous patients and dry eyes. However, whether limbal modifications depends on the known primary toxicity of medications versus ocular surface,[3-6,8] or are a consequence of the iatrogenic dry eye cannot be ascertained. The increase of limbal DC and the presence of punctate reflective elements within the LTE, which are important in vivo markers associated with ocular surface diseases leading to dry eye,[11,36-38] are shared features. Evidence that dry eye is one of the possible mechanisms involved in the development of signs and symptoms of the glaucoma related ocular surface disease (OSD), further supports the hypothesis that limbal changes at least in part could depend on dry eye.[39,40]

Notably, the significant correlations between DC density, LTE regularity, HLA-DR, and IL-6 with clinical parameters indicated that also limbal modifications might take part in the induction of the glaucoma related OSD.

A remarkable consideration is that worsening of all considered outcomes was seen from the transition between one and two drug therapies. The addition of further drugs did not present statistically significant differences. Therefore, this result, which was in accordance with recent reports on Meibomian glands,[14] indicates that the addition of a second drug (and, probably, the higher exposure to BAK) should be the most pondered step in therapy evolution.

Some limitations of this study have to be pointed out. First, this is a cross-sectional study with a similar duration of disease in all groups: thus, it is unknown the progressive order of morphologic changes during time. Second, we did not include control groups receiving vehicles at different BAK concentrations, which would have been useful in the definition of the exact role of preservative and active compounds in limbal modifications. However, in humans this arises ethical concerns. Third, we cannot identify the initial site of damage and the first CL modification. Further prospective studies evaluating the initial CL status in therapy-naïve eyes, and the impact of medications over time are warranted to clarify these points.

In conclusion, LSCM identified limbal modifications indicating an increased inflammatory response induced by antiglaucoma drugs. These modifications, more evident in patients on preserved or multitherapy, have evident implications in the overall ocular surface health, considering the limbus hosts corneal stem cells. Therefore, where available, the use of PF monotherapy or PF-fixed combinations is advisable.

Further in vivo studies in patients with a longer follow-up are required to evaluate whether evident macroscopic morphologic changes of POV occur. In addition, immune-histology of limbal specimens aimed at assessing stem cell markers, will clarify the potentiality of antiglaucoma drugs as a risk factor for limbal stem cell deficiency.

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