The Aging Meibomian Gland: An In Vivo Confocal Study

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Submitted: February 21, 2013
Accepted: June 2, 2013

PURPOSE. To evaluate age-related Meibomian gland (MG) changes by in vivo laser scanning confocal microscopy (LSCM).

METHODS. Asymptomatic healthy subjects (n = 100, age range 20–83 years) with an Ocular Surface Disease Index score of less than 13 were consecutively enrolled. Two additional groups, one composed of subjects under 40 years of age (n = 12) and one composed of subjects over 65 years (n = 12), were included without any inclusion or exclusion criteria. All subjects underwent a full ocular surface evaluation, and one eye of each subject was examined by LSCM to quantify the lower lid MG acinar unit diameters and densities, orifice diameters, secretion reflectivity, interstices inhomogeneity, and acinar wall inhomogeneity.

RESULTS. In the asymptomatic population, MG density and diameter decreased with age (P < 0.001 and P < 0.01, respectively), and secretion reflectivity and inhomogeneity of acinar walls increased (P < 0.001). For the under 40-year-old subjects and the over 65-year-old subjects included without any inclusion or exclusion criteria, acinar unit density decreased with age, and secretion reflectivity, and wall inhomogeneity increased (P < 0.01). There was no significant difference between the mean acinar diameters of these two groups.

CONCLUSIONS. In vivo LSCM imaging of age-related MG changes showed the histologic features underlying the clinically observed MG dropout. Asymptomatic older subjects mainly showed signs of atrophic, nonobstructive, age-related MG dysfunction. Comparing volunteers with and without ocular surface symptoms, LSCM can provide important information regarding the boundary between physiologic and pathologic MG aging.

Keywords: confocal microscopy, age-related, meibomian gland, ocular surface, dry eye

Meibomian glands (MGs) are holocrine lipid-secreting glands embedded in the tarsal plate of the upper and lower lids. Each MG is composed of clusters of secretory acini that are arranged circularly around a long central duct and are connected to it by short ductules. One end of the central duct is blind, and the other end opens at the lid margin. The glands are arranged in parallel in a single row throughout the length of the upper and lower lid tarsal plates. They presumably act in a coordinated fashion that is influenced by hormonal and neural regulation and by the mechanical forces of muscle contraction during the eye blink.1 Lipids from the MGs play an important role in the maintenance of the ocular surface tear film and form the most superficial layer that retards evaporation and protects against excessive dehydration.1

MG dysfunction (MGD) is a chronic, diffuse abnormality of the MGs, commonly characterized by terminal duct obstruction and/or qualitative/quantitative changes in the glandular secretion.2 Recent murine1 and human4–9 studies reported peculiar age-related MG changes, leading to the hypothesis that there is a primary, age-dependent form of MGD.1

In vivo laser scanning confocal microscopy (LSCM) is an emerging minimally invasive technology, recently applied to the armament of modalities used in the examination of MGs.7–9 It provides information on acinar density and diameter, secretion reflectivity, periglandular inflammation, and orifice features. In this study, we used LSCM to evaluate age-related changes of human MGs and periglandular tissue and to investigate the correlation between clinical and confocal findings.

METHODS

Patients

Written informed consent was obtained from all subjects before examination, and this study adhered to the tenets of the Declaration of Helsinki. Healthy volunteers (n = 100; 51 men and 49 women; average age 48.95 ± 18.3 years; age range, 20–83 years) were enrolled in this study. All of the subjects had an Ocular Surface Disease Index (OSDI) score less than 13. Exclusion criteria included blepharitis, ocular allergies, contact lens wear, continuous eye drop use, history of ocular trauma or surgery, or systemic or ocular diseases that would interfere with tear film production or function. Only the right eye of each subject was studied except when it was excluded. In those cases, the left eye was used.

The subjects were grouped by age according to two different schemes (Table 1). In the first scheme, two age groups were defined and composed of subjects under 50 years (n = 47) and those over 50 years (n = 53) of age. In the second scheme, six age groups, designated “A” through “E” were defined in 10-year intervals and each group contained 12 to 20 subjects (Table 1).
We also enrolled two more groups of subjects. Group G included subjects under 40 years (6 men; 6 women, age 31.9 ± 5.4 years), and Group H included subjects over 65 years (8 men and 4 women; average age 75.6 ± 8.5 years). The subjects in these groups were enrolled without clinical inclusion or exclusion criteria.

**Clinical Evaluation**

An accurate medical history was drawn up for each participant in the study. All of the subjects underwent a thorough ophthalmic examination, including biomicroscopic examination of the ocular adnexa and anterior segment.

MG morphology and function were evaluated on the basis of meibography and meibum expression, respectively. Meibography was conducted by transillumination of the lower eyelid, and the degree of MG dropout was scored as follows: grade 0, no gland dropout; grade 1, gland dropout in less than one-third of the inferior tarsus; grade 2, gland dropout between one-third and two-thirds; and grade 3 dropout in more than two-thirds of the inferior tarsus.

Assessment of obstruction in the MG orifices was conducted by applying digital pressure on the lower tarsus. The degree of case in expressing the meibum was evaluated semiquantitatively as follows: grade 0, clear meibum easily expressed; grade 1, cloudy meibum expressed with mild pressure; grade 2, cloudy meibum expressed with more than moderate pressure; grade 3, meibum not be expressed even with the hand pressure.

Tear film break-up time (BUT), corneal staining with fluorescein, and bulbar conjunctival staining with lissamine green were also performed. The ocular surface staining was scored according to the Collaborative Longitudinal Evaluation of Keratoconus (CLEK) scheme.12 Tear secretion was evaluated by the Schirmer test after topical oxybuprocaine chlorohydrate 0.4% and an ophthalmic gel (polyacrylic gel 0.2%) were separately instilled into the conjunctival fornix. After the lower eyelid was partly everted, the center of the Tomo-Cap was applanated onto the center of the eyelid margin, behind the eyelash line and before the mucocutaneous junction, halfway between the inner and outer canthi. The instrument focus was manually set with the microscope in the acquisition modality “Section Mode.” Image acquisition began at the most superficial tissues and progressed down to the deepest ones that could be visualized with a satisfactory resolution. Ten images were taken at every 10 μm of depth. Other images were taken midway to assess the quality of the different structures observed. This procedure was repeated on the nasal and temporal eyelid margins. The two-dimensional image sizes were 384 × 384 pixels with a 400 × 400 μm field of view. The length of a single LSCM examination session was approximately 4 to 5 minutes.

**Image Analysis.** For each variable examined, we analyzed three randomized nonoverlapping high quality digital images of the nasal, middle, and temporal lower eyelid margin. All images were randomized and encoded by a single independent masked observer. We quantified the following variables: (1) Diameter of the acinar units. The units were manually measured along the longest axis of each acinus. (2) Acinar density. The acini were manually marked inside each 400 × 400 μm frame, and the density was calculated automatically with the Cell Count software (included in the HRT II Corneal Rostock Module; Heidelberg Engineering GmbH). (3) Diameter of the glandular orifices. The diameter was manually marked along the longest axis of each orifice. (4) Meibum secretion reflectivity (grading 1–4).8 (5) Inhomogeneous appearance of the interstices (grading 1–4)8 and walls of the acinar units (grading 1–4).8

We tested the repeatability of confocal values randomly selecting 15 subjects from the asymptomatic population (n = 100) and repeating the masked procedure of randomization, analysis, and quantification of three nonoverlapping high quality digital images of the nasal, middle, and temporal lower eyelid margin. Wilcoxon test and Spearman’s index were used to check differences and correlations between the two measurements.

**Statistical Analysis**

All data were analyzed as means ± SDs. Correlations were analyzed with Spearman’s index of linear correlation. For each variable, the Mann-Whitney U test was used to study the differences between men and women and between young and older subjects. The Kruskal Wallis test was applied to test the statistical differences among Groups A to F, with the Mann-Whitney U test used as post hoc test. The minimum criterion for tests of significance was P less than 0.05.

The statistical analysis was conducted with commercial software (SPSS for Windows, ver. 12.0; SPSS Sciences, Chicago, IL).

**Results**

**Asymptomatic Population**

The 15 subjects selected to verify the repeatability of confocal examinations showed no differences (P > 0.05) and strong correlations between the two measurements for all the quantified parameters (P < 0.01; lower R: 0.84 for inhomoge-

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**Table 1.** Asymptomatic Population: Number of Subjects in Each Age Group

<table>
<thead>
<tr>
<th>Group</th>
<th>Age Range</th>
<th>Total</th>
<th>Men</th>
<th>Women</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>20–29</td>
<td>20</td>
<td>12</td>
<td>8</td>
</tr>
<tr>
<td>B</td>
<td>30–39</td>
<td>15</td>
<td>9</td>
<td>6</td>
</tr>
<tr>
<td>C</td>
<td>40–49</td>
<td>12</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>D</td>
<td>50–59</td>
<td>17</td>
<td>8</td>
<td>9</td>
</tr>
<tr>
<td>E</td>
<td>60–69</td>
<td>18</td>
<td>7</td>
<td>11</td>
</tr>
<tr>
<td>F</td>
<td>Over 70</td>
<td>18</td>
<td>9</td>
<td>9</td>
</tr>
</tbody>
</table>

Separation in two age groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Age Range</th>
<th>Total</th>
<th>Men</th>
<th>Women</th>
</tr>
</thead>
<tbody>
<tr>
<td>G</td>
<td>Under 50</td>
<td>47</td>
<td>27</td>
<td>20</td>
</tr>
<tr>
<td>H</td>
<td>Over 50</td>
<td>53</td>
<td>24</td>
<td>29</td>
</tr>
</tbody>
</table>
neous appearance of the walls of the acinar units, higher $R$: 0.95 for acinar units density).

The analysis of the entire asymptomatic population (OSDI < 13) showed significant negative correlations between age and tear film BUT ($R = -0.29$, $P < 0.05$; Spearman), Schirmer test ($R = -0.32$, $P < 0.01$), and acinar density ($R = -0.35$, $P < 0.01$). There was a positive correlation between age and meibography score ($R = 0.45$, $P < 0.001$). Additionally, there was a negative correlation between meibography score and acinar density ($R = -0.45$, $P < 0.001$), and a positive correlation between MG expressibility and inhomogeneous appearance of acinar wall scores ($R = 0.36$, $P < 0.01$).

The same significant correlations were found both in men and women.

Comparing subjects over 50 years to those under 50-years old (Table 2), we found significantly increased meibography and MGs expressibility scores in the older group ($P < 0.05$, Mann-Whitney $U$ test). Acinar density and diameter were significantly reduced ($P < 0.01$ and $P < 0.05$, respectively) and the acinar wall inhomogeneity score was increased ($P < 0.05$).

The comparison between men and women, performed for the entire population and for the under and over 50-year-old groups, showed no statistically significant differences.

### Table 3. Asymptomatic Population, Clinical and Confocal Data: Stratification on Six Age Groups

<table>
<thead>
<tr>
<th>Age range, y</th>
<th>Group A</th>
<th>Group B</th>
<th>Group C</th>
<th>Group D</th>
<th>Group E</th>
<th>Group F</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>20–29</td>
<td>48.5± 5.18</td>
<td>46± 4.87</td>
<td>40± 3.96</td>
<td>50–59</td>
<td>60–69</td>
<td>Over 70</td>
<td></td>
</tr>
<tr>
<td>OSOI</td>
<td>1.2 ± 2.1</td>
<td>1.8 ± 2.8</td>
<td>2.7 ± 2.5</td>
<td>2.4 ± 3.1</td>
<td>2.8 ± 3.0</td>
<td>2.6 ± 3.2</td>
<td>n.s.</td>
</tr>
<tr>
<td>BUT, s</td>
<td>10.15± 2.85</td>
<td>9.40± 2.41</td>
<td>10.08± 2.55</td>
<td>9.47± 3.50</td>
<td>9.22± 3.10</td>
<td>8.50± 3.13</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Schirmer, mm/5 min</td>
<td>17.25± 2.79</td>
<td>16.80± 2.78</td>
<td>16.67± 2.95</td>
<td>14.92± 3.92</td>
<td>13.28± 4.11</td>
<td>13.53± 3.74</td>
<td>n.s.</td>
</tr>
<tr>
<td>Fluorescein</td>
<td>0.00</td>
<td>0.15± 0.35</td>
<td>0.08± 0.29</td>
<td>0.12± 0.35</td>
<td>0.22± 0.55</td>
<td>0.22± 0.55</td>
<td>n.s.</td>
</tr>
<tr>
<td>Lissamine green</td>
<td>0.60± 0.78</td>
<td>0.60± 0.74</td>
<td>0.75± 0.75</td>
<td>0.76± 0.85</td>
<td>1.00± 0.97</td>
<td>1.33± 0.91</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Meibography</td>
<td>0.40± 0.60</td>
<td>0.75± 0.70</td>
<td>0.75± 0.62</td>
<td>0.82± 0.73</td>
<td>0.89± 0.76</td>
<td>0.94± 0.87</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>MG expressibility</td>
<td>0.40± 0.50</td>
<td>0.47± 0.83</td>
<td>0.25± 0.45</td>
<td>0.53± 0.62</td>
<td>0.67± 0.68</td>
<td>0.83± 0.71</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Acinar density, units/mm²</td>
<td>121± 42</td>
<td>114± 20</td>
<td>119± 48</td>
<td>112± 17</td>
<td>96± 24</td>
<td>85± 35</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Acinar diameter, μm</td>
<td>46± 8.57</td>
<td>49± 11.02</td>
<td>45± 6.53</td>
<td>43± 7.45</td>
<td>32± 8.92</td>
<td>34± 9.14</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Orifice diameter, μm</td>
<td>50± 5.23</td>
<td>52± 6.51</td>
<td>52± 7.08</td>
<td>35± 5.18</td>
<td>31± 4.73</td>
<td>29± 7.74</td>
<td>n.s.</td>
</tr>
<tr>
<td>Secretion reflectivity</td>
<td>1.45± 0.39</td>
<td>1.27± 0.46</td>
<td>1.25± 0.45</td>
<td>1.35± 0.49</td>
<td>1.66± 0.70</td>
<td>1.93± 0.86</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Interstic inhomogeneity</td>
<td>1.40± 0.50</td>
<td>1.27± 0.46</td>
<td>1.25± 0.45</td>
<td>1.35± 0.49</td>
<td>1.44± 0.61</td>
<td>1.55± 0.70</td>
<td>n.s.</td>
</tr>
<tr>
<td>Acinar wall inhomogeneity</td>
<td>1.30± 0.47</td>
<td>1.20± 0.41</td>
<td>1.25± 0.45</td>
<td>1.41± 0.51</td>
<td>1.72± 0.75</td>
<td>1.94± 0.62</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

Data expressed as mean ± SD. $P$ obtained by Kruskal Wallis test.

In comparing Groups A to F (Table 3), composed of asymptomatic subjects stratified on six age groups, we found that tear film BUT significantly decreased with age ($P < 0.05$, Kruskal Wallis test). For the same groups, lissamine green, meibography, and MG expressibility scores increased with age ($P < 0.01$, $P < 0.05$, respectively).

MG acinar diameter density decreased in Groups A to F ($P < 0.001$, Kruskal Wallis test), and post hoc analysis showed significant decreases in Groups E and F compared with Groups A to D ($P < 0.01$, Mann-Whitney $U$ test).

MG acinar diameter decreased with age (Figs. A, B) in Groups A to F ($P < 0.001$), and post hoc analysis showed significant decreases in Groups E and F compared with Groups A to D ($P < 0.01$, Mann-Whitney $U$ test).

Acinar secretion reflectivity and inhomogeneous appearance of the acinar walls were significantly increased with age in Groups A to F ($P < 0.05$ and $P < 0.01$, respectively, Kruskal Wallis test).

### Unrestricted Population

We also compared the clinical and confocal data between Groups G and H, which were composed of younger than 40- and older than 65-year olds, respectively, who did not necessarily meet the inclusion and exclusion criteria. The tear film BUT and Schirmer test were significantly greater for Group G than Group H ($P < 0.05$ each, Mann-Whitney $U$ test). The scores for OSDI, meibography, and MG expressibility were all significantly less for Group G than Group H ($P < 0.05$, $<0.01$, and $<0.01$, respectively).

MG acinar density was significantly decreased in Group H compared with Group G ($P < 0.001$), but the acinar diameter was not significantly different between the two groups. Group H included subjects with small, atrophic MGs, and subjects with enlarged MG diameters consistent with obstructive MGD. Between Groups G and H, acinar secretion reflectivity and inhomogeneous appearance of the acinar walls increased with age ($P < 0.001$ and $P < 0.01$, respectively).

### Discussion

The aim of this study was to analyze, by in vivo LSCM, age-related changes in the MGs of healthy subjects. We recruited 100 healthy, asymptomatic subjects using specific inclusion and exclusion criteria. Although population-based surveys are ideal, the subjects were deemed to be representative of the healthy population.
We performed two sub analyses. In the first, we compared subjects under 50-years old to those over 50. Nien et al.,\textsuperscript{13} analyzing meibocyte differentiation, cell cycling, and Peroxisome proliferator-activated receptors (PPAR) signaling, recently described major differences in acinar cell proliferation and localization of the lipogenesis factor in subjects older than 50 years of age. However the authors studied a population with an asymmetric age distribution, with unequal sampling, and with less information on younger subjects. Previous studies reported different data on the age range during which MG changes begin to develop, setting the cutoff at 20, 30, or 40 years of age.\textsuperscript{5,14,15} To get information on early MG changes, we performed a second sub analysis, stratifying the population into six 10-year age groups (Groups A–F). A similar approach was used by Arita et al. who used infrared meibography to document age-related MG changes.\textsuperscript{5}

The high prevalence of ocular surface symptoms and diseases in the older population is well known.\textsuperscript{16–18} Therefore, we thought it would provide a different, and perhaps important, perspective to recruit young (Group G) and older (Group H) subjects who did not necessarily meet the inclusion and exclusion criteria.

We found that tear film BUT decreased in an age-related manner, and MG abnormal clinical scores increased. However, the clinical approach was able to provide us with only limited information on changes of MG characteristics. The clinical evaluation of eyelid margin and MG orifices, including grading of MG expressibility, showed slight changes that were too nonspecific to understand their pathogenic and morphologic features. Meibography showed the extent of MG dropout, as already described by Arita et al.\textsuperscript{5} using a new noncontact meibography method. LSCM allowed us to perform an in vivo, minimally invasive, and steady-state respectful examination of the MGs, obtaining at the same time information on gland size and density, secretion, and periglandular tissue. We adopted previously published examination procedures,\textsuperscript{8,9} and we quantified confocal parameters previously tested in healthy and dry eye subjects.\textsuperscript{7,8,19,20} Confocal values showed a good reproducibility, with no differences and a strong correlation between repeated measures.

LSCM showed an age-related reduction in the density of MG acini and increases in secretion reflectivity and in the inhomogeneity of the acinar walls. The first finding suggests gland dropout, in accordance with meibography and with previously published data.\textsuperscript{21} Secretion reflectivity and acinar wall changes may be due to qualitative changes of the meibum. Increased reflectivity scores, indeed, were previously observed in MGD patients and correlated with the severity of the disease.\textsuperscript{8,20} The interpretation of the inhomogeneity of the acinar walls is more doubtful. In previous reports, we proposed that the increase most likely signified the presence of inflammatory processes and/or changes in MG secretion characteristics.\textsuperscript{8,20} However, the results of the present study, showing an age-related increased score, no correlations with inhomogeneity of the interstice or other signs of inflammation, and a positive correlation between MG expressibility and inhomogeneous appearance of acinar wall scores, suggest that this parameter is more related to changes in the secreted meibum than to inflammation. This is consistent with results by Sullivan et al. who reported that the polar and neutral lipid patterns of meibum undergo significant age-related changes.\textsuperscript{6}

We feel that our most interesting LSCM result may be the age-related reduction in MG acinar diameter. In contrast to a previous study of MGD patients that reported an increase in MG orifice size,\textsuperscript{8} we found no differences in the MGs orifice diameters among the groups. Previous studies reported MG age-related anatomical and physiologic changes, including acinar cell atrophy and loss, abnormal features such as hyperkeratinization of ductal epithelium, and reduced and viscous secretion.\textsuperscript{5,6} Age-dependent alterations in human MGs were reported by Obata et al.\textsuperscript{4,22} who described acinar atrophy without distinct dilatation as one of the pathologic findings. This suggests that primary acinar atrophy leads to a decrease in the MG secretion with aging. Age-dependent atrophic changes in mouse MGs have also been reported.\textsuperscript{3} They differ from those typically observed in human acinar atrophy due to obstructive MGD because in the mouse the well-known hyperkeratinization and dilatation of the ductal system and acini did not occur. A recent MGD workshop found that currently it is not clear if the murine findings are consistent with the proposed primary atrophy occasionally observed in humans.\textsuperscript{1,13} The workshop concluded that there may be a primary, age-dependent form of MGD that leads to a gradual decline of glandular function.\textsuperscript{1,13}

Our findings of age-related decrement of acinar size associated with the decrement of acinar density and the absence of obstructions and enlarged orifices provide support for the occurrence of primary, age-related MG atrophy.

Whether our subjects were divided into two groups of under and over 50 years of age or into six groups of 10 years
each, LSCM showed major MG age-related changes occurring at 50 to 60 years of age. These data are consistent with the recently reported timing of cellular changes that presumably lead to age-related acinar atrophy in humans.13 Earlier changes, between 20 and 30 years of age, were reported by Arita et al.,3 in a large study on MG aging. They interpreted these early changes to be the consequence of the high sensitivity of the noncontact infrared meibography method. In our opinion, in vivo LSCM is probably more sensitive, being able to detect acinar changes preceding the dropout. We think that this timing dissimilarity may be due to the potential differences in two populations enrolled in these studies that utilized different inclusion criteria. For instance, the usage of the OSDI score cutoff can play an important role in this regard.

Several studies reported an association between MG changes and sex hormones such as androgens, estrogen, and progesterone.23–28 However in our research, we did not find significant sex-related changes in MG aging. Further research designed to explore this issue is necessary to elucidate the clinical implications of the hormonal action on MG age-related changes.

Interestingly, comparing the younger (group G) with the older subjects (group H) enrolled without inclusion and exclusion criteria, we found no differences in mean acinar diameter, but very different SDs, 47±6 versus 9.80±45±20.15 μm, respectively, for the two groups. This result was due to the coexistence in the older group of subjects with small, atrophic glands and subjects with enlarged glands (Figure).

Based on the differences between asymptomatic and unrestricted populations, we hypothesize the existence of two different forms of age-related MGD. In one form, MGD exists due to the primary age-related atrophy of the MGs that results from acinar atrophy and reduced density. The second form of MGD occurs due to the presence of acinar obstruction. This “classic form” of MGD arises in MGs with low acinar density, but in which the acini become enlarged due to the obstruction. In older patients, both forms may occur. The primary atrophic changes seem to be less related to ocular surface symptoms, suggesting that it is a more physiologic way of MG aging.

In conclusion, we used LSCM to provide new in vivo information on human MG aging. Our data revealed age-related, nonobstructive changes that could not be discriminated in current clinical examinations. Larger population-based research could provide more evidence-based data on the features of MG aging.

Acknowledgments

Disclosure: E. Villani, None; V. Canton, None; F. Magnani, None; F. Viola, None; P. Nucci, None; R. Ratiglia, None

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