In Vivo Confocal Imaging of the Conjunctiva as a Predictive Tool for the Glaucoma Filtration Surgery Outcome

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PURPOSE. To examine the preoperative conjunctival dendritic cell density (DCD), goblet cell density (GCD), and stromal meshwork reflectivity (SMR) in glaucomatous patients undergoing filtration surgery, using in vivo confocal microscopy (IVCM).

METHODS. Sixty-six patients were enrolled. At baseline, IVCM was performed at the site planned for surgery, and was repeated after 12 months at bleb site. Surgery was successful when a one-third reduction of baseline IOP was obtained at the last follow-up. The main outcomes were baseline DCD, GCD, and SMR, and 12 months IOP. The relations between baseline confocal parameters and 12 months IOP were analyzed.

RESULTS. Filtration surgery was successful in 43 patients (group 1: complete success, 25; group 2: qualified success, 18), and unsuccessful in 23 patients (group 3). Baseline IOP (mm Hg) was 27.6 ± 2.8, 28.8 ± 4.1, and 27.7 ± 3.2 in groups 1 to 3, respectively. Preoperative DCD and SMR were lower in group 1 compared with groups 2 ($P < 0.001$, $P < 0.05$), and 3 ($P < 0.001$); preoperative GCD was higher in group 1 compared with groups 2 and 3 ($P < 0.001$). DCD and GCD were also different between groups 2 and 3 ($P < 0.05$, $P < 0.001$). At 12 months, IOP reduced by 43.3%, 38.4%, and 15.8% in groups 1 to 3. Twelve-month IOP reduction negatively correlated with baseline DCD and SMR ($P < 0.001$, $r = -0.786$; $P < 0.05$, $r = -0.618$), and positively with GCD ($P < 0.001$, $r = 0.752$).

CONCLUSIONS. Preoperative DCD, GCD, and SMR are parameters correlated with the filtration surgery outcome, with DCD presenting the strongest correlation. IVCM of the conjunctiva may represent an imaging tool to predict the surgical success in glaucoma.

Keywords: primary open-angle glaucoma, glaucoma filtration surgery, filtering bleb, conjunctiva, in vivo confocal microscopy

Filtration surgery, which still represents the most diffuse surgical procedure to control IOP in glaucoma, leads to the formation of an intrascleral fistula draining the aqueous humor (AH) from the anterior chamber into the subconjunctival space (filtration bleb).[1] After reaching this space, AH is removed by different routes, such as the vascular or the trans bleb-wall routes.[2,3] Because of this, the conjunctiva is considered the most important structure affecting the glaucoma filtration surgery outcome.[4]

It has been widely demonstrated that the development of an unbalanced fibrosis at bleb site during the postoperative period may hinder the AH resorption, resulting in inadequate IOP control.[4,5]

Critical risk factors for bleb-wall fibrosis are represented by the long-term use of IOP-lowering medications, previous surgical manipulations of the conjunctiva, inflammatory ocular surface diseases, younger age, and the presence of profibrotic components in the AH filling the bleb cavity.[4–7] All these conditions promote chronic inflammation of the conjunctiva, which deeply stimulates local myofibroblasts after surgery and leads to filtration failure.[5] Unfortunately, the preoperative clinical examination of the ocular surface does not provide sufficient information about the inflammatory status of the conjunctiva and, thus, is inappropriate to predict surgery outcome.

Ocular surface biomarkers may overcome this limitation, guiding clinicians in assessing the risk of filtration failure in a more accurate way. Conjunctival gel-forming mucins, such as trefoil factor family 1 (TFF1) and MUC5AC, HLA-DR, goblet cells (GCs), and immuno-inflammatory cells, have been proposed as potential predictive indicators for filtration surgery outcome.[3,8–10] Moreover, Chong et al.[11] reported a correlation of preoperative tear levels of monocyte chemoattractant protein-1 (MCP-1) and propensity to bleb fibrosis. Nevertheless each of these parameters needs to be measured in an invasive way and reflects only in part the elements involved in postoperative filtration ability of the bleb.

In vivo confocal microscopy (IVCM) may provide rapid information of the most crucial conjunctival components that condition and are involved in the filtration ability of a bleb after surgery, in a noninvasive way.[3,10,12–15] Dendritic cells (DCs), GCs, and the stromal fibrosis, which are indicators of inflammation, trans bleb-wall AH flow, and bleb-wall AH resistivity, respectively, represent some of these key components.

Recently, the in vivo preoperative density of GCs has been reported to positively correlate with the surgical success in patients who are candidates for glaucoma surgery.[10] These initial results stimulated the interest on IVCM as a useful tool to estimate the preoperative risk of failure, based on the conjunctival conditions.

The aims of the present study were to correlate the preoperative conjunctival dendritic cell density (DCD), goblet cell density (GCD), and stromal fibrous meshwork reflectivity (SMR), with the 12-month success in glaucomatous patients undergoing filtration surgery, using IVCM.

METHODS

Patients

This was a 12-month, prospective, single center, case-control study. Sixty-six consecutive patients (66 eyes) with uncontrolled primary open-angle glaucoma (POAG) and candidates to receive mitomycin-C augmented Ex-PRESS (Alcon Laboratories, Fort Worth, TX, USA) implantation were consecutively enrolled. Fifteen healthy subjects (15 eyes) were also enrolled and served as controls. The research adhered to the tenets of the Declaration of Helsinki and our institutional review board (Department of Medicine and Ageing Science of the University 'G. d'Annunzio' of Chieti-Pescara, Chieti, Italy) approved the study. Written informed consent was obtained from all subjects before enrollment, after explanation of the nature and possible consequences of the study.

Inclusion criteria for patients with glaucoma were as follows: best corrected visual acuity (BCVA) ≥8/10, diagnosis of POAG, uncontrolled IOP (>21 mm Hg, mean of three measurements acquired during a diurnal tonometric curve) under maximal tolerated medical therapy (including oral acetazolamide; therapy had to be unmodified during the past 3 months), central corneal thickness (CCT) between 530 and 580 µm, progression of the visual field (VF) damage confirmed on three consecutive examinations (Humphrey field analyzer [HFA] II 750; Carl Zeiss Meditec Inc., Dublin, CA, USA [30-2 test, full-threshold]). VF damage progression was assessed with the trend-based analysis of the HFA Guided Progression Analysis software: when the magnitude of visual field index slope was worse than 1% per year with a $P \leq 0.05$, the progression was considered clinically significant. In the case both eyes were eligible, the eye with the higher IOP or the more advanced perimetric damage (glaucoma staging system 2),[16] was included in the study.

Exclusion criteria were the following: diagnosis of angle-closure, secondary or end-stage glaucoma; previous ocular surgeries, such as surgical procedures for ocular surface diseases, penetrating keratoplasty, retinal detachment surgery, or filtration surgery; indication for phaco-trabeculectomy or cataract surgery planned within 12 months thereafter; history of ocular diseases other than glaucoma; or systemic or topical therapies in the past 6 months potentially affecting the ocular surface status other than topical therapy for glaucoma, contact lens wear, and pregnancy.

Healthy controls had to show a BCVA ≥8/10, mean IOP lower than 18 mm Hg, CCT ranging from 530 to 570 µm,

absence of glaucomatous optic neuropathy, and normal VF and retinal nerve fiber layer thickness (Cirrus, version 6.0; Carl Zeiss Meditec) examinations. Exclusion criteria were a history of systemic or topical therapy, ocular or systemic diseases in the past 6 months, pregnancy, and contact lens wear.

Briefly, the surgical procedure required the following steps: creation of a fornix-based conjunctival flap; hemostasis of bleeding scleral vessels; application of sponges soaked in 0.02% mitomycin-C on the sclera and under the conjunctiva for 3 minutes, followed by irrigation with physiological saline solution; creation of a 4×5-mm-wide half-layer scleral flap by using a crescent knife; and penetration into the anterior chamber with a 27-gauge needle, followed by the insertion of the Ex-PRESS P200 (Alcon Laboratories). The scleral flap was sutured with 10-0 nylon, and the conjunctiva with absorbable 8-0 vicryl.[17] As per protocol after filtration surgery, topical unpreserved steroids were tapered in 8 weeks (dexamethasone 0.15% eye drops four times a day for 4 weeks and two times daily for the following 4 weeks), whereas topical unpreserved antibiotics were prescribed for 2 weeks (levofloxacin 5 mg/mL four times daily). Postoperative bleb management procedures, such as laser suture lysis and bleb needling with subconjunctival injection of 5-fluorouracil, were performed when needed.

Ex-PRESS implantation was considered successful when at least 30% reduction from preoperative IOP was obtained at the last follow-up (12 months). A complete success was achieved whether baseline IOP reduced at least 30% without antiglaucoma medications (group 1); a qualified success was achieved whether baseline IOP reduced at least 30%, with the use of antiglaucoma medications (group 2); failure was considered an IOP reduction less than 30% with antiglaucoma medications, at the last follow-up (group 3).[18] In case of failure, patients abandoned the study and underwent a further additional surgical procedure to control the IOP.

Both patients who were candidates for surgery and healthy subjects underwent a complete baseline ophthalmological assessment including BCVA (Early Treatment Diabetic Retinopathy Study chart), IOP measurement (Goldmann applanation tonometry), slit lamp anterior segment and dilated fundus examination, VF test, and IVCM (24–36 hours later).

Patients undergoing surgery were scheduled to receive a weekly follow-up in the first month, and monthly in the following 11 months; those who required any medical, laser, or surgical therapies, or developed ocular surface diseases during follow-up were excluded from the study. Baseline and 12-month data were considered for the statistical analysis.

IVCM of the Conjunctiva Confocal Parameters

IVCM was performed to evaluate the DCD, GCD, epithelial microcysts (EMs), and SMR. DCs appears as hyperreflective elements, with a different morphological feature according to their maturity and activation: mature cells present an elongated body with long membrane processes resembling nerve dendrites, whereas immature cells present a large body with rare membrane processes, if any. DCs can be observed within the epithelium (10–30 µm of depth) or within the close subepithelium (50–100 µm), isolated or in clusters, and work by modulating the immune response of the ocular surface toward external pathogenic stimuli.[19–21] GCs are roundish cells located within the conjunctival epithelium (10–30 µm), filled with mucin granules, and with a peripherally displaced hyporeflective nucleus. They appear larger than surrounding epithelial cells, hyperreflective, singly dispersed or crowded in clusters, and produce the mucin components of the tear film.[10,13,19,20,22] In addition, GCs have been proposed also as carriers of the AH through the bleb-wall epithelium in patients who underwent filtration surgery for glaucoma.[3,10] EMs are
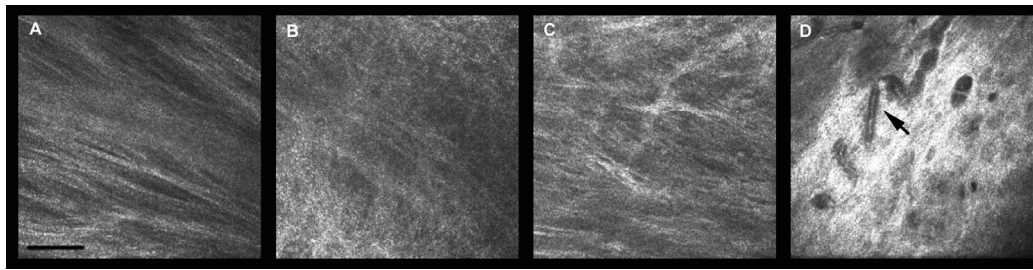
FIGURE 1. Arbitrary grading scale of the SMR. (**A**) Grade 0: normal SMR reflectivity (84.94), with thin collagen fibers presenting a regular and straight course. (**B**) Grade 1: mild reflectivity, with thin collagen fibers less regularly organized compared with Grade 0, and presenting different orientations (98.66). (**C**) Grade 2: moderate regularity, with collagen fibers appearing much thicker and with a less regular course compared with Grades 0 and 1 (111.88). (**D**) Grade 3: high reflectivity, with very thick and disorganized fibers, somewhere presenting as a dense, and irregularly homogeneous tissue (138.92); the *arrow* indicates a blood vessel. SMR images derived from representative glaucoma patients under medical therapy, not included in the sample. *Scale bar*: 100 μm.

round- or oval-shaped intraepithelial (10–30 μm) extracellular structures, isolated or sometimes confluent or clustered, with an inner hyporeflective feature. Sometimes they may host hyperreflective elements inside, such as presumed inflammatory cells or amorphous protein material. These structures have been considered in vivo hallmarks of AH percolation through the bleb-wall epithelium in successful filtration surgery.[3,6,10,14,15] The stroma of the conjunctiva presents a superficial adenoid layer (immediately posterior to the epithelium), which appears as a pale field with some collagen fibers and scattered cells, and a deep dense fibrous network with bundles of hyperreflective collagen fibers hosting blood vessels.[19] The definition and reference images of each confocal parameter were consistent with those reported in the literature.[3,10,13–15,19–23]

Confocal Microscopy Procedure

The technical characteristics of the laser scanning confocal microscope (HRT III Rostock Cornea Module, diode-laser 670 nm; Heidelberg Engineering, Heidelberg, Germany), and the details of conjunctival examination were reported elsewhere.[10,12,13,20] The device allows an automatic z-scan determination of depth of focus within the conjunctiva, provides a section thickness of approximately 10 μm, and a lateral and transverse resolution of 4 μm.

At baseline (48–72 hours before surgery) a 5 × 5-mm area of the upper bulbar conjunctiva, centered at 12 o'clock meridian (2–4 mm from the limbus) and corresponding to the bleb site after surgery, was analyzed. To explore this site, patients were instructed to direct the gaze downward for the entire duration of the confocal session. Images were acquired from the epithelium and subepithelium (10–50 μm of depth) to evaluate DCD and GCD, and from the deep stroma (50–150 μm of depth, where are located most parts of collagen fiber bundles) to evaluate the SMR.

After 12 months (or the last follow-up in failed cases), confocal microscopy was repeated at the bleb site to evaluate the mean microcysts density and area (MMD and MMA) taking particular attention to avoid bleb traumatisms in patients who underwent surgery.

Forty images (with a field of view of 400 × 400 μm) were acquired at the established areas and depths during baseline and 12-month sessions, and 10 randomly selected high-quality images without motion blur or compression lines were selected for the analysis. A single experienced IVCM operator performed the confocal examinations and selected the images (LB); a second experienced operator evaluated the images (VF). Both the operators were masked for the patient history and for grouping.

The Cell Count Software (Heidelberg Engineering GmbH) of the confocal microscope was used to determine the number of DCs and GCs (cells/mm² ± SD), and EM (cysts/mm² ± SD) in manual mode; the surface area of EM (μm² ± SD) was calculated using ImageJ software (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA).

To quantify the confocal image reflectivity of the deep stroma, we adopted a previously used arbitrary grading scale.[23] The SMR was calculated by determining the average gray value of the selected image using Image J software. 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 90.00 indicated a normal reflectivity (grade 0), from 90.01 to 105.00 mild reflectivity (grade 1), 105.01 to 125.00 moderate (grade 2), and greater than 125.01 high reflectivity (grade 3). Therefore, grades 0 to 3 corresponded to a loosely, mildly, dense, and very dense arranged stromal network, respectively (Fig. 1). The automatic brightness mode was selected during examination. The confocal examination lasted less than 5 minutes and no complications related to the procedure were reported.

The primary outcomes of the study were the baseline DCD, GCD, SMR, and 12-month IOP; the secondary outcomes were the 12-month MMD and MMA. The correlations between the baseline DCD with GCD and SMR; and the baseline DCD, GCD, and SMR with 12-month IOP, MMD, and MMA were evaluated.

Statistical Analysis

Analysis was performed by SPSS Advanced Statistical TM 13.0 Software and SPSS Sample Power Software (2005; Chicago, IL, USA). Student's t-test and χ^2 test were used to evaluate age, IOP, and sex differences between groups. Multivariate analysis of variance was used to confirm the prognostic value of variables among groups. Correlations among the variables were determined using a nonparametric measure by the Spearman's index. A $P < 0.05$ was considered statistically significant.

RESULTS

Patients

Sixty-six patients successfully completed the study. Serious intra- or postoperative complications were not reported in any case; none of the patients were treated with topical steroids to prepare the ocular surface for surgery or developed ocular surface diseases after surgery.

TABLE 1. Demography, Baseline, and Follow-Up Data

	Group 1	Group 2	Group 3	Controls
No. of patients	25	18	23	15
Age, y ± SD	58.32 ± 6.85	59.72 ± 7.24	62.09 ± 8.73	60.40 ± 6.43
Sex, Male/Female	12/13	10/8	11/12	8/7
Duration of disease, mo ± SD	45.23 ± 12.32	48.71 ± 10.10	46.14 ± 11.53	–
Baseline IOP, mm Hg, mean ± SD	27.60 ± 2.8	28.88 ± 4.1	27.74 ± 3.2	15.35 ± 2.9*
Last follow-up IOP, mm Hg, mean ± SD	15.64 ± 1.8†	17.79 ± 2.9†	23.35 ± 3.5	15.18 ± 2.7‡
Baseline no. of drugs	3.15 ± 0.41	2.95 ± 0.39	3.25 ± 0.47	–
Last follow-up no. of drugs	0§	1.80 ± 0.48‡	3.20 ± 0.50	–
Baseline MD, dB, mean ± SD	−15.21 ± 3.26	−16.43 ± 3.22	−14.58 ± 4.12	2.15 ± 0.45*
Last follow-up MD, dB, mean ± SD	−15.96 ± 3.02	−17.12 ± 1.99	−15.98 ± 3.41	1.95 ± 0.25*
Postoperative procedures¶	0.91 ± 0.28	1.12 ± 0.31	1.60 ± 0.42‖	–

MD, mean defect.
* $P < 0.001$ versus groups 1 to 3.
† $P < 0.05$ versus baseline and group 3.
‡ $P < 0.05$ versus group 3.
§ $P < 0.001$ versus groups 2 and 3.
‖ $P < 0.05$ versus groups 1 and 2.
¶ Laser suture lysis and needling with 5-fluorouracil.

Clinical Data

Table 1 summarizes the demographic information, and baseline and last follow-up clinical data. No significant differences were found between glaucoma patients and controls for demographic parameters. Medical therapy was not different between glaucomatous groups. All successful cases reached the 12-month follow-up, whereas the mean follow-up of failed patients was 210 ± 50 days.

At baseline, IOP did not differ between glaucoma groups but was significantly lower in controls ($P < 0.001$); at the last follow-up visit, IOP was significantly reduced in groups 1 and 2 ($P < 0.001$) without differences between them, whereas IOP did not change in group 3 and controls. Surgical success was obtained in 43 patients (65.2%), complete (group 1) in 25 patients, and qualified (group 2) in 18 (58.1% and 41.9%, respectively); surgery was unsuccessful (group 3) in 23 patients (34.8%). The number of postoperative procedures was significantly higher in group 3 compared with groups 1 and 2 ($P < 0.05$). At the last follow-up visit, IOP was reduced by 43.3%, 38.4%, and 15.8% in groups 1 to 3, respectively, whereas IOP did not change in controls.

In Vivo Confocal Microscopy

Qualitative Data. DCs, GCS, and EMs presented features similar to those described in previous confocal studies (Fig. 2).[3,8,10,19–23] DCs were mainly found within the subepithelial stroma or the basal epithelium. They were recognized both in the mature (slender nucleated cell body with associated medium to long dendrites) and immature (large cell body with fewer and shorter dendrites, or any) phenotypes dispersed among epithelial cells or in the subepithelium at various depths. In terms of morphology, no evident differences were observed among groups 1 to 3; compared with healthy subjects, glaucomatous patients displayed the typical branching phenotype of mature and activated cells. GCs were found both in glaucoma patients and controls within the epithelium, at 10 to 30 μm of depth, with the same morphological characteristics. EMs presented different features: whereas in healthy subjects they were small and scattered, with a surrounding capsule, in the bleb-wall epithelium they were larger, markedly hyporeflective, and frequently clustered or coalescent. The deeper fibrous layer of the stroma was recognizable in all subjects as a network of fiber bundles,

more evident and inhomogeneous in patients undergoing surgery with respect to healthy controls (Fig. 2).

Quantitative Data. In the multivariate analysis, no significant relations were found between age, sex, mean time on therapy, and number of medications and the surgical outcome. Conversely, analysis showed that DCD, GCD, and SMR were significantly associated with the surgery outcome.

Baseline DCD was significantly higher in glaucomatous groups compared with controls ($P < 0.001$), with values lower in group 1 with respect to groups 2 and 3 ($P < 0.001$). GCD was significantly lower in glaucomatous groups compared with controls ($P < 0.001$), with values higher in group 1 compared with groups 2 and 3 ($P < 0.001$) (Fig. 2). Group 2 showed DCD and GCD values significantly different compared with group 3 ($P < 0.001$, $P < 0.05$).

SMR was significantly higher in glaucoma groups compared with controls ($P < 0.001$), with values higher in group 3 compared with groups 1 ($P < 0.001$) and 2 ($P < 0.05$), and higher in group 2 compared with group 1 ($P < 0.05$). Conversely, no significant differences were found between groups 2 and 3 (Fig. 2). Baseline parameters are reported in Table 2.

At last follow-up, MMD and MMA were significantly higher in functioning cases compared with failed cases and controls ($P < 0.001$), with values significantly higher in group 1 compared with group 2 ($P < 0.001$) (Fig. 2).

Correlations

Spearman's correlation was used to test the strengths of association between baseline confocal parameters and last follow-up IOP, and the association between the number of postoperative bleb management procedures and the IOP reduction at the last follow-up.

At baseline, DCD correlated negatively with GCD and positively with SMR ($P < 0.001$, $r = −0.687$; $P < 0.05$, $r = 0.526$); no significant correlations were found between GCD and SMR. Baseline DCD and SMR negatively correlated with the IOP reduction at the last follow-up ($P < 0.001$, $r = −0.786$; $P < 0.05$, $r = −0.618$); baseline GCD positively correlated the IOP reduction at the last follow-up ($P < 0.001$, $r = 0.752$). The last follow-up MMD and MMA correlated negatively with the baselines DCD ($P < 0.001$, $r = −0.877$; $P < 0.001$, $r = −0.610$) and SMR ($P < 0.001$, $r = −0.716$; $P < 0.001$, $r = −0.510$) and positively with baseline GCD ($P < 0.001$, $r =$
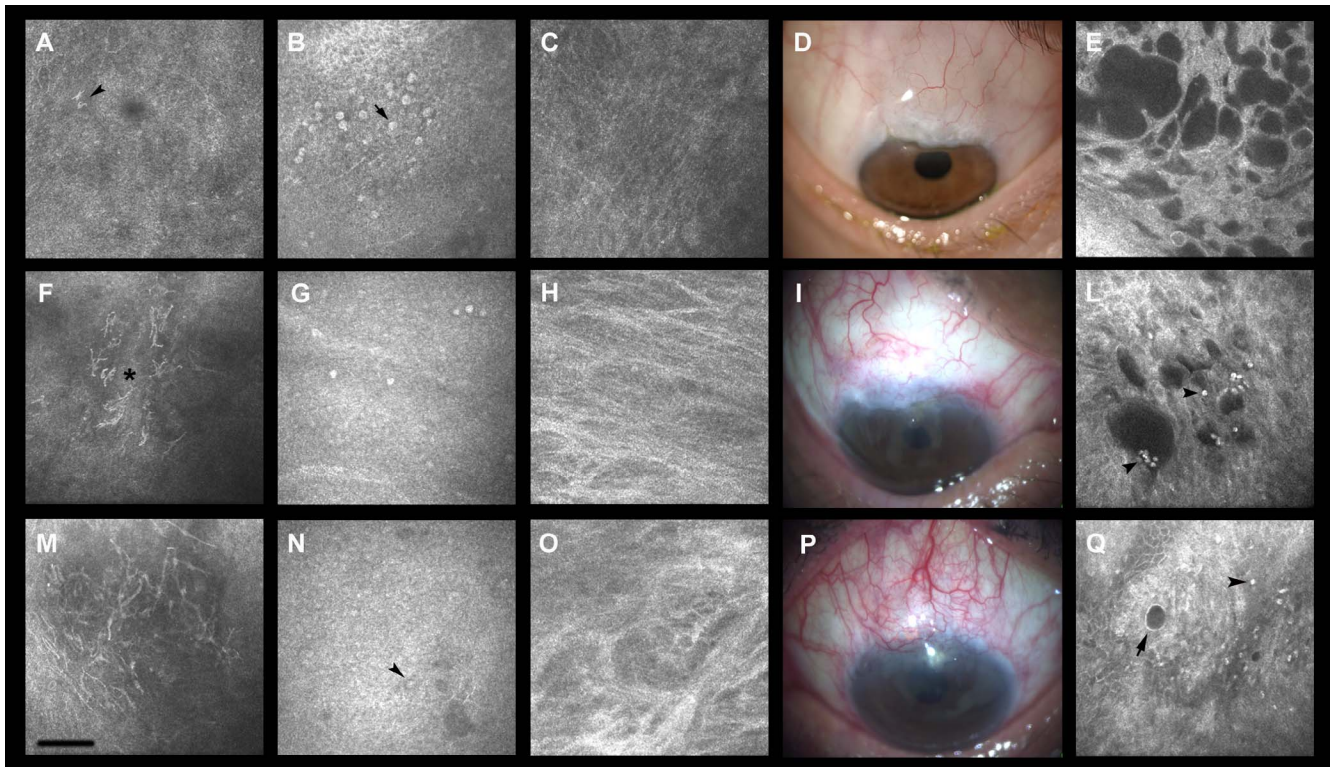
FIGURE 2. Preoperative IVCM of the conjunctiva (**A–C**; **F–H**; **M–O**). Group 1: (**A–C**) Rare DCs, more frequently in their immature phenotype (**A**; *arrowhead*), several GCs (**B**; *arrow*), and a loosely arranged hyporeflective stroma (**C**; SMR: 73.38) are the most common features. Group 2: (**F–H**) DCs are more common than in group 1, and show a mature phenotype, somewhat with a clustering tendency (**F**; *asterisk*); GCs are scattered and reduced (**G**), and the stroma is moderately hyperreflective with thicker collagen fibers (**H**; SMR: 124.04). Group 3: (**M–O**) Mature elongated DCs densely populate the subepithelial stroma (**M**); rare GCs are recognizable within the epithelium (**N**; *arrowhead*). The stroma appears highly reflective with a densely arranged feature, due to the presence of thick fibers bundles crossing each other (**O**; SMR; 132.90). Postoperative biomicroscopy of the bleb (**D**, **I**, **P**). In group 1, bleb presented either a diffuse or a cystic appearance (**D**), whereas in group 2 presented a diffuse feature, with a limited extension, reduced height, and increased vessels (**I**). In failed case (**P**), bleb showed a flat and hypervascularized feature. Postoperative IVCM of the bleb (**E**, **L**, **Q**). Groups 1 (**E**) and 2 (**L**) showed large and hyporeflective microcysts; these microcysts were much more numerous, and with a clustering tendency in patients with a complete success. Some hyperreflective elements, probably representing inflammatory cells, are recognizable inside or outside cysts (*arrowheads*) in group 2 (**L**). In failed cases (**Q**), EMs were rare (*arrow*), scattered and with smaller diameter compared with groups 1 and 2; hyperreflective elements, probably representing inflammatory cells (*arrowhead*) are also recognizable. *Scale bar*: 100 μm.

0.679; $P < 0.001$, $r = 0.756$). Mean number of postoperative procedures did not correlate with the IOP reduction in each group ($P = NS$). Baseline IVCM parameters did not correlated with the number of medications, number of preserved eye drops and duration of treatment ($P = NS$).

DISCUSSION

Besides demographic- and disease-related factors, the preoperative status of the conjunctiva represents an additional crucial factor for the outcome of filtration surgery.[4,18,24,25] Because of the long-term use of IOP-lowering medications, the conjunctiva undergoes deep alterations that reach the highest expression when medical therapy fails and surgery is needed.

The most common epithelial changes are represented by squamous metaplasia, immuno-inflammatory cell infiltration, DC activation, and GC loss, whereas the collagen deposition represents the main stromal modification.[4–6,9,10,25] All of these changes negatively affect the AH flow through the bleb wall after surgery.

IVCM has reached a consolidated position among imaging tools in ophthalmology, representing one of the elective techniques for ocular surface analysis. In glaucoma, IVCM proved essential to image tissue modifications induced by

medical therapy and to evaluate the bleb functionality after filtration surgery.[3,6,10,12–15,21,23,26]

In the present study, we found that in vivo imaging of the conjunctiva may predict the surgical outcome in patients who are candidates for filtration surgery. In detail, we observed that high preoperative levels of DCs, low levels of GCs, and a hyperreflective stroma at the site planned for surgery significantly increased the risk of bleb dysfunction and filtration failure. These parameters are an expression of the

TABLE 2. Baseline IVCM Parameters

	DCD, Cells/mm²	GCD, Cells/mm²	SMR, Arbitrary Scale
Group 1	87.44 ± 14.46*	36.00 ± 5.05*	1.04 ± 0.78†‡§
Group 2	113.66 ± 16.65||	26.22 ± 3.38¶	1.72 ± 0.67
Group 3	135.04 ± 19.92	22.95 ± 4.24	1.86 ± 0.75
Controls	24.15 ± 6.54	218.50 ± 28.43	0.68 ± 0.54

* $P < 0.001$ versus groups 2 and 3, and controls.
† $P < 0.05$ versus group 2.
‡ $P < 0.001$ versus group 3.
§ $P < 0.001$ versus controls.
\|\| $P < 0.001$ versus group 3.
¶ $P < 0.05$ versus group 3.

inflammatory status of the conjunctiva, the potential ability of the epithelium in vehiculating AH after surgery, and the stromal resistivity to the AH passage, respectively.[3,5,10] Therefore, they represent key structures because they directly or indirectly affect the filtration ability of a bleb.

DCs are antigen-presenting cells that populate the entire ocular surface and work by modulating the immune response toward local stimuli. They were considered a hallmark of inflammation in different ocular surface diseases (OSD), including the glaucoma therapy–related OSD.[19–21,23,26–28] The high preoperative DCD observed in patients with a poor surgical outcome was in line with numerous evidence, which reported that an inflamed conjunctiva represents a strong risk factor for bleb dysfunction.[4,5,7,9,10,25,29,30]

Different OSDs, such as immunological disorders, infections, chemical injuries, or the chronic instillation of topical drugs, can cause stromal fibrosis.[31] Between drugs, antiglaucoma medications are well recognized promoters of collagen deposition, because they increase TGF-β levels and stimulate stromal myofibroblasts.[4–6,25,30–32]

Our study supported this evidence, because a hyper-reflective stroma (expression of fibrosis) was commonly observed in patients under medical therapy compared with healthy controls. Moreover, the significant positive correlation between DCD and SMR supports that the iatrogenic inflammation represents the promoter of the conjunctival scarring processes. In terms of surgical outcome, a higher preoperative stromal reflectivity negatively correlated with the IOP reduction, this suggesting that a thick and densely arranged stroma before surgery reduces the AH filtration ability of a bleb. These findings are in accordance with clinical studies that reported a significant relation between preoperative fibrosis and filtration failure.[4,5,25,29,30,32]

Considering GCs, we found that high preoperative levels of these cells were associated with a good surgical outcome. This was also supported by the positive correlation between preoperative GCD and postoperative MMD and MMA, which are consolidated confocal markers of AH filtration through the bleb wall. The significant relation between preoperative GCD and the surgical outcome is in line with immunocytological and confocal studies in which GCs and GC-derived mucins were found to correlate with the filtration ability after trabeculectomy.[3,8,10] This evidence further supports the already proposed theory that GCs are cytological carriers of AH through the bleb wall.[3,10]

Between confocal parameters, DCD presented the strongest correlation with IOP reduction and, therefore, with surgical success. This is not surprising, because inflammation represents the first step in the development of conjunctival changes induced by drugs, and in initiating and maintaining bleb scarring processes after surgery.[4,7,14,23,25,29,30,33] This correlation is also in line with numerous evidence reporting a robust association between the surgical success and the number of medications, the preoperative daily doses of benzalkonium chloride (BAK), and the duration of treatment.[25,29,34,35] In fact, all these parameters act as strong inflammatory triggers.

The primary impact of preoperative conjunctival status on surgical outcome seems also supported by the absence of correlation between postoperative procedures and IOP reduction; this may rule out the possibility that the high success rate in groups 1 and 2 could be related to a more intensive bleb management.

As common experience in the daily practice, the clinical observation of the conjunctiva or the use of routine ocular surface tests does not accurately reflect the real tissue conditions. Therefore, the only clinical assessment before surgery cannot provide accurate information to define the best time for surgery; to adopt the most appropriate measures before, during, and after surgery, aimed at controlling the bleb fibrosis; and to predict the final outcome.

In the attempt to overcome this limitation, different molecular biomarkers, such as HLA-DR, GCs, MUC5AC, TFF1, MCP-1, or immuno-inflammatory cells markers (CD3, CD4, CD8, CD20, CD38, CD68) have been proposed over the years.[8–10,29,33,36] However, all these biomarkers are indicators of the epithelial inflammation and GC status.

Our results support the potential role of these molecular biomarkers because the in vivo DCD and GCD can be considered as epithelial indicators of the surgical outcome; in addition, we introduced a stromal indicator of the conjunctiva, which is the confocal reflectivity. In this way, to predict surgical outcome, we proposed full-thickness, multiparametric, in vivo imaging of the conjunctiva that concomitantly considered all the elements conditioning the AH passage through the bleb wall. In addition, with respect to ex vivo techniques, IVCM permits imaging of all conjunctival layers in a rapid, noninvasive, and cost-effective way.

The present study has some limitations. First, although GCs and DCs are objectively measurable parameters, the stromal reflectivity is an arbitrary index. Nevertheless, the selection of the automatic brightness mode during the image acquisition, and the calculation of the average gray value of the image according to numeric values provided by ImageJ, significantly reduce the arbitrariness of the method. Second, the study cannot elucidate whether the DCD, GCD, and SMR before medical therapy was different between groups. Thus, prospective studies following patients from initial diagnosis to surgery are required. Third, given that the number of medications and the duration of therapy before surgery were similar between groups, we cannot clarify why group 1 presented higher levels of GCD, and lower levels of DCD and SMR compared with the other groups. Nevertheless, medical therapy–related factors are the most probable candidates: in fact, the use of preservative-free IOP-lowering formulations, associative fixed combinations, lubricants, or short periods of steroids, could have contained the OSD. Even though patient charts did not methodically report this information, it could be hypothesized that patients belonging to group 1 could have had a more preserved ocular surface during medical management of the disease. Fourth, there is also the possibility of normal interindividual variability in the pool of DCs and GCs, and in the stromal density of the conjunctiva, as previously proposed.[10,20]

Even if promising, at this stage, IVCM cannot be proposed as a predictive imaging biomarker because validation steps defining the sensitivity, specificity, and repeatability of considered parameters in larger patient populations are required. In addition, prospective confocal studies testing the potential impact of the preoperative use of anti-inflammatory agents in improving the conjunctival status and the surgical outcome could further corroborate these initial results.

In closing, our study confirmed that inflammatory conjunctival alterations play a critical role in patients undergoing filtration surgery. In this optic, strategies aimed at reducing the ocular surface inflammation before surgery are strongly recommended, because they may positively affect the bleb functionality-related factors. Whether these results will be validated, IVCM could be considered as an imaging tool to stage the iatrogenic damage of the ocular surface, and the in vivo confocal status of the conjunctiva proposed as a predictive biomarker of the surgical outcome. This will guide clinicians in determining the best time for surgery, and in adopting the most appropriate perioperative strategies to contain the bleb fibrosis.

Acknowledgments

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