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Microdot Accumulation in the Anterior Cornea with Aging – Quantitative Analysis with in Vivo Confocal Microscopy

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ABSTRACT

Purpose: Degenerative ‘microdot’ deposits in healthy and hypoxic corneas are believed to represent lipofuscin-like material aggregation in the stroma. To accurately assess microdot deposits in a clinical setting, we sought to quantify these deposits for the first time using the non-invasive clinical imaging technique of in vivo confocal microscopy (IVCM).

Methods: The corneas of 102 healthy subjects aged 15–88 years were examined by IVCM and microdot density was quantified using a 6-point grading scale by two masked, trained examiners. Microdot density was analyzed with respect to age, sex and stromal depth, and inter-eye and inter-observer differences were evaluated.

Results: In healthy subjects, microdot density decreased from the anterior to posterior stroma, with the greatest accumulation observed in the most anterior stroma (subepithelial region). In this region, microdot density correlated strongly with age (P < 0.0001), with increased microdot deposition in older subjects (>60 years) relative to younger ones (<45 years) (P < 0.001). Microdot density between eyes of the same subject was highly correlated (r = 0.92, P < 0.0001), while no association with sex was noted (P ≥ 0.05). The mean inter-observer difference in microdot assessment was 0.62 ± 0.09 grades, with a high correlation of grading between observers (r = 0.77, P < 0.0001).

Conclusions: IVCM can be used to non-invasively quantify microdot deposits in the subepithelial corneal stroma with good inter-observer reproducibility. Microdot assessment may provide a novel means of quantifying age-related or pathologic degeneration of the corneal stroma in a clinical setting.

Introduction

In vivo confocal microscopy (IVCM) is a noninvasive technique that enables real-time, high-resolution imaging of the living cornea at the cellular level. Its clinical use has grown substantially over the years. Some of the earliest studies that applied confocal microscopy to the cornea described small, highly reflective stromal point-like structures, referred to as “microdots”.

Later, these microdots were considered a type of corneal degenerative disease, and their incidence increased with contact lens wear. Microdots have also been reported in patients following Thygeson’s superficial punctate keratopathy, amiodarone/Fabry keratopathy, chronic and delayed mustard gas keratopathy, Reis-Bücklers’ corneal dystrophy, and corneal surgery. It has been hypothesized that they may be lipofuscin-like material that accumulates as a result of oxidative stress due to chronic hypoxia of the cornea.

Knowledge of the effects of aging on corneal morphology is essential for differentiating normal aging from degenerative disorders or pathologic processes. IVCM has been used to study the effects of aging on corneal epithelial cells, the sub-basal nerve plexus, stromal keratocytes, endothelial cells, and central corneal thickness. Hillenaar et al. found an increased incidence of corneal microdots in older relative to younger subjects. There are no quantitative measurements, however, of microdots with respect to age, and microdot distribution in relation to depth within the corneal stroma remains unknown.

Bönhke and Masters developed a scoring system ranging from 0 to 4+ to quantitatively evaluate the microdot density measured by scanning slit confocal microscopy in the corneas of subjects with contact lens–wearing history. They found no stromal microdots in the eyes in the healthy control group. The laser-scanning IVCM used in the present study yields images with superior contrast and an axial resolution of 4 µm, relative to the 26-µm axial resolution with slit-scanning systems, which may allow better detection of stromal microdots.

Aging is known to result in degeneration of ocular tissues, and the cornea, in particular, is exposed to potential stressors from the environment due to its exposed anatomical location. A prior study in rabbit eyes suggested that the aged cornea might be more susceptible to oxidative injury caused by an imbalance between antioxidant and prooxidant enzymes. There is a lack, however, of means to quantify aging or stromal degeneration in the human cornea in a clinical context.
setting. Here, we aimed to develop a reliable means of grading microdot density in the cornea using laser-scanning IVCM. We used this method to determine the age and stromal depth dependence of central corneal microdots in the healthy cornea, to establish an age-controlled baseline against which corneal microdots in various pathologies can be assessed in the future, as a surrogate indicator of ocular degenerative processes.

Materials and methods

Subjects and examinations

With ethical approval from the Linköping Regional Ethical Review Board, Sweden (Application No. M172-09), 102 healthy volunteers accompanying patients at the Linköping University Ophthalmology Department were recruited to the study. The exclusion criteria were diabetes, history of contact lens wear, use of ocular medication, prior corneal surgery or pathology, and dry eye symptoms. A full ophthalmic examination of both eyes that included slit lamp biomicroscopy, intraocular pressure, visual acuity, IVCM, and anterior segment optical coherence tomography was performed to rule out corneal or other potentially confounding ocular pathology. Before the examination, each subject provided signed informed consent to participate in the study, and the study was conducted according to the tenets of the Declaration of Helsinki.

In vivo confocal microscopy

IVCM was performed using the Heidelberg Retina Tomograph 3 (HRT3) laser scanning in vivo confocal microscope with the Rostock Cornea Module (RCM) (Heidelberg Engineering, Heidelberg, Germany). The microscope was outfitted with a × 63/0.95 NA (numerical aperture) immersion objective lens (Carl Zeiss SMT GmbH, Oberkochen, Germany) to yield images representing an enface view of a 400 × 400 μm corneal area. Axial depth was adjusted using motorized joystick control.

During the examination, care was taken to minimize TomoCap (Heidelberg Engineering) pressure on the cornea to avoid pressure-induced striation artifacts in the images.20 Volume scan mode scanning was initiated when the epithelial wing cell layer appeared in the real-time display window; consequently, 40 images were acquired automatically over an axial depth of 80 μm. In a subgroup of 16 healthy eyes (four eyes in each age group: 15–30, 31–45, 46–60, and >60 years), 100 consecutive images (eight frames per second) were acquired from the epithelium through the entire corneal stroma to the endothelium. In all cases, the automatic brightness adjustment setting on the HRT3–RCM system was used.

Image selection and grading

Two observers masked to study subject’s identifying information analyzed the IVCM images. Microdots were defined based on size, brightness/reflectivity, and degree of circularity. The two observers graded the number of point-like (1–2 μm), highly reflective and distinct, relatively circular structures within each 400 × 400 μm image as follows: Grade 0 (<5 dots), Grade 1 (5–10 dots), Grade 2 (11–25 dots), Grade 3 (26–50 dots), Grade 4 (51–75 dots), and Grade 5 (>75 dots) (Figure 1).

IVCM images acquired using the ‘sequence scan’ mode were viewed to compare the microdot grades according to stromal depth. Five stromal layer categories were defined: subepithelial (0–10% of the stromal depth), anterior (11–30% of the stromal depth), mid (31–60% of the stromal depth), posterior (61–90% of the stromal depth), and pre-endothelial (91–100% of the stromal depth).

To assess the age dependence of anterior stromal microdots, we analyzed the three most anterior stromal images acquired by volume scan just under Bowman’s layer. Each observer manually assessed six images per healthy subject (three each from the left and right eye).

Quantitative and statistical analysis

Statistical analyses were performed with IBM SPSS Statistics v22 (IBM Corp., Armonk, NY). Data normality was tested with the Kolmogorov–Smirnov test.21 Microdots across different age groups in healthy eyes were compared using the average microdot value of six images per subject. Differences between age groups and between stromal depths were tested using one-way analysis of variance (ANOVA) after averaging the grading from the two independent observers. The associations of microdots with age and inter-eye correlations were tested with the Spearman correlation test; the difference in microdots between male and female subjects was tested with the Mann–Whitney U test. In all cases, results were considered significant where \( P < .05 \). All values in the figures and tables are the mean ± SE (standard error). Inter-observer difference of microdot density was assessed using the Bland–Altman method, and the 95% limits of agreement (LOA) were calculated.22

Results

Stromal depth dependence of central corneal microdots

Subjects aged over 30 years had a clear pattern of microdot deposition in the stroma, with accumulation occurring in the subepithelial region and decreasing with increasing stromal depth \(( P < .001, \text{ Table 1, Figure 2})\). This pattern, however, was not observed in younger subjects, who had relatively unchanged microdot grade with corneal stromal depth \(( P = .09, \text{Figure 2, Table 1})\). There were age-dependent increases in microdot grade in the subepithelial, anterior, mid-, and posterior stromal regions \(( P < .05)\) but not in the pre-endothelial region. Careful inspection was performed in stromal images from the pre-endothelial region in all 16 subjects where full-thickness IVCM scans were available. It was observed that posterior keratocytes did not appear enlarged, nor did the microdots exhibit a clear localization to the cell nucleus or cell body (regardless of microdot grade), contrary to findings described in cases of pre-Descemet’s membrane corneal dystrophy,23–25 (Figure 3).
Age dependence of anterior stromal microdots

As microdots appeared most frequently in the subepithelial region, the remainder of the analysis focused on this region. The three most anterior stromal images just under Bowman’s layer were analyzed, with the grade taken as the mean of three images. Table 2 presents the subject characteristics. Based on the averaged values from observers I and II, microdots in the right and left eye of the same healthy subjects were highly correlated ($r = 0.92$, $P < .0001$). Microdot grade correlated strongly with age ($r = 0.79$, $P < .0001$) (Figure 4), with a mean grade of 1.84 ± 0.12 in subjects aged <45 years and 3.95 ± 0.12 in subjects aged >60 years ($P < .0001$). No subjects aged <60 years had microdot grade 5, while no subjects aged >60 years had microdot grade 0 or 1. Independent of the observer, there was no association between sex and microdot grade ($P > .05$).

Inter-observer agreement on anterior stromal microdots

The manual grading by observers I and II was highly correlated ($r = 0.77$, $P < .0001$). The inter-observer mean difference was 0.62 ± 0.09 grades (95% LOA: ±1.65 grades) (Figure 5).

Discussion

The recognition of small, microscopic changes in the cornea from a normal, healthy appearance can be important for the detection of pathology. Features that are not discernible under slit lamp observation may be visualized and readily quantified in the cornea using IVCM. Stromal microdots are highly reflective, and increased microdots in the anterior stroma produce higher levels of backscatter. Here, microdots in healthy corneas could for the first time be quantified using a grading scale which exhibited inter-observer consistency. Using this scale, significant stromal depth dependence was found in healthy eyes over the age of 30, with the greatest microdot accumulation occurring in the subepithelial stromal region, just posterior to Bowman’s layer. Microdot grade in both eyes of the same subject was highly correlated and increased with age, while sex had no influence.

Using slit-scanning confocal microscopy, Bönkhke and Masters found microdots of approximately 0.3–0.6 µm in size, with round-to-polygonal shape, throughout the corneal stroma in 24 contact lens wearers with a contact lens–wearing history of >5 years, while none of the 29 age-matched healthy controls had microdot deposits. The results were supported by
Trottibach et al., who observed microdots (mean size, 3.04 ± 0.92 µm; range, 1.5–5.0 µm) throughout the entire depth of the corneal stroma in all contact lens wearers, but not in healthy control subjects. Yagmur and associates using slit-scanning confocal microscopy reported microdots in 39.4% of contact lens–wearing patients, while no microdots were evident in the control group. Efron et al., however, observed small, dense, highly reflective microdots about 1–2 µm in diameter throughout the stroma in virtually all corneas of 119 subjects aged 10–80 years using slit-scanning confocal microscopy. In line with the findings in the Efron study, we observed microdots in most of the eyes examined, who had no history of contact lens wear. Only three eyes (age: 15, 23, and 27 years) and one eye (age: 15 years) were graded 0 by both observers. The lack of consensus on the definition of microdots (size, reflectivity, shape) may be a possible source

**Figure 2.** Top: Illustration of the mean microdot grade versus stromal depth for five age ranges: all ages, 15–30 years, 31–45 years, 46–60 years, and >60 years. Microdot grade was significantly depth-dependent from age >30 years. There was significant age dependence in a 0–90% depth of the stroma. Bottom: IVCM images obtained from a 68-year-old woman demonstrating decreased microdots with increased scanning depth. Left: Subepithelial layer, microdot Grade 5. Middle: Anterior stroma, microdot Grade 2. Right: Posterior stroma, microdot Grade 0.

**Figure 3.** Representative images taken from the pre-Descemet stromal layer in subjects with various microdot grades. In all cases, keratocytes did not appear enlarged or hyper-reflective, nor did the microdots preferentially localize to the keratocyte nuclei. All images 400 × 400 µm.
found in both groups were not counted. Different confocal microscope imaging capabilities and varying image acquisition techniques also likely contributed to the reported discrepancies. Nevertheless, in the present study using high-contrast laser-scanning confocal microscopy, even with the same images and microdot definition, there was a mean observer-dependent grading difference of approximately 0.62 ± 0.09 grades. These results may be explained in part by variations in the microdot contrast/visibility in some of the images. Moreover, it is especially difficult to count the microdot density when a group of microdots is close to a network of keratocytes. Appropriate image enhancement and consensus on manual microdot grading as well as automated microdot detection using new algorithms may increase the measurement reliability.

Hillenaar et al. using slit-scanning confocal microscopy demonstrated statistically significantly increased anterior stromal microdot incidence (presence/absence) in normal eyes, from 0% in the 20–29-year age group to 80% in the 70–79-year age group. In the present study, linear regression revealed quantitative age-dependent anterior stromal microdot deposition. Therefore, it is important to use age-matched control groups in future studies in which different subject groups are compared.

In subjects aged over 30 years, the subepithelial region contained the greatest density of microdots, thereafter decreasing with stromal depth. Furthermore, the age-dependent microdot density increase was observed in all but the pre-endothelial stroma. The anterior stromal keratocytes contain twice as many mitochondria as that in the mid- or posterior stroma. Moreover, keratocyte density is greater in the anterior than posterior stroma and anterior keratocyte density is significantly reduced with age. Whether microdot density and keratocytes are correlated and the clinical implications of microdot density remain unclear; however, future studies can be designed to examine stromal microdot presence and density in degenerative ocular pathologies.

There is a limited understanding of the origin and production of the microdot deposits. One theory is that microdots consist of lipofuscin granules of intracellular origin. It has been suggested that this lipofuscin-like material within the corneal stroma of long-term contact lens wearers forms as a result of chronic oxygen deprivation and chronic microtrauma to the cornea. Böhnke and Masters hypothesized that microdots might be an early stage of irreversible corneal stromal alteration that may require treatment in the future. By contrast, Efron et al. believed that microdots might represent dysgenic or apoptotic cellular remnants lying dormant in the stroma, and should be considered a normal feature of human corneal morphology that perhaps could be altered by contact lens wear, which is in line with our findings of increased stromal microdot production and deposition in healthy aging corneas.

IVCM is a promising tool for identifying corneal pathology and monitoring treatment efficacy. However, the realization of its full potential requires a reliable baseline characterization of morphological structures to enable the detection and documentation of abnormal findings. Currently, there is a lack of information on microdot density of inconsistency among prior studies. For example, in the study by Trittibach et al., only clearly visible, hyper-reflective, round, or near-round structures were considered, whereas dust-like, low-reflective structures in the sub-micron range

### Table 2. Subject demographic characteristics indicating the number of subjects (N) in each age and gender category.

<table>
<thead>
<tr>
<th>Age Groups</th>
<th>Total (N)</th>
<th>Male (N)</th>
<th>Female (N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15–30 years</td>
<td>46 55 34 15 19</td>
<td></td>
<td></td>
</tr>
<tr>
<td>31–45 years</td>
<td>21 5 16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>46–60 years</td>
<td>24 16 8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;60 years</td>
<td>23 11 12</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Figure 4.** The mean microdot grade across observers, with respect to age, for microdots in the most anterior stromal region (subepithelial layer). The regression line indicates that microdots strongly accumulate with age in this region (r = 0.79; P < .0001).

**Figure 5.** Bland–Altman analysis of inter-observer agreement in healthy subjects. The mean inter-observer difference was 0.62 ± 0.09 grades (black line), with 95% LOA (upper and lower grey lines) of ±1.65 grades.
in the normal, healthy central cornea. The present study quantified microdot density in the human cornea in a large cohort, for the first time using high-resolution laser-scanning IVCM. The analysis revealed associations between age, depth of deposits and microdot density. One limitation of this study, however, is the potential inclusion of subjects with possible conditions not detected by the general ophthalmic examinations, such as mild endothelial dystrophy or asymptomatic dry eye, which may have influenced the microdot grading. Excluding subclinical alterations via additional clinical examinations and tests of the corneal endothelium, epithelium, and tear film could reduce the degree of variability of microdot density in future studies. A further limitation is that the methods used in this study did not allow for the immunohistological labeling of the corneal tissue. Antibody-based studies of excised corneal tissue are recommended for determining the precise nature of the microdot deposits; however, new methods may be required to visualize these submicron-sized features in standard corneal sections. Nevertheless, the in vivo images presented herein can aid in distinguishing normal corneal aging from keratopathy using IVCM, and the proposed grading system could be used in future studies to investigate differences between healthy and diseased populations.

In conclusion, we report an accumulation of microdots in the aging cornea, primarily in the subepithelial stromal region, that may have implications for corneal assessment. Further avenues for research include identifying the proteins present in microdots, confirming their intra- and/or extracellular origins, as well as examining and quantifying the presence of microdots in different pathologies.

Disclosure Statement

None of the authors have any competing proprietary/financial interest to disclose.

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Data availability

The data used to support the findings of this study are included within the article. All raw data are available upon request.

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