In vivo confocal microscopy of meibomian glands in glaucoma

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ABSTRACT

Aim To investigate, using laser scanning confocal microscopy (LSCM), the morphological changes of meibomian glands (MGs) in patients with glaucoma.

Methods A total of 80 patients who were glaucomatous were enrolled, and 20 healthy subjects were used as controls. After completing an Ocular Surface Disease Index (OSDI) questionnaire, all subjects underwent tear film break-up time (BUT), fluorescein staining, Schirmer test I (STI) and LSCM examination of the MGs. The main outcome measures were: eyelid margin epithelial cell density, mean acinar density (MAD) and area (MAA), glandular orifice area, secretion reflectivity and inhomogeneous appearance of interstice and acinar wall.

Results According to the number of anti-glaucoma medications they were taking, patients were divided into three groups: group 1 (30 eyes), one drug; group 2 (23 eyes), two drugs; group 3 (27 eyes), three or more drugs. LSCM showed lower MAD and MAA, greater secretion reflectivity and glandular orifice area in groups 2 and 3 than in controls (p<0.05). The inhomogeneity of the interstice and acinar wall was significantly greater in all groups compared to controls (p<0.05). Preserved prostaglandin analogues (PGAs) induced more pronounced modifications of all parameters than preservative free (PF)-PGAs (p<0.05). No significant differences were found between preserved and PF-β-blockers. Significant relations were found among MAD, MAA, secretion reflectivity and OSDI score, BUT and ST (p<0.05) and between secretion reflectivity and orifice area (p<0.001).

Conclusions In vivo LSCM is an effective tool in revealing morphological changes of MGs induced by anti-glaucoma medications. Given the key role in the ocular surface health, the evaluation of MG status in patients who are glaucomatous is worthwhile.

INTRODUCTION

Meibomian glands (MGs) are holocrine glands embedded in the tarsal plate of the eyelids. These glands synthesise meibum, a lipoid secrete that forms the superficial layer of the tear film, preventing its excessive evaporation and functioning as a lubricant for the eyelids during blinking. MG dysfunction is one of the most diffuse causes of dry eye. 1

In glaucoma, long-term treatment induces modifications of ocular surface tissues and adnexa, such as the conjunctiva, cornea, eyelids, periculisk skin and MGs. 2–5 To date, the impact of anti-glaucoma medications on MGs has been investigated using clinical methodologies such as meibometry and non-contact meibigraphy. 5–8 These studies reported that anti-glaucoma drugs induced morphological changes and dysfunction of MGs, leading to dry eye.

In vivo laser scanning confocal microscopy (LSCM) can non-invasively characterise structural tissue changes in many ocular surface diseases. 9 In glaucoma, LSCM is used to analyse ocular surface alterations induced by treatment, filtering bleb functionality 4, 10 and trans-scleral aqueous outflow modifications induced by medical and surgical approaches. 11–14

Recently, LSCM was used to investigate MGs in contact lens (CL) wearers, in Sjögren syndrome and in MG dysfunction. 15–18 However, to date, no previous study has evaluated MGs in glaucoma and the effects of topical medications by using in vivo confocal microscopy. The aim of this study was therefore to describe the features of MGs in medically controlled glaucoma by means of LSCM, in order to elucidate modifications induced by treatment.

MATERIALS AND METHODS

Patient enrolment

This was a case-control study. The study adhered to the tenets of the Declaration of Helsinki, and our institutional review board (Department of Medicine and Ageing Science, G d’Annunzio University of Chieti-Pescara, Chieti, Italy) approved the project. Written informed consent was obtained from all patients prior to enrolment. We examined 80 consecutive Caucasian patients (80 eyes) with medically controlled primary open angle glaucoma referred to our clinic; 20 healthy Caucasian subjects (20 eyes) were used as controls.

For patients who were glaucomatous, inclusion criteria were: corrected visual acuity ≥8/10, refractive error ≤3 dioptres, mean intraocular pressure (IOP) at the time of diagnosis ranging from 22–34 mm Hg and medically controlled at enrolment (IOP<18 mm Hg: mean of three measurements taken at 9:00, 12:00 and 16:00), central corneal thickness (CCT) (ultrasound pachimetry: Altair; Optikon 2000, Rome, Italy) ranging from 530–570 μm, visual field (VF) test (Humphrey field analyser II 750 (Carl Zeiss Meditec Inc, Dublin, California, USA) (30–2 test, full threshold)) 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 classic ophthalmoscopic signs of glaucomatous optic consistent with the VF alterations. Topical treatment had to be the same in both eyes without variation during the 18 months immediately prior to enrolment.

Exclusion criteria were history of ocular or systemic diseases or treatments in the last 12 months that could have modified the MG status, previous ocular surgery and ocular trauma, end-stage
glaucoma, pregnancy and CL wear. History of MG dysfunction
(according to criteria reported by Matsumoto et al.15) and dry
eye prior to glaucoma diagnosis and initiating treatment were
also considered exclusion criteria.

Controls showed a best-corrected visual acuity ≥8/10, a
refractive error ≤±3 dioptries, mean IOP lower than 18 mm Hg,
cCT ranging from 530–570 µm, absence of signs of glaucomat-
ous optic neuropathy and a normal VF examination. None of
the healthy subjects had either a history of topical or systemic
treatment nor were they affected with any ocular or systemic
diseases in the last 12 months. Pregnant women and CL wearers
were also excluded.

In patients who were glaucomatous and healthy controls,
both eyes were evaluated, but only one eye per subject was ran-
domly chosen (using a computer generated random number list)
for the statistical analysis.

Ocular Surface Disease Index (OSDI) questionnaire and tear
film function tests

After enrolment, all subjects were asked to complete the OSDI
questionnaire.19 Subsequently, tear film break-up time (BUT) tests
and the Schirmer test I (STI) with topical anaesthesia (20 min after
eyelid was summed to obtain a score for each eye.

The degree of ease in expressing meibomian secretion (meibum score or
grade 0, clear meibum easily expressed; grade 1, cloudy
expression with more than moderate pressure; and grade 2,
cloudy meibum not expressed even with hard pressure.

Meibography

Transillumination of the MGs through the inferior lid was
achieved by using an illuminated probe placed on the skin of
the everted eyelid. The glandular morphology was observed and
photographed along the entire length of the eyelid using a slit
lamp. This allowed quantification of the meibomian glandular
rows and their loss.

The degree of MG dropout (meibo score) was evaluated as
follows: grade 0, no dropout; grade 1, dropout in less than half of
the inferior tarsus; and grade 2, dropout in more than half of
the inferior tarsus.15 16 The meibo score for the lower eyelid was summed to obtain a score for each eye.

In vivo confocal microscopy

The day after the tear film function assessment, LSCM was per-
formed using HRT III Rostock Cornea Module (Heidelberg
Engineering GmbH, Dossenheim, Germany).

Briefly, after the lower eyelid was partly everted, the centre of
the sterile Tomo-Cap was applied onto the centre of the
eyelid margin. The instrument was manually focused while the
microscope was in ‘section mode’ of acquisition modality.

Scanning was started at the most superficial tissues and pro-
gressed down to the deepest tissue that could be visualised with
a satisfactory resolution. The procedure was repeated by moving
the lens along the entire lid length (from nasal to temporal
canthus) with minute horizontal movements. A total of 15
images (5 each for the middle, nasal and temporal areas) were
acquired per lid at every 10 µm of depth for a maximum depth
of 30 µm. Images were also acquired in the mid-depth range in
order to manually assess the quality of the different structures
found in this area. Images were 384×384 pixels with a
400×400 µm field of view. Typical sessions for LSCM examin-
ation lasted between 3–5 min. A single operator (VF) performed
cnfocal examinations and selected the images, which were evalu-
ated by a second operator (LA). Both operators were masked
for patient history and to the group.

Three randomly selected, non-overlapping high-quality
images of the nasal, middle and temporal lower eyelid margins
were analysed. LSCM parameters considered were: (1) cellular
density of the superficial and basal epithelium of the eyelid
margin (calculated automatically at the largest available region
(Cell Count software; Heidelberg Engineering)); (2) the mean
acinar area of MG (MAA) (area of each acinar unit calculated
using ImageJ, an open source software program (http://rsb.info.
nih.gov/ij), as described elsewhere);21 (3) mean acinar density
(MAD) (density of the acinar unit manually marked for each
400×400 µm frame and calculated automatically using Cell
Count); (4) area of glandular orifice (calculated by using
ImageJ); (5) meibum secretion reflectivity; (6) inhomogeneity
of periglandular interstices and (7) acinar unit wall. An arbitrary
grading scale of 1–4 was adopted to quantify secretion reflectiv-
ity and inhomogeneity.15 16

To validate the sectorial findings, we manually created a
planar reconstruction of the central part of the lower eyelid
(3 mm in length) in two healthy and four glaucomatous eyes
two under monotherapy and two under multitherapy).

Statistical analysis

Analysis was performed using SPSS V13.0 Software (SPSS Inc.;
Chicago, Illinois, USA). Student t and χ² tests were used to
evaluate age and sex differences, respectively, among healthy
and patients who were glaucomatous.

The Mann–Whitney U test was used to determine differences
among groups of subjects. 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. p Values less than 0.05 were considered statistically
significant.

Multivariate regression analysis was performed with stepwise
selection of contributing variables (probability of F to enter
≤0.05, probability of F to remove ≥0.100) including age,
LSCM parameters, OSDI score, BUT, STI value and the duration
of topical treatment.

RESULTS

The demographic and clinical data in glaucoma groups are
shown in table 1. Patient treatment is shown in table 2. No
patients in group 1 had their treatment modified from treatment onset.

OSDI questionnaire score, STI and BUT were significantly different among glaucoma groups and controls and between group 1 with groups 2 and 3 (p<0.05). Corneal staining and meibum scores did not differ among treatment groups.7

LSCM of eyelid epithelium

Epithelial cellular densities were significantly lower in groups 2 and 3 compared to controls (p<0.05) and in group 2 compared to group 1. No significant differences were found between controls and group 1 and between groups 2 and 3 (table 4); (figure 1). No significant differences were found among drug classes in group 1 (table 5).

LSCM of MGs

MAD and MAA were significantly lower in groups 2 and 3 than in controls (p<0.05). Significantly lower values were found between all preserved drugs of group 1 and controls and between preserved prostaglandin analogues (PGAs) and controls (p<0.05) (tables 4 and 5); (figure 2). In group 1, MAD and MAA were lower in preserved PGAs than in preservative free (PF)-PGAs (p<0.05) (table 5). Secretion reflectivity was significantly greater in groups 2 and 3 than in group 1 and controls (p<0.001). In group 1, patients treated with preserved PGAs showed higher reflectivity with respect to controls (p<0.05) (figure 2). No statistically significant differences were found between preserved and PF-β-blockers.

The glandular orifice area appeared significantly greater in groups 2 and 3 with respect to controls and group 1 (p<0.05). No significant differences were found among drugs in group 1 (figure 1). MAD, MAA, reflectivity and orifice area were not significantly different between groups 2 and 3 and between overall group 1 and controls.

The inhomogeneity of interstice and MG wall was significantly higher in all groups than in controls, with values higher in group 3 compared to groups 2 and 1 (p<0.05). In group 1, overall preserved monotherapy induced higher inhomogeneity compared to PF formulations; only preserved PGAs showed values significantly higher compared to controls (p<0.05). Preserved PGAs showed higher inhomogeneity than PF-PGAs (p<0.05), whereas no significant differences were found between preserved and PF-β-blockers (figure 3).

The global distribution of MGs (central part of the inferior eyelid margin) in healthy subjects and modifications induced by monotherapy and multitherapy in patients who were glaucomatous are shown in figure 4 (figure 4A–C, respectively).

The OSDI score, STI and BUT correlated with MAD, MAA and secretion reflectivity (p<0.05, Spearman). The inhomogeneity of the interstice correlated with secretion reflectivity and BUT (p<0.05, Spearman); secretion reflectivity and glandular orifice area were strongly correlated (p<0.001). Multivariate regression analysis indicated that LSCM parameters did not significantly correlate with age in neither healthy nor glaucoma groups (p>0.05).

DISCUSSION

Recent studies have shown that anti-glaucoma medications induce MG loss and low secretion expressibility.5–8 These findings correlated with BUT and ocular surface symptoms. Notably, the glandular dropout was not significantly different between preserved PGAs and preserved β-blockers.7 8 However, these studies did not consider the eyes of patients taking PF drugs and, therefore, the question of whether the gland modifications were induced by preservative compounds, active compounds or by both was not clarified.8 Unexpectedly, MG modifications did not correlate with the number of daily drugs since the meibum and meibo scores did not differ among treatment groups.7

Our study, which analysed MGs in glaucoma using in vivo confocal microscopy for the first time, showed an overall

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Patient treatment</th>
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<tbody>
<tr>
<td><strong>Group</strong></td>
<td><strong>Treatment</strong></td>
</tr>
<tr>
<td>Group 1</td>
<td>Single</td>
</tr>
<tr>
<td></td>
<td>β-blockers</td>
</tr>
<tr>
<td></td>
<td>Preserved timolol 0.5%</td>
</tr>
<tr>
<td></td>
<td>PF-timolol 0.5%</td>
</tr>
<tr>
<td></td>
<td>PGAs</td>
</tr>
<tr>
<td></td>
<td>Latanoprost 0.005%</td>
</tr>
<tr>
<td></td>
<td>PF-tafluprost 0.0015%</td>
</tr>
<tr>
<td>Group 2</td>
<td>Double</td>
</tr>
<tr>
<td></td>
<td>Latanoprost-timolol fixed combination</td>
</tr>
<tr>
<td></td>
<td>Bimatoprost and timolol unfixed combination</td>
</tr>
<tr>
<td></td>
<td>Brimonidine and timolol unfixed combination</td>
</tr>
<tr>
<td></td>
<td>Dorzolamide-timolol fixed combination</td>
</tr>
<tr>
<td>Group 3</td>
<td>Triple or more</td>
</tr>
<tr>
<td></td>
<td>Bimatoprost 0.03%, brimonidine and timolol 0.05%</td>
</tr>
<tr>
<td></td>
<td>Latanoprost 0.005%, timolol and dorzolamide</td>
</tr>
<tr>
<td></td>
<td>Bimatoprost 0.03%, brimonidine, timolol 0.05% and dorzolamide</td>
</tr>
</tbody>
</table>

| Pf, preservative free; PGAs, prostaglandin analogues. |

<table>
<thead>
<tr>
<th>Table 3</th>
<th>Clinical characteristics</th>
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</thead>
<tbody>
<tr>
<td><strong>OSDI score</strong></td>
<td><strong>BUT</strong></td>
</tr>
<tr>
<td>Controls</td>
<td>9.5±4.8*</td>
</tr>
<tr>
<td>Group 1</td>
<td>16.4±5.4**</td>
</tr>
<tr>
<td>PF drugs</td>
<td>9.4±3.5***</td>
</tr>
<tr>
<td>Preserved drugs</td>
<td>18.7±4.1****</td>
</tr>
<tr>
<td>Group 2</td>
<td>30.8±6.7</td>
</tr>
<tr>
<td>Group 3</td>
<td>32.6±7.2</td>
</tr>
</tbody>
</table>

*p<0.05 vs groups 1, 2 and 3.  
**p<0.05 vs groups 2 and 3.  
***p<0.05 vs group 1 preserved drugs and vs groups 2 and 3.  
****p<0.05 vs groups 2 and 3.  
BUT, break-up time; OSDI, Ocular Surface Disease Index; PF, preservative free; STI, Schirmer test I.
reduction of the MAD and MAA, particularly in patients in multitherapy. In contrast, overall group 1 did not differ compared to controls. Lower MAD and MAA values are expressions of glandular loss and reduced meibum production, respectively. This could explain the higher secretion viscosity in groups 2 and 3, which indicated increased secretion density and the duct blockage induced by treatment. Consequently, the higher glandular oriﬁce area in groups 2 and 3 was probably an adaptive mechanism to overcome the high secretion density and the duct blockage induced by treatment.

These findings were supported by the correlation between the secretion reﬂectivity and glandular oriﬁce area. These results have an evident clinical impact since MAD, MAA and reﬂectivity correlated with OSDI, BUT and ST.

All groups showed an inhomogeneous appearance of the interstice and glandular wall compared to controls. The inhomogeneity may be seen as a sign of tarsum and MG inﬂammation. Interestingly, these were the only parameters that also differed between group 1 and controls and between groups 2 and 3. This appears consistent with the dose-dependent inﬂammatory effects of anti-glaucoma medications on ocular surface. Nevertheless, in our cases, it was difﬁcult to identify inﬂammatory cells with the typical dendritic shape. Nevertheless, in our cases, it was difﬁcult to identify inﬂammatory cells with the typical dendritic shape.

The comparison of preserved and PF agents of the same drug class showed that preserved PPCAs were more toxic than PF-PGAs, without signiﬁcant differences between preserved and PF-PGAs, without signiﬁcant differences between preserved and PF-PGAs. Table 4 Meibomian gland parameters in healthy controls and glaucoma groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Cell density of eyelid margin, cells/mm²</th>
<th>Acinar unit</th>
<th>Density, units/mm²</th>
<th>Secretion reﬂectivity</th>
<th>Glandular oriﬁce area, μm²±SD</th>
<th>MG wall: inhomogeneous appearance of MG wall</th>
<th>Periglandular interstice: inhomogeneous appearance of MG interstice</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Controls</strong></td>
<td>1810.27±34.19*</td>
<td>Acinar</td>
<td>219.31±50.62**</td>
<td>1.2±0.22**</td>
<td>1894.44±336.65**</td>
<td>1.33±0.37***</td>
<td>1.11±0.25***</td>
</tr>
<tr>
<td>Group 1</td>
<td>1789.70±208.76**</td>
<td>Acinar</td>
<td>195.33±58.55**</td>
<td>1.23±0.30***</td>
<td>1847.66±818.42**</td>
<td>1.63±0.50****</td>
<td>1.65±0.64****</td>
</tr>
<tr>
<td><strong>PF drugs</strong></td>
<td>1863.66±156.93</td>
<td>Acinar</td>
<td>222.40±45.25**</td>
<td>1.08±0.13</td>
<td>1956.32±855.78</td>
<td>1.27±0.42*</td>
<td>1.31±0.32****</td>
</tr>
<tr>
<td><strong>Preserved drugs</strong></td>
<td>1752.84±157.07</td>
<td>Acinar</td>
<td>1495.24±679.02***</td>
<td>1.32±0.35</td>
<td>1779.03±809.91</td>
<td>1.85±0.42*</td>
<td>1.86±0.71****</td>
</tr>
<tr>
<td><strong>Group 2</strong></td>
<td>1427.5±203.46</td>
<td>Acinar</td>
<td>1220.81±771.18</td>
<td>2.49±0.46</td>
<td>2413.70±621.69</td>
<td>2.66±0.60</td>
<td>1.95±0.69</td>
</tr>
<tr>
<td><strong>Group 3</strong></td>
<td>1380.46±169.44</td>
<td>Acinar</td>
<td>100.47±33.23</td>
<td>2.87±0.61</td>
<td>2322.03±339.77</td>
<td>3.17±0.53</td>
<td>2.73±0.66</td>
</tr>
</tbody>
</table>

*p<0.001 vs group 2; p<0.05 vs group 1, preserved drugs. **p<0.001 vs groups 1 and 3. ***p<0.05 vs groups 2 and 3. ****p<0.05 vs preserved drugs. *****p<0.05 vs group 1. MG, Meibomian gland; PF, preservative free.

Figure 1: Epithelium of basal eyelid margin and glandular orifice. The basal epithelial cell appeared reduced and glandular orifice area greater in group 2 and 3. (A) Controls; (B–D) groups 1 to 3, respectively. In controls, overall group 1 did not differ compared to controls.

Table 5  Meibomian gland parameters in group 1

<table>
<thead>
<tr>
<th>Drug class</th>
<th>Cell density of eyelid margin, cells/mm²</th>
<th>Acinar unit</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Superficial epithelium</td>
<td>Basal epithelium</td>
</tr>
<tr>
<td>Controls</td>
<td>1810.27±234.19</td>
<td>4066.94±284.54</td>
</tr>
<tr>
<td>Preserved β-blockers</td>
<td>1759.50±135.4</td>
<td>3926.16±451.64</td>
</tr>
<tr>
<td>PF β-blockers</td>
<td>1850.50±245.2</td>
<td>4089.75±413.37</td>
</tr>
<tr>
<td>Preserved PGA</td>
<td>1743.07±157.27</td>
<td>3840.23±238.37</td>
</tr>
<tr>
<td>PF-PGA</td>
<td>1903.75±96.00</td>
<td>4017.37±121.56</td>
</tr>
</tbody>
</table>

*p<0.05 vs preserved PGA.
**p<0.02 vs preserved PGA.
***p<0.01 vs preserved PGA.
****p<0.001 vs preserved β-blockers.

MG, Meibomian gland; PF, preservative free; PF-β-blockers, preservative-free timolol 0.5%; PF-PGA, preservative-free tafluprost; PGA, prostaglandin analogue; preserved β-blockers, timolol 0.5%; preserved PGA, preserved latanoprost.
Finally, we cannot clearly identify the initial site of damage and the first MG modification, though the high inhomogeneity also seen in group 1 indicated that the interstice and acinar unit wall might be altered early. Further prospective studies evaluating the initial MG status in treatment-naïve eyes and the impact of medications over time are mandatory to clarify these points.

In conclusion, LSCM proved valuable in identifying MG modifications induced by anti-glaucoma drugs, possibly more accurate than just a clinical approach. These modifications present evident implications for the ocular surface health; therefore, where available, the use of PF formulations is advisable. Further studies evaluating the relation between MG changes and...
adherence to and persistence of treatment could clarify the role of MG damage on worsening patient compliance.

Contributors LA: conception and design; writing the article; critical revision of the article; VF: provision of patients; performing examinations; CCi: writing the article; final approval of the article; CCi: provision of patients; data collection; PE: literature research; data collection; PC: literature research; critical revision of the article; LM: conception and design, final approval of the article.

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Competing interests None.

Patient consent None.

Ethics approval The study adhered to the tenets of the Declaration of Helsinki and our institutional review board (Department of Medicine and Ageing Science, G d’Annunzio University of Chieti-Pescara, Chieti, Italy) approved the project.

Provenance and peer review Not commissioned; externally peer reviewed.

REFERENCES