Correlations of Long-term Matrix Metalloproteinase Localization in Human Corneas After Successful Laser-Assisted In Situ Keratomileusis With Minor Complications at the Flap Margin

Pierre R. Fournié, MD; Gabriel M. Gordon, BS; Daniel G. Dawson, MD; Henry F. Edelhauser, PhD; M. Elizabeth Fini, PhD

Objective: To determine whether matrix metalloproteinases (MMPs) are present long-term in human corneas after successful laser-assisted in situ keratomileusis (LASIK).

Methods: Eighteen postmortem corneas from 10 patients with postoperative intervals of 2 to 8 years after LASIK surgery and 4 normal control corneas from 2 patients were collected from US eye banks and processed for histologic analysis and immunolocalization with antibodies to MMP-1, MMP-2, MMP-3, MMP-7, MMP-8, MMP-9, MMP-10, and MMP-14.

Results: Matrix metalloproteinase 7 was present in the epithelium of all corneas. Other MMPs were localized to the wound margin in some post-LASIK corneas. Matrix metalloproteinase 9 was detected around epithelial cells trapped in the lamellar scar in 5 of 6 corneas with epithelial ingrowth. Various MMPs were detected in fibrotic tissue at the wound margin in 2 of 2 corneas with flap retraction.

Conclusions: The presence of MMPs in post-LASIK corneas correlates with an ongoing wound healing process associated with minor post-LASIK complications. Matrix metalloproteinases might contribute to instances of ongoing flap instability, and if so, judicious use of MMP inhibitors could provide benefit.

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ase expression is transcriptionally up-regulated in resident tissue cells during wound healing, regeneration, and remodeling; MMPs are also brought in by invading leukocytes, which both synthesize and store MMPs.2,15-18

The cornea has been an important experimental model for defining mechanisms of MMP expression and role in normal and pathologic repair processes, including epithelial regeneration and failure to heal, fibrotic repair and scar remodeling, infection, and angiogenesis.2,16,19-37 These studies have revealed that specific MMPs appear in specific locations in the repairing cornea correlated with events occurring on the cellular and tissue level. For example, MMP-9 is produced in the corneal epithelium regenerating across the intact basement membrane after abrasion injury and then disappears once regeneration is complete.19 However, in situations involving chronic epithelial defects, MMP-9 levels remain elevated, causally contributing to failure to heal.21,22 Similarly, a number of different MMPs are produced by the fibrotic repair tissue deposited in response to keratectomies that penetrate through the epithelium and into the stroma.20 In this case, MMP expression continues long-term, at least 1.5 years, as documented in a rabbit penetrating keratectomy model, correlated with the lengthy process of repair tissue remodeling. Some of the reports evaluating MMP expression in more specific models of photo-refractive keratectomy and LASIK,23-26 and corneal button specimens obtained after penetrating keratoplasty for photorefractive keratectomy model, correlated with the lengthy process of repair tissue remodeling. Some of the reports evaluating MMP expression in more specific models of photo-refractive keratectomy and LASIK,23-26 and corneal button specimens obtained after penetrating keratoplasty for photorefractive keratectomy or post-LASIK complications,27-30 have supported similar conclusions in the short-term, but the long-term studies have not been done.

The purpose of the present study was to determine whether MMPs continue to be expressed long-term in human corneas after reportedly uncomplicated successful LASIK. All corneas were obtained postmortem from US corneal eye bank donors at varying intervals after LASIK surgery. We also investigated factors, including patient age, postoperative interval, and histopathologic findings, that could affect MMP expression.

METHODS

SOURCE OF THE CORNEAS

After approval by the Emory University institutional review board, 18 postmortem corneoscleral buttons from 10 corneal eye bank donors with a history of LASIK surgery were obtained from various eye banks in North America. A number was assigned to each cornea according to the order of inclusion. The specimens were received in Optisol-GS solution (Bausch & Lomb Surgical, Irvine, California) within 6 days of death (mean [SD] time of preservation, 3.51 [1.4] days). Review of preoperative, intraoperative, and postoperative clinical records, when available, was performed. Four postmortem normal corneas stored in Optisol-GS (mean [SD] time of preservation, 2.95 [0.35] days) from 2 patients were obtained from the Georgia Eye Bank (Atlanta) and the Lions Eye Bank (Miami) as controls.

CORNEAL PROCESSING

The corneoscleral buttons were oriented with the hinge superiorly and then trisected. The central portion was immediately snap-frozen in liquid nitrogen, embedded in optimal cutting temperature compound (Tissue-Tek-II; Miles Inc, Elkhart, Indiana), and stored at −70°C until sectioned. Sections (8 µm) were cut in a cryostat microtome (Leica 1850 cryostat; Leica, Deerfield, Illinois) and mounted on adhesive-coated glass slides for conventional and immunofluorescent histologic processing. Normal control corneas were sectioned centrally and processed identically as LASIK corneas.

HISTOLOGIC EXAMINATION

The sections were fixed and stained with hematoxylin-eosin according to standard procedures. Histopathologic findings from light microscopic examination of the peripheral lamellar wound at the flap margin and central lamellar wound, flap thickness (taken from a representative area with the fewest artifacts and containing all 3 distinct types of epithelial cells: basal, wing, and superficial epithelial cells), and residual stromal bed thickness measurements using a Zeiss Axiosvert 200M inverted microscope (Carl Zeiss Meditec, Jena, Germany) coupled to a Zeiss AxioCam MRc5 camera (Carl Zeiss Meditec) were recorded.

ANTIBODIES

Polyclonal antibodies to MMPs were purchased from Triple Point Biologics (Forest Grove, Oregon): rabbit antibodies to interstitial collagenase (RH MMP1), gelatinase A (RH MMP2), stromelysin 1 (RP2MMP3), MMP-7 (RP2MMP7), neutrophil collagenase (RP1MMP8), gelatinase B (RP3MMP9), stromelysin 2 (RP2MMP10), and membrane-type MMP-1 (RP2MMP14). Rabbit monoclonal anti–CD11b (Mac-1) (clone M1/70.15) was obtained from Cedarlane Laboratories (Burlington, North Carolina). CD11b (Mac-1) was used as a marker for leukocytes, including macrophages and granulocytes. Rabbit polyclonal antiantimamin (L9393) and mouse monoclonal α-smooth muscle actin (α-SMA) (clone 1A4) were purchased from Sigma-Aldrich (St Louis, Missouri). Rabbit IgG and mouse IgG (Chemicon, Temecula, California) were purchased as negative controls. Secondary antibodies used were Alexa Fluor 488 conjugated goat antimouse IgG (A-11001) and donkey antirabbit IgG (A-21206) from Invitrogen Molecular Probes (Carlsbad, California).

INDIRECT IMMUNOLOCALIZATION

Slides to be stained were air-dried for 20 minutes at room temperature and then fixed in 100% cold acetone (−20°C) for 20 minutes. They were washed with phosphate-buffered saline (PBS) 3 times for 5 minutes each, followed by a 1-hour incubation in a humidified level chamber in 10% normal donkey (D9663, Sigma-Aldrich) or goat (G9023, Sigma-Aldrich) sera

Table 1. Clinical Data

<table>
<thead>
<tr>
<th>Patient/ Age, y</th>
<th>No. of Corneas Harvested</th>
<th>Postoperative Interval, y</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/51</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td>2/24</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>3/55</td>
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<td>5</td>
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<td>4/51</td>
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<tr>
<td>5/51</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>6/59</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>7/48</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>8/55</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>9/62</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>10/48</td>
<td>1</td>
<td>8 (first flap)/4 (second flap)</td>
</tr>
</tbody>
</table>
in PBS to block nonspecific staining. Primary antibodies were used in a dilution of 1:100 and incubated at 4°C overnight. After 3 additional washes with PBS for 5 minutes each, the Alexa Fluor 488 conjugated secondary antibody was applied for 1 hour. Samples were mounted with the Vectashield mounting medium with 4',6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, California) for nuclear counterstaining. Negative control sections were processed identically but incubated

**Table 2. Histologic Findings**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Margin of the Flap</th>
<th>Flap Thickness, µm</th>
<th>Residual Stromal Bed Thickness, µm</th>
</tr>
</thead>
<tbody>
<tr>
<td>2Ra</td>
<td>No specificity, good alignment of BL ends</td>
<td>110</td>
<td>345</td>
</tr>
<tr>
<td>4R</td>
<td>No specificity, good alignment of BL ends</td>
<td>115</td>
<td>385</td>
</tr>
<tr>
<td>1</td>
<td>Major epithelial ingrowth, good alignment of BL ends with a small gap at the break</td>
<td>145</td>
<td>335</td>
</tr>
<tr>
<td>4Lb</td>
<td>Minor epithelial ingrowth with islands of epithelial cells in the scar, good alignment of BL ends</td>
<td>155</td>
<td>345</td>
</tr>
<tr>
<td>7L</td>
<td>Major epithelial ingrowth, depressed BL end with a small gap at the break</td>
<td>145</td>
<td>315</td>
</tr>
<tr>
<td>9R</td>
<td>Major epithelial ingrowth, good alignment of BL ends with a small gap at the break</td>
<td>170</td>
<td>330</td>
</tr>
<tr>
<td>9L</td>
<td>Minor epithelial ingrowth with microscopic foci of epithelial cells in the scar, good alignment of BL ends</td>
<td>140</td>
<td>295</td>
</tr>
<tr>
<td>10</td>
<td>First flap: epithelial ingrowth, good alignment of BL ends; second flap: no specificity, good alignment of BL ends</td>
<td>First flap: 135; second flap: 40</td>
<td>330</td>
</tr>
<tr>
<td>3R</td>
<td>Epithelial hyperplasia, depressed BL end</td>
<td>140</td>
<td>350</td>
</tr>
<tr>
<td>3L</td>
<td>Epithelial hyperplasia, depressed BL end</td>
<td>125</td>
<td>318</td>
</tr>
<tr>
<td>5L</td>
<td>Epithelial hyperplasia, good alignment of BL ends with a small gap at the break</td>
<td>120</td>
<td>460</td>
</tr>
<tr>
<td>6R</td>
<td>Epithelial hyperplasia, elevated BL end</td>
<td>110</td>
<td>335</td>
</tr>
<tr>
<td>6L</td>
<td>Epithelial hyperplasia, depressed BL end</td>
<td>100</td>
<td>350</td>
</tr>
<tr>
<td>7R</td>
<td>Minor epithelial hyperplasia, good alignment of BL ends</td>
<td>110</td>
<td>405</td>
</tr>
<tr>
<td>8R</td>
<td>Minor epithelial hyperplasia, good alignment of BL ends</td>
<td>125</td>
<td>340</td>
</tr>
<tr>
<td>8L</td>
<td>Minor epithelial hyperplasia, depressed BL end</td>
<td>120</td>
<td>390</td>
</tr>
<tr>
<td>2L</td>
<td>BL end curled inward with mild flap retraction</td>
<td>120</td>
<td>335</td>
</tr>
<tr>
<td>5R</td>
<td>BL end curled inward with significant flap retraction</td>
<td>115</td>
<td>390</td>
</tr>
</tbody>
</table>

**Abbreviation:** BL, Bowman’s layer.

a Right cornea.

b Left cornea.

**Figure 1.** Light microscopy of laser-assisted in situ keratomileusis (LASIK) flaps (hematoxylin-eosin, original magnification × 200). Histopathologic findings of the peripheral LASIK wound margin regions (arrowheads) display varying healing modalities. A, “Normal healing” with good alignment of Bowman’s layer ends (patient 4R); B, Epithelial hyperplasia with depressed Bowman’s layer end (patient 3L); C, Epithelial ingrowth (patient 1); D, Bowman’s layer end curled inward with a flap retraction (patient 5R); E, Double-cut LASIK with the second cut deeper and wider (patient 10).
with strain-specific IgG as primary antibodies. Samples were examined using a Zeiss Axiovert 200M inverted fluorescence microscope and images were captured using a Zeiss AxioCam MRc5 camera attached to the microscope. Camera and microscope settings were controlled by Axiovision software version 4.1 (Carl Zeiss Meditec). To evaluate the effect of LASIK, 4 regions were evaluated: (1) the LASIK flap wound margin, (2) the corneal stroma in the LASIK flap, (3) the paracentral and central lamellar wound regions, and (4) the residual stromal bed. Normal control corneas were evaluated in the central, paracentral, and peripheral regions. The LASIK flap wound margin was also examined using a Leica TCS SP2 confocal microscope (Leica Microsystems, Bannockburn, Illinois) with a total magnification of 400.

**DATA ANALYSIS**

The slides were evaluated for staining intensity, cellular localization of staining, and the presence of any distinctive staining patterns. Immunoreactivity intensity was evaluated using a semi-quantitative scale with the negative control as baseline (− = none or same as background, + = weak [trace or slightly perceptible above background], ++ = moderate, and +++ = strong). Reliability was assessed by having 2 evaluators independently rate the staining intensities at different times. The average of the 2 observers’ scores was used. Concordance among the observers was high.

**RESULTS**

**CLINICAL FINDINGS**

A complete history of the LASIK surgeries could not be obtained for the majority of the 18 specimens. Clinical data that were available for every specimen included patient age, cause of death, a history of uncomplicated LASIK, and the postoperative interval after LASIK (Table 1). Patient 10 underwent LASIK with 2 enhancements by flap relifting 6 and 12 months after initial surgery and a third enhancement by recutting a flap (Figure 1E). The 10 patients ranged in age from 34 to 62 years (mean [SD], 50.30 [7.15] years). The postoperative interval after LASIK ranged from 2 to 8 years.

**HISTOLOGIC FINDINGS**

Hematoxylin-eosin–stained light microscopy evaluations revealed a lamellar interface scar that was relatively easy to find (Figure 1) in all the specimens. The mean (SD) central thickness of the LASIK flap was 130 (21) µm. The mean (SD) residual stromal bed thickness
was 353 (39) µm (Table 2). At the LASIK flap wound margin, some corneas had variable amounts of epithelial ingrowth into the lamellar wound (Figure 1C), microscopic foci or islands of epithelial cells actually in the scar, and variability in the alignment of the cut end of Bowman’s layer at the wound margin (eg, good alignment with a small gap at the break [Figure 1C], depressed or elevated ends [Figure 1B], and ends curled inward with various flap retraction and stromal defects [Figure 1D]). Epithelial ingrowth into the flap margin was observed in 6 of the 18 corneas (33.3%) examined using serial sections. Variable amounts of epithelial hyperplasia (Figure 1B) were commonly present at the wound margin, filling the gaps in Bowman’s layer. The histologic findings in the wound margin are summarized in Table 2. Corneas were grouped according to their main histopathologic finding at the wound margin.

**IMMUNOSTAINING FINDINGS**

Staining of normal control corneas was the same in the central, paracentral, and peripheral regions: only MMP-7 was detectable in 2 of the 4 normal control corneas with at least ++ staining intensity, localized in the epithelium (Table 3). A number of different MMP proteins were immunodetected in the wound margin of LASIK corneas. Semiquantitative staining intensity results in the wound margin are summarized in Table 3. No staining
was observed at the paracentral or central scar regions. All other regions in the corneal stroma were unstained. The negative control serving as reference for immunofluorescence intensity evaluation is shown for each figure. Matrix metalloproteinase 9 immunostaining with a level of at least H11001/H11001 was observed in 5 (patients 1, 7L, 9R, 9L, and 10) of the 6 corneas with epithelial ingrowth. Immunoreactive MMP-9 protein was detected around epithelial cells trapped in the lamellar scar (Figure 2C and Figure 3B). The most MMPs within the defect area at the wound margin were detected in corneas from the 2 patients (patients 2L and 5R) (Figure 4) with the ends of Bowman’s layer curled inward with flap retraction. This area was also positive for CD11b (Mac-1), a marker for granulocytes or macrophages, in the corneas of these 2 patients (Figures 4E). In 3 corneas, α-SMA, a marker for myofibroblast differentiation, was detected within the margin defect of the right cornea of patient 5 (Figure 4I) and of the left cornea of patient 2 and in keratocytes between both flap cuts of patient 10 (Figure 3E-F).

**COMMENT**

Matrix metalloproteinases are expressed by resident tissue cells in response to injury and remodeling stimuli and are also produced by inflammatory cell types that invade the tissue during remodeling events. Collagenase is the prototype member of the MMP family, its activity first demonstrated in the involuting tadpole tail.41,42 When applied shortly thereafter to the cornea, this discovery gave the first insight into the enzymatic basis of corneal ulceration.43-45 Much subsequent work has defined complex roles for MMPs in normal and pathologic corneal repair and remodeling processes.2,46 The results of studies using transgenic mice (knockouts or overexpression) emphasize the high functional redundancy of MMPs.48 The ablation of a particular MMP can lead to higher expression levels of other MMPs, presumably to compensate for the loss. In view of this fact, it is thought that the multiplicity of MMP forms is an indicator of the extreme importance of these enzymes for the maintenance and repair of tissues.8

With the molecular cloning of collagenase, and expansion of the MMP family, and development of molecular and antibody probes,10 investigation in the cornea was renewed and expanded into new territory.37-47 Much subsequent work has defined complex roles for MMPs in normal and pathologic corneal repair and remodeling processes.2,46 The results of studies using transgenic mice (knockouts or overexpression) emphasize the high functional redundancy of MMPs.48 The ablation of a particular MMP can lead to higher expression levels of other MMPs, presumably to compensate for the loss. In view of this fact, it is thought that the multiplicity of MMP forms is an indicator of the extreme importance of these enzymes for the maintenance and repair of tissues.8

This study of adult human corneas that underwent uneventful LASIK 2 to 8 years before evaluation revealed that MMPs, when present, are localized exclusively to the flap margin. Matrix metalloproteinase presence was correlated with specific histopathologic findings identified at the wound margin. There was no observable correlation between patient age or postoperative interval with the severity or type of MMP activity in corneas that underwent LASIK.

Of the 33% (n=6) of corneas with epithelial ingrowth observed in the current series, 83% (n=5) had at least ++ immunostaining for MMP-9, which was positively correlated with basement membrane interruption or irregularities visualized via laminin staining. This is consistent with the capacity for MMP-9 to cleave epithelial basement membrane components, such as collagen types IV and VII and laminin.8,51 New basement membrane components such as laminin and type IV collagen are deposited underneath migrating corneal epithelial cells19 and around corneal epithelial cells implanted into
the stroma. A causal relationship between overexpression of MMP-9 in the corneal epithelium and basement membrane dissolution/failure to heal has been demonstrated in experimental models. In these studies, the corneal epithelium gave the appearance of an invading front, dissolving the basement membrane and subsequently penetrating the underlying stroma in its path. Matrix metalloproteinase 9 associated with a disrupted basement membrane around ingrowing epithelium suggests a similar chronic, ongoing remodeling and invasion process and might help explain the previous findings identifying the weakest wound margin scars as those with epithelial ingrowth.

Matrix metalloproteinase 7 immunostaining is observed around trapped epithelial cells with the same intensity as in control corneas. Matrix metalloproteinase 7 was immunolocalized in previous studies to the epithelial layers of unwounded and wounded corneas in rat. In this study, MMP-7 was also found in the epithelium of corneas from groups 1 and 3 but not specifically at the stromal wound margin. This is in comparison with corneas of groups 2 and 4 where MMP-7 was localized under the epithelial surface: around trapped epithelial cells in epithelial ingrowth (group 2) and in the overlying wound stroma in group 4. We thus did not mention MMP-7 in the wound margin of groups 1 and 3 in Table 3.

Figure 4. Immunofluorescence of the laser-assisted in situ keratomileusis flap margin of patient 5R with flap retraction. A-E, Positive staining for matrix metalloproteinase (MMP) 3 (B), MMP-7 (C), MMP-8 (D), and Mac-1 (CD11b) (arrows) (E) was observed within the margin defect compared with the negative control (rabbit IgG) (A). F and G, No MMP-9 staining (F) or continuous epithelial basement membrane laminin (G) were observed. I and H, Note the presence of α-smooth muscle actin (I) within the defect compared with the negative control (mouse IgG) (H) (A-I, original magnification ×400).
Unlike most MMPs, MMP-7 (matrilysin) is constitutively present in uninjured epithelia, including in the intestine, airways, and cornea. In the small intestine, MMP-7 has been shown to function in host defense by activating the latent form of defensins, a family of antimicrobial peptides that are also found in the cornea. In models of airway and corneal injury, MMP-7 expression is up-regulated in migrating epithelial cells, and the MMP-7 activity is required for repair of airway wounds. These observations indicate that matrilysin serves key functions in both epithelial defense and repair.

Various MMPs were detected at flap margins with flap retraction (patients 2L and 3R). In both cases, Mac-1 (CD-11b), used as a marker for macrophages and granulocytes, was colocalized, suggesting a chronic inflammatory process. This can explain the expression of various MMPs, particularly the presence of the neutrophil collagenase (MMP-8), which is synthesized specifically by polymorphonuclear neutrophils. Also, α-SMA-positive cells, a marker of the “myofibroblast,” were observed in the stroma layer near the edge of the flap in these 2 corneas. A similar α-SMA expression pattern was reported in human corneas up to 6 years after LASIK surgery. Myofibroblasts expressing α-SMA are associated with a highly fibrotic wound phenotype characterized by a significant deposition of repair-type extracellular matrix, significant hypercellularity, and extensive repair tissue contraction. In the cornea, myofibroblasts are also associated with repair tissue opacity. When repair tissue deposition reaches its peak, the fibrotic phenotype and myofibroblast markers first begin to resolve. In a fully healed wound, there are few if any myofibroblasts. Long-term expression in some post-LASIK corneas emphasizes the chronic, ongoing nature of the fibrotic wound healing process.

Our study represents the first investigation, to our knowledge, of MMP expression in a series of clinically uneventful human LASIK refractive procedures. Reports of MMP expression following complicated LASIK procedures are also rare and consist mainly of case reports. Maguen et al studied 2 corneas that underwent a complicated LASIK procedure. They reported anterior stromal expression of MMP-1, MMP-2, and MMP-7 in the epithelium in a torn flap and an ectatic cornea. They did not report presence of MMPs following a single uneventful LASIK procedure performed several years earlier. This is consistent with our study as we found that MMPs are present only in corneas with specific histopathologic findings.

In the absence of keratoconus or forme fruste keratoconus, progressive post-LASIK keratectasia might be the result of biomechanical instability or a chronic disease process with progressive enzymatic degradation. An imbalance in proteolytic breakdown and repair could lead to a progressive stromal melting. The spatial localization of MMPs at the wound margin seen in our study does not support a role in ectasia, a process that primarily involves central changes in the post-LASIK cornea. On the other hand, epithelial ingrowth and cicatrization changes at the flap edges could relate to reduced resistance of some LASIK corneas to shearing trauma, resulting in late flap displacements.

In conclusion, to our knowledge, this is the first study to report that MMP proteins can be detected in some post-LASIK corneas even after many years. The presence of MMPs correlates with an ongoing, highly localized wound healing process at the flap margin, associated with minor post-LASIK complications. We cannot say with certainty whether epithelial ingrowth, flap retraction, or the ongoing repair process this signifies is the cause or the consequence of MMP presence. However, we propose that minor defects in the surgically created flap might interfere with perfect alignment with the underlying cornea following surgery. This would predispose to slippage, creating space for epithelial ingrowth and a chronic requirement for fibrotic repair. Like other chronic repair processes, MMP expression might become excessive because of ever-amplifying feedback loops, and this could gradually evolve into a contributing factor in failure to heal. In such situations, judicious and timely use of appropriate MMP inhibitors might provide some benefit, inhibiting epithelial ingrowth and enabling fibrotic repair tissue to accumulate sufficiently to “tack” the flap in place.

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Correspondence: M. Elizabeth Fini, PhD, McKnight Vision Research Center, Bascom Palmer Eye Institute, Miller School of Medicine, University of Miami, 1638 NW 10th Ave, Miami, FL 33136 (efini@med.miami.edu).

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REFERENCES


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