

# In Vivo Goblet Cell Density as a Potential Indicator of Glaucoma Filtration Surgery Outcome

Luca Agnifili,<sup>1</sup> Vincenzo Fasanella,<sup>1</sup> Rodolfo Mastropasqua,<sup>2</sup> Paolo Frezzotti,<sup>3</sup> Claudia Curcio,<sup>4</sup> Lorenza Brescia,<sup>1</sup> and Giorgio Marchini<sup>2</sup>

<sup>1</sup>Ophthalmology Clinic, Department of Medicine and Aging Science, University G. d'Annunzio of Chieti-Pescara, Chieti, Italy

<sup>2</sup>Ophthalmology Unit, Department of Neurological, Neuropsychological, Morphological and Movement Sciences, University of Verona, Verona, Italy

<sup>3</sup>Department of Ophthalmology, University of Siena, Siena, Italy

<sup>4</sup>Excellence Eye Research Center, CeSI, University G. d'Annunzio of Chieti-Pescara, Chieti, Italy

Correspondence: Luca Agnifili, Ophthalmology Clinic, Via dei Vestini, 66100, Chieti (CH), Italy; l.agnifili@unich.it.

Submitted: January 31, 2016

Accepted: April 25, 2016

Citation: Agnifili L, Fasanella V, Mastropasqua R, et al. In vivo goblet cell density as a potential indicator of glaucoma filtration surgery outcome. *Invest Ophthalmol Vis Sci*. 2016;57:2928-2935. DOI:10.1167/iivs.16-19257

**PURPOSE.** We analyzed the preoperative conjunctival goblet cell density (GCD), MUC5AC, and HLA-DR in glaucomatous patients undergoing trabeculectomy, using laser scanning confocal microscopy (LSCM) and impression cytology (IC).

**METHODS.** We enrolled 57 patients undergoing trabeculectomy. At baseline LSCM and IC were performed at the site planned for surgery; LSCM was repeated after 12 months at the bleb site. The main outcomes were: GCD, mean microcyst density (MMD) and area (MMA) at LSCM, MUC5AC, and HLA-DR positivity at IC, and IOP. The relationships between baseline GCD, and 12-month IOP, MMD, and MMA were analyzed.

**RESULTS.** Trabeculectomy was successful in 39 patients (complete success in 27, Group 1; qualified in 12, Group 2), and unsuccessful in 18 (Group 3). At baseline IOP (mm Hg) was  $27.2 \pm 3.12$ ,  $27.5 \pm 2.23$ , and  $27.7 \pm 1.90$  in Groups 1 to 3, respectively; GCD and MUC5AC positivity were higher in Group 1 compared to Groups 2 and 3 ( $P < 0.05$ ); HLA-DR, MMD, and MMA were not significantly different among the groups. At 12 months, IOP reduced by 45.3%, 35.4%, and 12.8% in Groups 1 to 3, respectively. Goblet cell density did not change in Group 1, whereas it was reduced in Groups 2 and 3 ( $P < 0.05$ ), with values lower in Group 3. Mean microcyst density and MMA increased in Groups 1 and 2 ( $P < 0.05$ ), with values higher in Group 1 ( $P < 0.05$ ). Baseline GCD positively correlated with 12-month IOP reduction ( $P < 0.001$ ,  $r = 0.641$ ), MMD ( $P < 0.05$ ,  $r = 0.454$ ), and MMA ( $P < 0.001$ ,  $r = 0.541$ ).

**CONCLUSIONS.** Goblet cells positively affect the filtration ability after trabeculectomy; therefore, preoperative GCD could be considered as a potential in vivo biomarker of surgical success.

**Keywords:** conjunctival goblet cells, MUC5AC, HLA-DR, trabeculectomy, in vivo confocal microscopy, impression cytology

Filtration surgery is one of the most effective surgical procedure for lowering IOP in patients with medically uncontrolled glaucoma.<sup>1,2</sup> Successful surgery leads to an elevation of the conjunctiva at the surgical site, which is referred to as a filtering bleb. The aqueous humor (AH) flows into bleb spaces and then is removed by several routes, such as the transconjunctival routes.<sup>3-5</sup>

The transconjunctival pathway was documented after intracameral injection of fluorescein in patients undergoing trabeculectomy.<sup>6</sup> Laser scanning confocal microscopy (LSCM) confirmed the existence of this pathway in vivo,<sup>7</sup> also in patients who did not undergo previous surgery (as the terminal portion of the uveoscleral AH outflow).<sup>8</sup> In functioning blebs LSCM observed optically clear microcysts within the conjunctival epithelium, which were expressions of AH percolation toward the ocular surface; conversely, in failed blebs these structures were rare or absent. Thus, microcysts were interpreted as transcellular vesicles transporting AH through the bleb wall. Amar et al.<sup>9</sup> clarified the nature of epithelial microcysts (EM): based on the topographic correspondence between MUC5AC-positive cells and epithelial empty spaces in

functioning blebs, the authors proposed that EM corresponded to goblet cells (GC) containing AH. Therefore, the transconjunctival AH pathway through the bleb wall epithelium was hypothesized to occur at the level of GC.

Goblet cell density (GCD) and MUC5AC, the main mucin product of GC, were found significantly overexpressed in successful compared to failed surgeries: histology and confocal microscopy showed that a higher postoperative GCD was related to a good IOP control and a higher success rate.<sup>9-11</sup> On this basis, the preservation of GC seems critical because the final filtering ability of a bleb could depend on the number of GC available before, and surviving surgery. The aim of the present study was to correlate the preoperative conjunctival GCD and MUC5AC positivity, using in vivo LSCM and impression cytology (IC), with the 12-month success in patients undergoing filtration surgery.

## METHODS

### Patient Selection

This was a 12-month, prospective, interventional, case-control study. The patients were treated in accordance with the criteria



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 enrolled 57 consecutive Caucasian patients (57 eyes) with uncontrolled open angle glaucoma scheduled to undergo MMC augmented trabeculectomy.

A total of 15 consecutive age- and sex-matched healthy subjects were used as controls. 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 who were surgical candidates were the following: diagnosis of open angle glaucoma (including primary open-angle, pseudoexfoliative, and pigmentary glaucoma), uncontrolled IOP ( $>20$  mm Hg, mean of three measurements at 9 AM, 12 PM, and 4 PM) under maximal tolerated medical therapy (MTMT; unmodified during the last 3 months), progression of glaucomatous damage confirmed on three consecutive visual fields (VF; 30-2 test, full-threshold, Humphrey field analyzer II 750; Carl Zeiss Meditec, Inc., Dublin, CA, USA). Maximum tolerated medical therapy required the use of all available classes of IOP lowering eye-drops and oral acetazolamide, without significant side effects. Visual field 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  $P$  value  $\leq 0.05$ , the progression was considered clinically significant.

Subjects were included if they had histories of laser trabeculectomy or cataract surgery, performed at least 6 months before enrollment.

If both eyes were eligible, the eye with the higher IOP level or the more advanced stage of glaucoma (according to the glaucoma staging system 2 [GSS2]),<sup>12</sup> was included in the study.

Exclusion criteria were diagnosis of angle-closure, normal tension, neovascular or uveitic glaucoma; history of previous incisional glaucoma surgery, penetrating keratoplasty, extracapsular cataract extraction, cataract extraction planned at the time of filtering surgery or within 12 months thereafter; history of ocular diseases in the operated eye other than glaucoma and cataract; systemic or topical therapies in the last 6 months that could have modified the AH hydrodynamics or the ocular surface contact lens wear; and pregnancy. Patients who received medical, laser, or surgical therapy, or who had ocular surface diseases during the follow-up were excluded from the study. Surgery was considered successful when at least 30% reduction from preoperative IOP was obtained at 12 months. Eyes that met the above criteria and were not on supplemental anti-glaucoma medical therapy were defined as achieving a complete success, whereas eyes that met the criteria with supplemental medical therapy were defined as achieving a qualified success.<sup>13</sup>

Inclusion criteria for controls were best-corrected visual acuity greater than or equal to 8/10, mean IOP lower than 18 mm Hg, absence of glaucomatous optic neuropathy, and a normal visual field examination. 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 6 months. Pregnant women and contact lens wearers were excluded. Both eyes were evaluated, but one eye per control subject was chosen randomly (using a computer generated random number list) for the statistical analysis.

Baseline evaluations included measurement of visual acuity and IOP (Goldmann applanation tonometry; mean of three measurements), slit-lamp anterior segment and fundus examination, visual field, and LSCM, and IC.

Patients underwent a weekly follow-up in the first month after surgery, and monthly afterwards; baseline and 12-month data were considered for the statistical analysis.

## LSCM of the Conjunctiva

We performed LSCM to evaluate GC density and EM density and area. For the examination of presumed GC and microcysts, we adopted a definition and reference images of GC and EM consistently with those reported in the literature.<sup>7-9,14-19</sup>

Presumed GC were defined as large to giant oval-shaped cells with central or peripherally displaced hyporeflexive nuclei, hyperreflexive and two/three times larger than the surrounding epithelial cells, dispersed within the epithelium or crowded in groups. Microcysts were defined as round- or oval-shaped extracellular structures, optically clear because of the low internal reflectivity, dispersed or clustered within the epithelium, occasionally containing amorphous material or inflammatory cells.

For days before filtration surgery the upper bulbar conjunctiva was examined using a digital confocal laser-scanning microscope (HRT III Rostok Cornea Module [RCM]; Heidelberg Engineering, Heidelberg, Germany). The technical characteristics of this instrument and the details of conjunctival examination have been described previously.<sup>7-9</sup>

The confocal laser-scanning device was equipped with a water immersion objective (633/N.A. 0.95 W; Carl Zeiss Meditec, Inc.) and permitted an automatic z-scan determination of depth of focus within the conjunctiva. Thus, high-contrast digital images of epithelium (15–30  $\mu$ m of depth; 2 mm from the limbus at the site of the planned surgery in downward gaze) with a field of view of  $400 \times 400$   $\mu$ m were acquired. The automatic brightness mode was selected during examination.

To analyze the same conjunctival portion with LSCM and IC, a  $5 \times 5$  mm area of the upper bulbar conjunctiva, centered at 12 o'clock meridian of the limbus, was marked using methylene blue. In eyes that were scheduled for surgery, this area corresponded to the site where a bleb would be positioned. Particular attention was adopted in maintaining the RCM (and the strip for IC afterwards) within the marked area.

We acquired 48 images for each eye, and 12 randomly selected high-quality images without motion blur or compression lines were selected for the analysis. The Cell Count Software in manual mode was used to determine the number of GC and EM.

The surface area of EM was calculated using ImageJ software (<http://imagej.nih.gov/ij/>; provided in the public domain by the National Institutes of Health [NIH], Bethesda, MD, USA).

Eyes that underwent surgery received unpreserved topical steroids for 4 weeks (unpreserved dexamethasone 0.15% eye-drops four times a day for 2 weeks and three times daily for the following 2 weeks) and topical antibiotic for the initial 2 weeks (unpreserved levofloxacin 5 mg/mL 4-fold daily). Postoperative procedures, such as subconjunctival injections of 5-fluorouracil (5-FU), laser suture lysis, and bleb needling, were performed when needed. Laser scanning confocal microscopy was repeated after 12 months at the bleb site to evaluate GCD, mean microcyst density (MMD), and mean microcyst area (MMA).

A single operator (LB) performed all confocal examinations and selected the images, which were evaluated by a second operator (VF). Operator VF and operator LB during the image selection were masked for the subject history and for grouping. Confocal session lasted less than 5 minutes, and none of the patients reported visual symptoms and complications related to the procedure.

Two masked investigators (VF, investigator 1; LB, investigator 2) independently calculated the GCD, MMD, and MMA to assess the interobserver variability.

## IC of the Conjunctiva

Impression cytology was performed to evaluate the expression of HLA-DR and MUC5AC in the conjunctival epithelium, at the site planned for surgery, within the marked area, at baseline. Since IC is a more invasive procedure with respect to IVCM, the examination was not repeated after 12 months to avoid any potential bleb damage. Impression cytology sampling was performed from 36 to 48 hours after confocal microscopy to avoid misinterpretation due to the mechanical pressure during execution of the LSCM. After instillation of 1 drop of 0.4% oxybuprocaine, a strip (previously cut in  $5 \times 5$  mm to match the marked area) was applied on the upper bulbar conjunctiva (with the eye in downward gaze), and then pressed gently by a glass rod to obtain a cytological biopsy. Impression cytology samples were collected using Millicell-CM 0.4 mL (12 mm of diameter; Millipore, Bedford, MA, USA) membrane. The Millicell 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 2 washes with Wash Buffer (Dako, Glostrup, Denmark) of 2 minutes each. Then, Ribonuclease A (Sigma-Aldrich Corp., St. Louis, MO, USA) diluted 1:290 in PBS was incubated for 20 minutes at room temperature. The specimens were washed and PBS-BSA 1% was added for 1 hour at room temperature. Finally, each membrane was cut in two pieces and incubated overnight at  $4^{\circ}\text{C}$  with MUC5C or HLADR diluted 1:50 (Dako).

Samples were washed and anti-mouse IgG1 Alexa fluor 488 (Invitrogen, San Giuliano Milanese, Italy) diluted 1:200 and propidium iodide at 1:150 were added and incubated for 1 hour at room temperature. Membranes were mounted with a drop of Fluorescent Mounting Medium (Dako) and Zeiss Confocal LSM 510 (Carl Zeiss MicroImaging GmbH, Vertrieb, Germany) was used to visualize the cells. Five different fields for each IC sample were evaluated. Positive (red nucleus and green cytoplasm) and negative (red nucleus) cells were counted. Minor irregularities that may have been present after cutting the membranes were normalized by reporting the percentage of positive cells for HLA-DR or MUC5AC.

Two independent observers (CC and AM) masked to the details of the staining technique performed evaluations of IC specimens. Digital images of representative areas were taken.

None of the patients reported complications related to IC sampling. Healthy control subjects underwent IC with the same modality.

The primary outcomes were the baseline GCD at LSCM and 12-month IOP; the secondary outcomes were the baseline positivity of MUC5AC and HLA-DR at IC, and 12-month GCD, MMD, and MMA at LSCM.

The correlations between GCD and MUC5AC, GCD, and HLA-DR, and MUC5AC and HLA-DR at baseline were evaluated; the correlations between baselines GCD, MUC5AC, and HLADR and 12-month IOP, MMD, and MMA also were evaluated.

## Statistical Analysis

Analysis was performed by SPSS Advanced Statistical TM 13.0 Software and SPSS Sample Power Software (2005; SPSS, Inc., Chicago, IL, USA). Student's *t*-test and  $\chi^2$  test were used to evaluate age and sex differences between groups. A 1-way ANOVA with post hoc Tukey for multi-comparison was used to assess differences among groups. ANOVA repeated measures, was used to assess differences between baseline and 12 months.

The sample size was established calculating a rate difference of at least 50% between groups for  $\chi^2$  or Fisher's

exact tests and of 52% for Mann-Whitney *U* and Wilcoxon tests, for a power of 80%.

Correlations among the variables were determined using a nonparametric measure by the Spearman's index. A *P* value  $< 0.05$  was considered statistically significant. The values obtained by investigator 1 (VF) were used for the statistical analysis, and the values obtained by investigator 2 (LB) were used to assess the interobserver agreement.

## RESULTS

The demographic and clinical data of the enrolled patients are shown in Table 1. All patients completed the study and no major intra- or postoperative complications occurred. No patient received steroids before surgery or had ocular surface diseases during follow-up.

### Clinical Data

The surgical procedure was successful in 39 patients (68.4%), with complete (Group 1) and qualified (Group 2) success in 27 and 12 patients (69.2% and 30.7%), respectively; surgery was unsuccessful (Group 3) in 18 patients (31.6%).

Baseline IOP was not significantly different between glaucoma groups, but was significantly lower in controls; after 12 months, IOP significantly reduced in Groups 1 and 2 ( $P < 0.05$ ) without differences between Groups, whereas it did not change in Group 3 and controls. The 12-month IOP reduction was 45.3% and 35.4% in Groups 1 and 2, respectively, and 12.8% in Group 3. Table 2 reports the baseline therapy of the groups.

### Laser Scanning Conformal Microscopy

Baseline and 12-month LSCM parameters are reported in Table 3. Goblet cells and EM were observed in all glaucoma patients and in controls, and were morphologically similar to those described previously.<sup>7-9,15-17</sup> Goblet cells were readily observed within the epithelium at 20  $\mu\text{m}$  of depth, and appeared as large cells, hyperreflective, and oval-shaped with hyporeflexive nuclei, larger than the surrounding epithelial non-goblet cells, crowded in groups or dispersed within the epithelium (Figs. 1-3A).

Microcysts appeared as round- or oval-shaped extracellular structures, ranging from 10 to 90  $\mu\text{m}$  in size, with an optically clear aspect due to the low internal reflectivity, located at the intermediate layer of the epithelium (10-30  $\mu\text{m}$  in depth Figs. 1-3C, 3F). Occasionally, they were filled with amorphous material or mononucleate hyperreflective elements probably corresponding to inflammatory cells.

At baseline GCD was significantly higher in Group 1 compared to Groups 2 and 3 ( $P < 0.05$ ), but significantly lower compared to controls ( $P < 0.001$ ; Figs. 1-3A). Mean microcyst density and MMA were not significantly different between glaucoma groups, and were significantly lower in controls ( $P < 0.05$ ; Figs. 1-3C).

After 12 months, GCD did not significantly change in Group 1, whereas it was significantly reduced in Groups 2 and 3 ( $P < 0.05$ ), with values lower in Group 3 (Figs. 1-3E). Mean microcyst density and MMA significantly increased in Groups 1 and 2 ( $P < 0.05$ ), with values significantly higher in Group 1 ( $P < 0.05$ ; Figs. 1-3F). Mean microcyst density and MMA presented a 7- and an 8-fold increase in Group 1, respectively, and a 3-fold increase in Group 2. Microcyst parameters did not significantly change in Group 3.

For the purpose of determining interobserver variability, all three groups of patients and healthy controls were combined.

TABLE 1. Demographics and Clinical Characteristics of Glaucomatous Groups and Controls

	Group 1	Group 2	Group 3	Controls
Patients	27	12	18	15
Age, y ± SD	64.8 ± 10.16	61.7 ± 8.65	63.6 ± 9.88	62.4 ± 6.43
Sex, M/F	14/13	7/5	9/9	8/7
Preoperative mean time of therapy, mo ± SD	48.23 ± 8.32	51.71 ± 10.10	49.14 ± 9.52	-
Baseline IOP, mean mm Hg ± SD	27.2 ± 3.12	27.5 ± 2.23	27.7 ± 1.90	14.5 ± 3.90*
12-mo IOP, mean mm Hg ± SD	15.1 ± 1.79†	17.7 ± 1.88†	24.1 ± 1.46	15.1 ± 2.75‡
Baseline N° of drugs, mean ± SD	3.1 ± 0.41	2.9 ± 0.39	3.3 ± 0.47	-
12-mo N° of drugs, mean ± SD	0§	1.90 ± 0.48‡	2.90 ± 0.50	-
Baseline MD, mean dB ± SD	-9.21 ± 3.34	-8.43 ± 2.22	-9.89 ± 4.12	1.1 ± 0.4*
12-mo MD, mean dB ± SD	-9.96 ± 3.02	-8.12 ± 1.99	-10.98 ± 3.81	1.3 ± 0.2*
Postoperative procedures	0.9 ± 0.3	1.2 ± 0.7	1.9 ± 0.5	-

\* *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.

The baseline GCD, MMD, and MMA estimated by investigator 1 (VF) were not significantly different (*P* > 0.05) from that estimated by the investigator 2 (LB) (Table 4).

**Impression Cytology**

Baseline IC results are reported in Table 5. Human leukocyte antigen DR (HLA-DR) positivity did not show significant differences between groups, but was significantly lower in controls (*P* < 0.001; Figs. 1-3D). Positivity for MUC5AC was significantly higher in Group 1 with respect to Groups 2 and 3 (*P* < 0.05), and in Group 2 compared to Group 3 (Figs. 1-3B). In healthy controls HLADR was found in traces, whereas MUC5AC positivity was markedly higher compared to glaucomatous groups (*P* < 0.001).

**Correlations**

At baseline, Spearman's correlation analysis indicated that GCD correlated significantly with MUC5AC positivity, whereas it correlated negatively with HLADR positivity (*P* < 0.001, *r* = 0.556; *P* < 0.05, *r* = -0.402). Baseline MUC5AC and GCD correlated positively with IOP reduction (*P* < 0.001, *r* = 0.725; *P* < 0.001, *r* = 0.641), MMD (*P* < 0.05; *r* = 0.325; *P* < 0.05, *r* = 0.454), and MMA at 12 months (*P* < 0.001, *r* = 0.615; *P* < 0.001, *r* = 0.541). Baseline HLADR positivity correlated negatively with 12-month IOP reduction (*P* < 0.001; *r* = -0.734), MMD (*P* < 0.05; *r* = -0.235), and MMA (*P* < 0.05; *r* = -0.215). The 12-month GCD correlated positively with the IOP reduction (*P* < 0.001, *r* = 0.456), MMD (*P* < 0.001, *r* = 0.815), and MMA (*P* < 0.001, *r* = 0.672).

TABLE 2. Therapy of Glaucomatous Patients

	Group 1	Group 2	Group 3
PGA	(9) 14.06%	(5) 17.24%	(7) 18.42%
PF - PGA	(9) 14.06%	(3) 10.34%	(5) 13.15%
β-blockers	(5) 6.25%	(2) 6.89%	(3) 7.89%
PF - β-blockers	(4) 7.81%	(2) 6.89%	(3) 7.89%
Brimonidine	(8) 12.50%	(4) 13.79%	(5) 13.15%
Topical CAI	(14) 18.75%	(5) 17.24%	(6) 15.78%
Fixed combinations	(17) 26.56%	(8) 27.5%	(9) 23.68%

*P* > 0.05 between Groups for all medications. PGA, prostaglandin analogues; CAI, carbonic anhydrase inhibitors; PF, preservative-free.

**DISCUSSION**

Topical medications are responsible for several ocular surface changes during medical management of glaucoma, with the conjunctival alterations being the most critical for the surgical outcome.<sup>20</sup> Inflammatory cell infiltration, fibroblasts proliferation and connective deposition within the stroma, squamous metaplasia, dendritic cell infiltration, and GC loss represent the most common iatrogenic alterations.<sup>15,21-23</sup> All of them may negatively affect the AH flow through the bleb wall.<sup>11,20,21</sup>

In our study, patients with a complete surgical success presented with higher preoperative GCD and MUC5AC expression, compared to patients with a qualified success or failure. This was in accordance with a previous IC study, where higher MUC5AC and trefoil factor family (TFF) 1 (mucin-forming peptides produced by GC) expressions were observed in successful cases.<sup>10</sup> Even though this study did not specifically evaluate GC, it was hypothesized that the mucus overexpression could result from a higher GCD. This hypothesis also was supported by the positive correlation between MUC5AC and TFF1 positivity. The authors proposed that this mucin overexpression could lead to a protective mechanism against the conjunctival aggression during medical therapy. In failed blebs this mucin protection could be

TABLE 3. Baseline and 12-Month LSCM Parameters

	GCD, Cells/mm <sup>2</sup>	MMD, Cysts/mm <sup>2</sup>	MMA, mm <sup>2</sup>
Group 1			
Baseline	44.37 ± 16.71*	29.29 ± 34.80	2089.57 ± 825.44
12-mo	49.11 ± 12.23*	217.51 ± 234.69†	17189.00 ± 4182.15†
Group 2			
Baseline	25.63 ± 7.97	33.33 ± 28.92	1932.41 ± 602.79
12-mo	22.46 ± 6.90‡	95.75 ± 107.07	5039.00 ± 1673.85
Group 3			
Baseline	22.33 ± 8.38	28.88 ± 27.35	1894.38 ± 596.60
12-mo	18.09 ± 7.14‡	14.88 ± 20.23	1917.77 ± 463.13
Controls			
Baseline	205.49 ± 21.44§	16.12 ± 30.78	1621.34 ± 408.18
12-mo	215.22 ± 32.45	18.01 ± 27.33	1701 ± 477.20

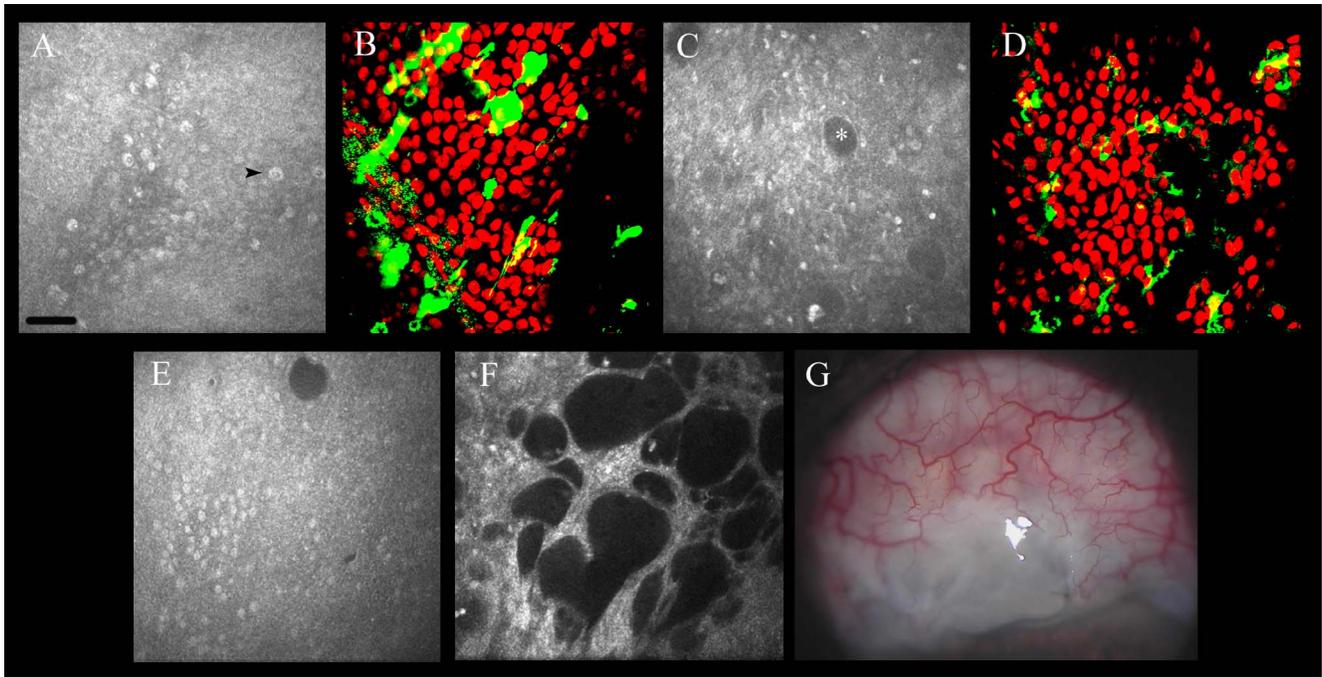
\* *P* < 0.05 versus Groups 2 and 3.

† *P* < 0.05 versus Groups 2 and 3 and controls.

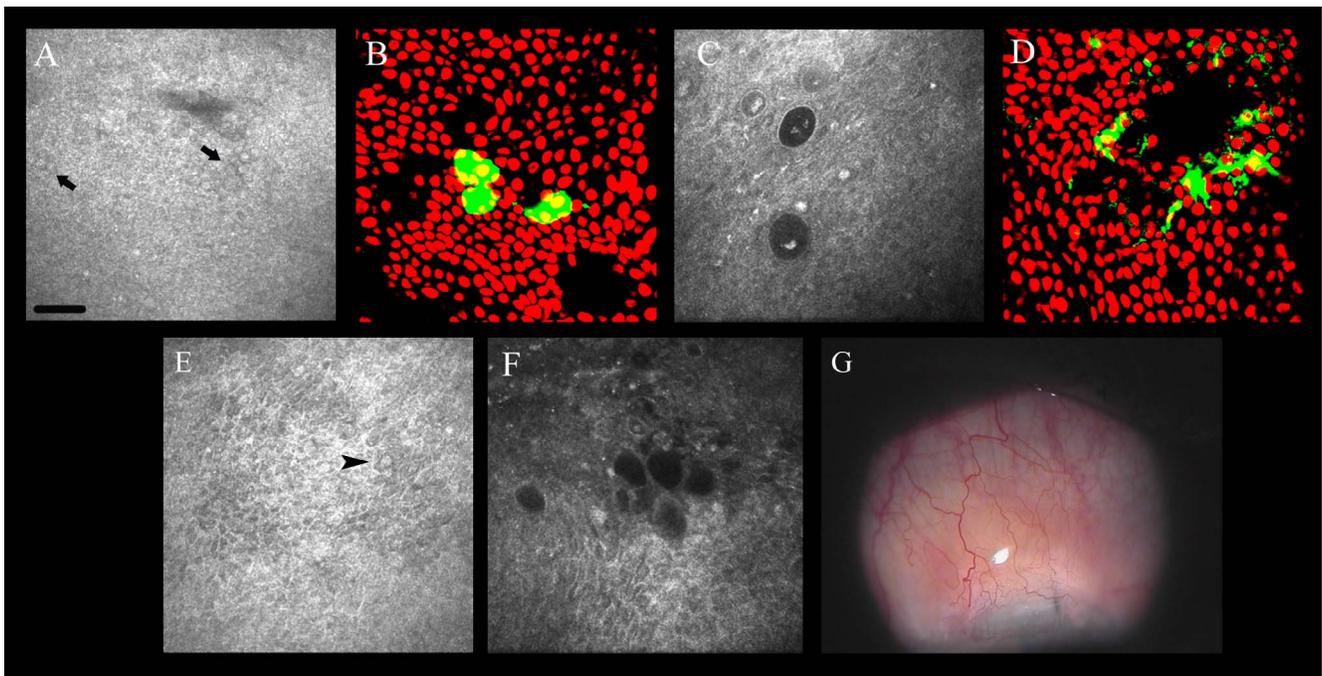
‡ *P* < 0.05 versus baseline.

§ *P* < 0.001 versus Groups 1 to 3.

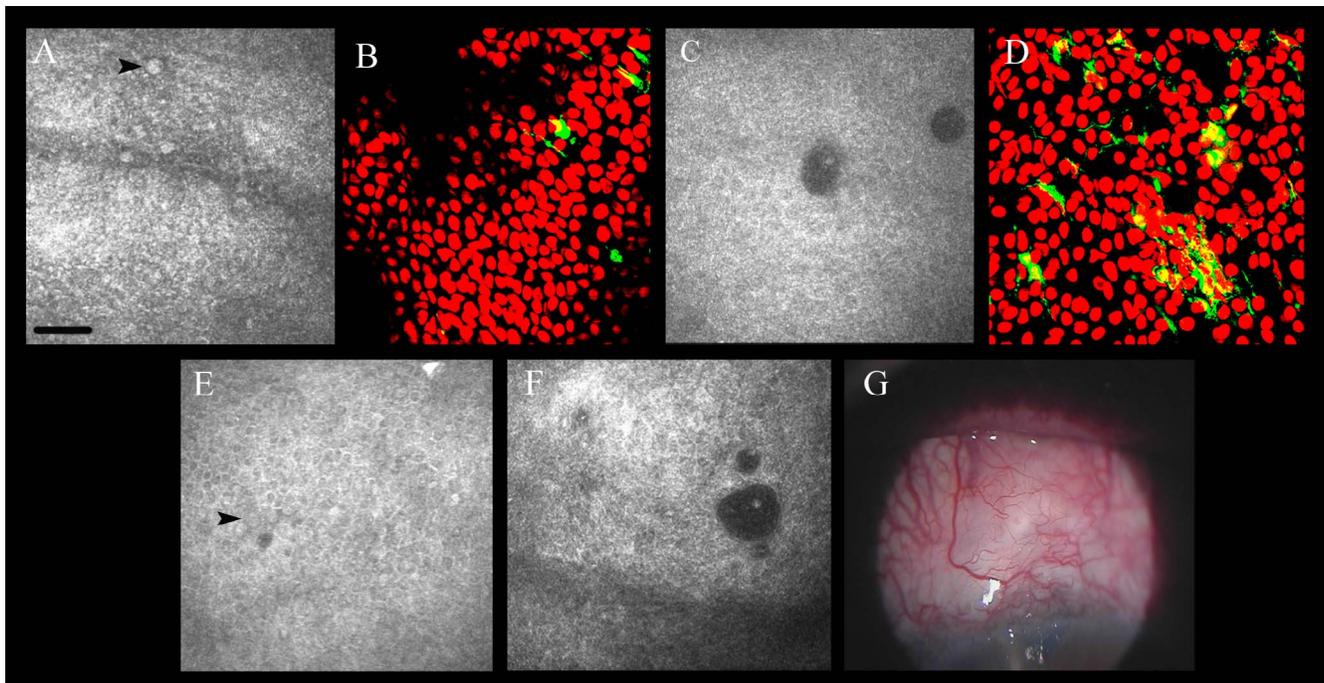
|| *P* < 0.05 versus Groups 1 to 3.



**FIGURE 1.** Group 1 (Complete success). (A–D) Laser scanning confocal microscopy and IC of the conjunctiva at baseline. Laser scanning confocal microscopy shows several GC (A, *arrowhead*) and some scattered microcysts (C, *asterisk*) within the epithelium; IC shows the MUC5AC (B) and HLA-DR (D) immune staining positivity (*green*). Nuclei of cells were stained with propidium iodide (*red*). Magnification:  $\times 630$ . (E, F) Laser scanning confocal microscopy of the bleb at 12 months. Goblet cells (E) did not change compared to baseline, whereas microcysts increase their density and area (F). (G) Slit-lamp photograph. Functional filtering bleb at 12 months, with a mixed feature (diffuse/multi-cystic). *Scale bar:* 50  $\mu\text{m}$ .



**FIGURE 2.** Group 2 (Qualified success). (A–D) Laser scanning confocal microscopy and IC of the conjunctiva at baseline. Laser scanning confocal microscopy shows a lower density of GC (A) compared to Group 1, with scattered microcysts (C) within the epithelium (*arrows* indicate clusters of GC); at IC the MUC5AC (B) immune staining positivity is lower compared to Group 1, whereas HLA-DR (D) presents a similar staining. Magnification:  $\times 630$ . (E, F) Laser scanning confocal microscopy of the bleb at 12 months. Goblet cells (E) reduced with respect to baseline (*arrowheads* indicate scattered GC); microcysts increased, though were much less evident compared to Group 1 (F). (G) Slit-lamp photograph. Functional filtering bleb at 12 months, with a diffuse/encapsulated feature. *Scale bar:* 50  $\mu\text{m}$ .



**FIGURE 3.** Group 3 (Failure). (A–D) Laser scanning confocal microscopy and IC of the conjunctiva at baseline. Laser scanning confocal microscopy shows some scattered GC (arrowhead) and epithelial microcysts (A, C). MUC5AC (B) immune staining positivity is lower compared to Group 1 and 2 (B), whereas the HLA-DR positivity (D) is similar to Groups 1 and 2. Magnification:  $\times 630$ . (E, F) Laser scanning confocal microscopy of the bleb at 12 months. GC density (E) appears further reduced with respect to baseline (arrowhead indicates a scattered GC), whereas microcysts did not change 1 (F). (G) Slit-lamp photograph. Failed filtering bleb at 12 months, with a fibrotic and flat feature. Scale bar: 50  $\mu\text{m}$ .

profoundly altered and ineffective, due to a reduced GC population. Our results confirmed the hypothesis that the higher MUC5AC is expression of a higher GCD, since both parameters were higher in Group 1 and positively correlated between them.

The results of our study also supported the hypothesis of Amar et al.,<sup>9</sup> who supposed that GC vehiculates the AH through the bleb-wall epithelium. In addition, based on the role of microcysts, the 12-month positive correlation between GCD, MMD, and MMA further support this hypothesis.

Different factors could affect the preoperative GC density, most of them related to medical therapy. While preservatives and  $\beta$ -blockers have a toxic effect, prostaglandin analogues (PGA) positively stimulate GC.<sup>15,22–24</sup> Thus, patients treated with preservative-free formulations and/or PGA for longer periods, or receiving fixed combinations, could maintain a larger pool of GC.

In our study, the post hoc correlation between mean number of medications and surgical outcome was not statistically significant. However, though the difference was not statistically significant and therapy could have changed during the course of the disease, a slightly higher percentage of patients was treated with preservative-free formulations or

fixed-combinations in Group 1 compared to the other groups. On the other hand, by reviewing patient charts, it was not possible to ascertain whether Group 1 used PGA for longer periods, compared to Groups 2 and 3.

One also may consider that patients receiving sodium hyaluronate-containing artificial tears to contain the iatrogenic dry eye, could have a better behavior for GC.<sup>25</sup> Unfortunately, patient charts did not systematically report the use of lubricants.

Another possible explanation is the interindividual variability of GC: as reported, GC varies significantly between normal subjects (338–520 cells/mm<sup>2</sup>) independently by age.<sup>18</sup> On these bases, one may hypothesize that patients reaching a complete surgical success could have had a higher GCD before initiation of medical therapy.

Positivity values for HLA-DR were consistent with the literature, since they were markedly higher in glaucomatous groups compared to controls, and correlated negatively with GCD and MUC5AC production.<sup>21–24</sup> As expected, HLA-DR did not show significant differences between glaucomatous groups. Despite the fact that iatrogenic inflammation strongly reduces GC,<sup>15,21–24</sup> the higher GCD and MUC5AC positivity in Group 1 supported the hypothesis that differences in GCD are not exclusively related to the conjunctival inflammation.

Nonetheless, it is not possible to rule out definitely that Group 1 had a more preserved ocular surface. In fact, despite the fact that HLA-DR is the most widely used biomarker to evaluate the drug-induced conjunctival inflammation, it cannot represent, alone, the real ocular surface status.

In line with previous reports,<sup>7,9</sup> patients with a successful surgery showed higher postoperative MMD and MMA values compared to failed cases, expression of a higher AH flow through the bleb wall. Moreover, the strong correlation between 12-month MMD and MMA and baseline GCD and

**TABLE 4.** Baseline Interobserver Agreement for GCD and MMD

	GCD	MMD
Investigator 1 (VF)	42.14 $\pm$ 9.21	28 $\pm$ 7.30
Investigator 2 (LB)	43.27 $\pm$ 11.33	25 $\pm$ 9.78
<i>P</i> <sup>*</sup>	>0.05	>0.05
<i>P</i> <sup>†</sup>	<0.05	<0.001

\* Paired *t*-test.

† Spearman correlation analysis.

TABLE 5. Baseline IC Parameters

	Group 1	Group 2	Group 3	Controls
HLADR (%)	54.92 ± 7.24	55.79 ± 9.41	57.88 ± 8.54	4.43 ± 2.25*
MUC5AC (%)	32.75 ± 5.65†	24.48 ± 6.25‡	18.80 ± 4.35	88.21 ± 8.24

IC, impression cytology.

\*  $P < 0.001$  versus Groups 1 to 3.

†  $P < 0.05$  versus Group 2 and 3;  $P < 0.001$  versus controls.

‡  $P < 0.05$  versus Group 3.

MUC5AC, suggested a direct active role of GC in vehiculating the AH. This confirmed the initial results of Amar et al.<sup>9</sup>

The present study has some limitations. First, the study cannot elucidate whether the GCD before medical therapy was different between Groups. Thus, prospective studies following patients from initial diagnosis are mandatory. Second, GCD was not determined with IC, which is the gold standard method to identify these cells, because it would have required an additional sampling on the same site. However, previous evidences found positive correlations between GCD measured by LSCM and IC, even though GCD assessed by LSCM seems higher.<sup>16,17</sup> Further studies aimed at quantifying MUC5AC transcripts on IC samples might significantly improve the sensitivity of MUC5AC analysis and further confirm our results.<sup>26</sup> Third, GC and microcysts should be interpreted carefully, since they may present a significant interobserver variability. However, in the present study, GCD, MMD, and MMA values were not significantly different between two experienced confocal microscopy executors. Fourth, since EM also can be found in healthy subjects and in some ocular surface diseases, their significance should be clarified. However, several studies indicated that in glaucoma they are a hallmark of AH outflow.<sup>7-9,14</sup>

In closing, GC seems to have a critical role for the bleb functionality after filtration surgery. This should be considered strongly during the medical management of glaucoma, because a significant proportion of patients will undergo filtration surgery to control the damage progression. Thus, strategies aimed at preserving GC before surgery, by reducing the ocular surface inflammation, are recommended.

Whether these results will be confirmed in further studies, GC could be proposed as a potential in vivo biomarker to predict the surgical outcome and to guide clinician in determining the appropriate time of surgery, the intraoperative strategy, and in the postoperative bleb management.

### Acknowledgments

Disclosure: **L. Agnifili**, None; **V. Fasanella**, None; **R. Mastropasqua**, None; **P. Frezzotti**, None; **C. Curcio**, None; **L. Brescia**, None; **G. Marchini**, None

### References

1. Parc CE, Johnson DH, Oliver JE, Hattenhauer MG, Hodge DO. The long-term outcome of glaucoma filtration surgery. *Am J Ophthalmol*. 2001;132:27-35.
2. Molteno AC, Bosma NJ, Kittelson JM. Otago glaucoma surgery outcome study: long-term results of trabeculectomy-1976 to 1995. *Ophthalmology*. 1999;106:1742-1750.
3. Galin MA, Baras I, McLean JM. The mechanism of external filtration. *Am J Ophthalmol*. 1966;61:63-68.
4. Tenner A, Jaeger W. Demonstration of the drainage of aqueous humor following various glaucoma operations by means of injection of fluorescein into the anterior chamber [in German]. *Ber Zusammenkunft Dtsch Ophthalmol Ges*. 1977;74:853-855.
5. Kronfeld PC. The chemical demonstration of transconjunctival passage of aqueous after antiglaucomatous operations. *Am J Ophthalmol*. 1952;35:38-45.
6. Benedikt O. The effect of filtering operations [in German]. *Klin Monatsbl Augenheilkd*. 1977;170:10-19.
7. Ciancaglini M, Carpineto P, Agnifili L, et al. Conjunctival characteristics in primary open-angle glaucoma and modifications induced by trabeculectomy with mitomycin C: an in vivo confocal microscopy study. *Br J Ophthalmol*. 2009;93:1204-1209.
8. Ciancaglini M, Carpineto P, Agnifili L, et al. Conjunctival modifications in ocular hypertension and primary open angle glaucoma: an in vivo confocal microscopy study. *Invest Ophthalmol Vis Sci*. 2008;49:3042-3048.
9. Amar N, Labbé A, Hamard P, Dupas B, Baudouin C. Filtering blebs and aqueous pathway: an immunocytological and in vivo confocal microscopy study. *Ophthalmology*. 2008;115:1154-1161.
10. Souchier M, Buron N, Lafontaine PO, Bron AM, Baudouin C, Creuzot-Garcher C. Trefoil factor family 1, MUC5AC and human leucocyte antigen-DR expression by conjunctival cells in patients with glaucoma treated with chronic drugs: could these markers predict the success of glaucoma surgery? *Br J Ophthalmol*. 2006;90:1366-1369.
11. Gwynn DR, Stewart WC, Pitts RA, McMillan TA, Hennis HL. Conjunctival structure and cell counts and the results of filtering surgery. *Am J Ophthalmol*. 1993;116:464-468.
12. Brusini P, Filacorda S. Enhanced Glaucoma Staging System (GSS 2) for classifying functional damage in glaucoma. *J Glaucoma*. 2006;15:40-46.
13. Edmunds B, Bunce CV, Thompson JR, Salmon JF, Wormald RP. Factors associated with success in first time trabeculectomy for patients at low risk of failure with chronic open-angle glaucoma. *Ophthalmology*. 2004;111:97-103.
14. Mastropasqua L, Agnifili L, Salvat ML, et al. In vivo analysis of conjunctiva in canaloplasty for glaucoma. *Br J Ophthalmol*. 2012;96:634-639.
15. Mastropasqua L, Agnifili L, Fasanella V, et al. Conjunctival goblet cells density and preservative-free tafluprost therapy for glaucoma: an in vivo confocal microscopy and impression cytology study. *Acta Ophthalmol*. 2013;91:e397-e405.
16. Le QH, Wang WT, Hong JX, et al. An in vivo confocal microscopy and impression cytology analysis of goblet cells in patients with chemical burns. *Invest Ophthalmol Vis Sci*. 2010;51:1397-1400.
17. Hong J, Zhu W, Zhuang H, et al. In vivo confocal microscopy of conjunctival goblet cells in patients with Sjögren's syndrome dry eye. *Br J Ophthalmol*. 2010;94:1454-1458.
18. Zhu W, Hong J, Zheng T, et al. Age-related changes of human conjunctiva on in vivo confocal microscopy. *Br J Ophthalmol*. 2010;94:1448-1453.
19. Villani E, Beretta S, Galimberti D, Viola F, Ratiglia R. In vivo confocal microscopy of conjunctival roundish bright objects:

- young, older, and Sjögren subjects. *Invest Ophthalmol Vis Sci.* 2011;52:4829-4832.
20. Yu DY, Morgan WH, Sun X, et al. The critical role of the conjunctiva in glaucoma filtration surgery. *Prog Retin Eye Res.* 2009;28:303-328.
  21. Mastropasqua L, Agnifili L, Mastropasqua R, Fasanella V. Conjunctival modifications induced by medical and surgical therapies in patients with glaucoma. *Curr Opin Pharmacol.* 2013;13:56-64.
  22. Baudouin C, Labbé A, Liang H, Pauly A, Brignole-Baudouin E. Preservatives in eye drops: the good, the bad and the ugly. *Prog Ret Eye Res.* 2010;29:312-334.
  23. Baudouin C, Brignole E, 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.
  24. Pisella PJ, Debbasch C, Hamard P, et al. Conjunctival proinflammatory and proapoptotic effects of latanoprost and preserved and unpreserved timolol: an ex vivo and in vitro study. *Invest Ophthalmol Vis Sci.* 2004;45:1360-1368.
  25. Aragona P, Papa V, Micali A, Santocono M, Milazzo G. Long term treatment with sodium hyaluronate-containing artificial tears reduces ocular surface damage in patients with dry eye. *Br J Ophthalmol.* 2002;86:181-184.
  26. McKenzie RW, Jumblatt JE, Jumblatt MM. Quantification of MUC2 and MUC5AC transcripts in human conjunctiva. *Invest Ophthalmol Vis Sci.* 2000;41:703-708.