Confocal Microscopy Analysis of Corneal Changes After Photorefractive Keratectomy Plus Cross-Linking for Keratoconus: 4-Year Follow-up

GIOVANNI ALESSIO, MILENA L’ABBATE, CLAUDIO FURINO, CARLO SBORGIA, AND MARIA GABRIELLA LA TEGOLA

• PURPOSE: To analyze corneal confocal microscopy changes after combined photorefractive keratectomy (PRK) plus the cross linking (CXL) procedure.
• DESIGN: Prospective interventional case series.
• METHODS: At the Department of Basic Medical Sciences, Neuroscience, and Sense Organs of the University of Bari, Bari, Italy, 17 eyes of 17 patients with progressive keratoconus underwent confocal microscopy examination before and after 1, 3, 6, 12, 18, and 48 months following PRK plus the CXL procedure. The main outcome measures were mean superficial epithelial cell density; mean basal epithelial cell density; mean anterior, mid and posterior keratocyte density; qualitative analysis of stromal backscatter; sub-basal and stromal nerve density parameters; and mean endothelial cell density.
• RESULTS: During the 4-year follow-up, the mean superficial epithelial cell density, mean basal epithelial cell density and mean endothelial cell density remained unchanged (P > 0.05). The anterior mid-stromal keratocyte density showed a significant decrease (P < 0.05) as compared with preoperative values, and the posterior stromal keratocyte density showed a significant increase at 1 and 3 months of follow-up. Sub-basal and stromal nerve density parameters were significantly decreased until postoperative month 6 (P < 0.05 at 1, 3, and 6 months) and then tended to increase up to preoperative values by the 18th postoperative month.
• CONCLUSION: Corneal changes after the PRK plus CXL procedures seem to be pronounced and long lasting as far as keratocyte density of the anterior and mid stroma is concerned. Sub-basal nerve densities tend to reach preoperative values 6 months after surgery. (Am J Ophthalmol 2014;158:476–484. © 2014 by Elsevier Inc. All rights reserved.)

CORNEAL CROSS-LINKING (CXL) WITH RIBOFLAVIN and ultraviolet-A (UVA) is currently the predominant treatment for mild and moderate progressive keratoconus. It is generally preferred to keratoplasty surgeries because it is noninvasive and requires a shorter visual rehabilitation time and, in particular, because there is no risk for graft failure. However, there are instances in which keratoplasty is preferred to CXL for the treatment of patients with severe keratoconus with corneal scarring.

Since CXL was introduced, the technique has been enhanced by combining certain elements of photorefractive keratectomy (PRK) to improve the refractive outcomes. Instead of mechanical removal of the epithelium, according to the technique described by Wollensak and associates,1 ablation of the epithelium and superficial stroma is used in the PRK plus CXL procedure to remodel the corneal surface and reduce irregular astigmatism. In our experience, as compared with standard CXL, the PRK plus CXL procedure offers better visual outcomes in terms of lower keratometric values, changes in the elevation of the anterior corneal surface and greater reductions in root mean square values.2 Some studies have reported changes in corneal structure investigated by in vivo confocal microscopy after CXL alone,3–6 but only a few studies have been made of the outcomes of the PRK plus CXL procedure,7 and a follow-up of 4 years has never been reached previously.

The aim of this study was to analyze morphologic changes in all corneal layers, as examined by confocal microscopy, over a 4-year follow-up period after PRK plus CXL for keratoconus.

METHODS

• SUBJECTS: The study protocol was approved and monitored by the Local Ethics Committee of Azienda Ospedaliera Policlinico di Bari (protocol no. 839/C.E.) and conformed to the tenets of the Declaration of Helsinki. Informed consent was obtained from all patients taking part in the study. This is a registered clinical trial: clinical trial registration at http://www.controlled-trials.com/ISRCTN57262986.

Between July 2008 and June 2009, 17 patients with keratoconus (34 eyes) (mean age, 31.17 ± 8.12 years; range, 21–46 years) were recruited at the Cornea Service of the Department of Basic Medical Sciences, Neuroscience, and Sense Organs of the University of Bari to take part in this prospective nonrandomized clinical study. The 34

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eyes were graded as stage I-III according to the Aliò and Shabayek classification and were assigned to 1 of 2 groups: the worse eye underwent PRK followed by CXL (17 eyes), and the better eye (fellow eye) underwent CXL alone (17 eyes). The diagnosis of keratoconus was based on corneal topography (Orbscan IIz; Bausch & Lomb, Rochester, New York, USA) as an asymmetric bowtie pattern, with or without skewed axes, and a paracentral inferior-superior dioptric difference of more than 1.4 diopters (D). Enrolment depended on documented progression of keratoconus in the previous 6 months, a corneal thickness of at least 450 μm at the thinnest point in the worse eye, hard contact lens and full spectacle correction intolerance, and age older than 18 years. Progression of keratoconus was defined based on serial differential topography, as an increase in the apex keratometry by more than 1.0–1.5 D and a corresponding change (>1.0–1.5 D) in the refractive cylinder in the previous 6 months. Exclusion criteria were a corneal thickness of less than 450 μm at the thinnest point in the worse eye, corneal scarring or Vogt striae, any ocular disorder other than keratoconus, any history of eye surgery, any systemic disease, pregnancy, and contact lens wear. Patients who failed to attend the follow-up visits were excluded.

Visual acuity, refractive, topographic, and corneal higher order aberrations outcomes after PRK plus CXL and after CXL alone up to 24 months of follow-up have already been reported. The current study investigated the confocal microscopy findings in 17 corneas of 17 patients after the PRK plus CXL procedure over a 4-year follow-up period. In this trial the eyes treated only by CXL were not taken into consideration because there are already many publications concerning confocal microscopy analyses in these eyes, reporting similar results in all cases.

**TOPOGRAPHIC-GUIDED PRK PLUS CXL PROCEDURE:** The steps of topographic-guided PRK plus CXL are reported briefly below. After acquisition of the corneal shape by means of the Scheimpflug-based Precisio tomographer (Ligi Tecnologie Medicali, Taranto, Italy), elevation data together with the patient’s subjective refraction were imported into Corneal Interactive Programmed Topographic Ablation (CIPTA) software to plan the ablation procedure. To ensure the maximum reduction of irregularities with minimum tissue consumption, the Restored Morphological Axis strategy of the CIPTA software was used. The center of the planned ablation was the corneal apex in all treated eyes. The stromal ablation depth was between 18 and 49 μm (mean, 31.1 ± 9.5 μm). A supplementary depth of 50 μm was selected by the surgeon for epithelium ablation because of transepithelial PRK. The corneal epithelium was removed by laser within a 9 mm diameter. Transepithelial topography-guided PRK was performed with the high-resolution 1-KHz flying spot laser iRES (Ligi Tecnologie Medicali). Immediately after PRK, riboflavin 0.1% solution (10 mg riboflavin-5-phosphate in 20% dextran T500 solution; Ricrolin; SOOFT Italia, Montegiorgio, Italy) was administered topically every 2 minutes for 30 minutes, under topical anesthesia. Riboflavin absorption throughout the corneal stroma and anterior chamber was confirmed on slit-lamp examination. Then the cornea was exposed to UVA 365 nm light at an irradiance of 3.0 mW/cm² for 30 minutes. During UVA exposure, riboflavin drops were continued every 2 minutes. At the end of the procedure, a soft bandage contact lens was applied and ofloxacin and 0.1% indomethacin drops were administered until re-epithelialization was complete. After epithelial healing and removal of the contact lens, 0.1% fluorometholone was instilled 4 times daily for the first month. The dosage was tapered by 1 drop monthly over the next 3 months.

**CONFOCAL MICROSCOPY AND OUTCOMES:** The Confoscan 4 (NIDEK Technologies, Padova, Italy) confocal microscope equipped with a 40× objective lens was used in all eyes. After the instillation of local anesthetic (benoxinate hydrochloride 0.4 g eye drops, Alfa Intes Industria Terapeutica Splendore, Casoria, Italy), the instrument lens was advanced until the high-viscosity gel (hydroxypropyl methylcellulose 2.5%) contacted the cornea. A fixed device setting was used for all examinations: full-thickness mode, 72% light intensity; 7 μm scan step; autoalignment function. The Z-ring was used in all cases. Each image represented a coronal section of 460 × 345 μm with magnification of ×500. A total of 4 scans were obtained at the optical center of each cornea for all patients at each follow-up visit. The confocal microscopy examination was performed by the same researcher in all study participants. The best-focused 3 frames per layer were selected for analysis of each cornea. Evaluations were repeated at 1, 3, 6, 12, 18, and 48 months postoperatively. Image parameters were:

1. Superficial epithelial cell density (cells/mm²); a minimum of 35 cells were counted manually by Confoscan 4 NAVIS analysis software in a randomly selected rectangular area. The software highlights the results as unreliable if fewer than 35 cells are present in the selected rectangle.
2. Basal epithelial cell density (cells/mm²). At least 75 cells were counted manually by Confoscan 4 NAVIS analysis software in a randomly selected polygonal area.
3. Keratocyte density (cells/mm²) in the anterior, mid, and posterior thirds of stroma. The stroma was divided into 3 equal-thickness anteroposterior regions, and 3 frames were selected from each of these 3 regions, expressed as percent stromal depth (1%–33%, 34%–66%, and 67%–100%). Images of the anterior boundary of the stroma immediately posterior to the Bowman layer (the layer with the highest concentration of keratocyte nuclei) were included in the analysis of each eye at each follow-up time. At least 35 keratocyte
nuclei were counted manually, marking each cell or nucleus inside a randomly selected rectangular area. Cells that touched the top or left edge of the rectangle were counted, but cells that touched the bottom or right edge were not.

(4) Sub-basal nerve density, defined as the mean total length (μm) within the region of interest (by default, 81,972.17 μm²), mean total length of the nerves per image (μm/mm²), and mean total number of nerves within the region of interest (including main nerve trunks and branches). The semiautomated Nerves Tracking Tool of NAVIS software was used in all cases.

(5) Stromal nerve density, defined as the total number of nerves.

(6) Endothelial cell density (cells/mm²). In a randomly selected polygonal area, at least 75 cells were counted by Confoscan 4 NAVIS software.

Qualitative analysis of corneal haze was also performed. Evaluation of stromal backscatter served to identify the corneal layer associated with pathologic scatter and for follow-up of corneal haze.

All counts were performed independently, by 2 experienced examiners blinded to the study identifiers. Comparisons between preoperative and postoperative values at each time point were performed for all parameters by paired-samples t test. A P value of less than 0.05 was considered statistically significant.

RESULT

THE CLINICAL FEATURES OF THE SUBJECTS ARE SHOWN IN Table 1.

The mean values and P values for comparisons of the preoperative and the postoperative results are shown in Table 2.

Compared with baseline values, mean superficial epithelial density, mean basal epithelial cell density, and mean endothelial cell density were not significantly different at each postoperative time point. Anterior stromal keratocyte nuclei were present in no eyes (n = 0) at 1, 3 and 6 postoperative months; in 23.5% of eyes (n = 4) at 12 months; in 35.2% of eyes (n = 6) at 18 months; and in 41.1% of eyes (n = 7) at 48 months. Mid stromal keratocyte nuclei were present in no eyes (n = 0) at 1, 3 and 6 postoperative months; in 23.5% of eyes (n = 4) at 12 months; and in 35.2% of eyes (n = 6) at 18 and 48 months. The mean anterior and mid stromal keratocyte densities were significantly lower at each follow-up as compared with the preoperative values and in no case reached the preoperative value. Compared with baseline values, mean posterior keratocyte density at 1 and 3 months was significantly increased, while at 6, 12, 18, and 48 months it was not statistically different. Postoperatively, sub-basal nerves were present in no (n = 0) eyes at 1 and 3 months; in 82.3% of eyes (n = 14) at 6 months; in 94.1% of eyes (n = 16) at 12 months; and in 100% of eyes (n = 17) at 18 and 48 months. Postoperatively, stromal nerves were present in no eyes (n = 0) at 1 and 3 months; in 88.2% of eyes (n = 15) at 6 months; and in 100% of eyes (n = 17) at 12, 18, and 48 months. The mean total length of nerves, mean total length of nerves per image, mean total number of sub-basal nerves, and mean total number of stromal nerves were drastically lower and undetectable at 1 and 3 months postoperatively and then tended to increase up to 18 months postoperatively. By 4-year follow-up, the preoperative values for mean total length of nerves, mean total length of nerves per image, mean total number of sub-basal nerves, and mean total number of stromal nerves were restored.

Confocal images of corneal stroma at different time points in a 32-year-old patient are illustrated in Figure 1 and Figure 2. Confocal microscopy findings in keratoconic eyes before treatment are not reported because they have already been described11 and are very similar to ours.

DISCUSSION

THIS STUDY INVESTIGATED CONFOCAL MICROSCOPY changes in corneal layers after the PRK plus CXL procedure up to 4 years after surgery. The most noteworthy structural changes concerned the anterior and mid stromal keratocyte density and the sub-basal and stromal nerve density parameters. The mean anterior and mean mid keratocyte densities were markedly decreased in the early postoperative months, then showed a slow, gradual increase until 4 years
<table>
<thead>
<tr>
<th></th>
<th>Preoperatively</th>
<th>1 mo.</th>
<th>3 mo</th>
<th>6 mo</th>
<th>12 mo</th>
<th>18 mo</th>
<th>48 mos.</th>
</tr>
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<tbody>
<tr>
<td><strong>Superficial epithelial cell density (cells/mm²)</strong></td>
<td>1481 ± 597.5</td>
<td>1414.4 ± 448.6</td>
<td>1414 ± 484.4</td>
<td>1294.3 ± 465.9</td>
<td>1385.6 ± 423</td>
<td>1234.8 ± 371.4</td>
<td>1308.7 ± 414.3</td>
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<tr>
<td>P value</td>
<td>0.74</td>
<td>0.74</td>
<td>0.21</td>
<td>0.48</td>
<td>0.15</td>
<td>0.28</td>
<td></td>
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<tr>
<td><strong>Basal epithelial cell density (cells/mm²)</strong></td>
<td>5759.5 ± 1785.3</td>
<td>5459.3 ± 1300.3</td>
<td>5412.7 ± 1324.4</td>
<td>5653.4 ± 2057.5</td>
<td>6150.1 ± 1513.2</td>
<td>5735.7 ± 1467.8</td>
<td>6020.7 ± 1094.5</td>
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<tr>
<td>P value</td>
<td>0.60</td>
<td>0.51</td>
<td>0.87</td>
<td>0.58</td>
<td>0.96</td>
<td>0.65</td>
<td></td>
</tr>
<tr>
<td><strong>Anterior stromal keratocyte density (cells/mm²)</strong></td>
<td>880.9 ± 199.7</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>138.3 ± 247.4</td>
<td>210.3 ± 262.3</td>
<td>253 ± 295.3</td>
</tr>
<tr>
<td>P value</td>
<td>1.98 ± 10^-9</td>
<td>1.98 ± 10^-9</td>
<td>1.98 ± 10^-9</td>
<td>3.88 ± 10^-7</td>
<td>2.81 ± 10^-6</td>
<td>8.08 ± 10^-5</td>
<td></td>
</tr>
<tr>
<td><strong>Mid stromal keratocyte density (cells/mm²)</strong></td>
<td>618.3 ± 90.7</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>68.54 ± 127.5</td>
<td>99.2 ± 139.2</td>
<td>102.2 ± 143.5</td>
</tr>
<tr>
<td><strong>Posterior stromal keratocyte density (cells/mm²)</strong></td>
<td>736.2 ± 97.6</td>
<td>831.3 ± 86.3</td>
<td>830.2 ± 88.7</td>
<td>782.8 ± 139.7</td>
<td>695.9 ± 73.8</td>
<td>740.6 ± 157.5</td>
<td>647.2 ± 167.2</td>
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<tr>
<td>P value</td>
<td>0.04</td>
<td>0.02</td>
<td>0.35</td>
<td>0.20</td>
<td>0.93</td>
<td>0.06</td>
<td></td>
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<tr>
<td><strong>Endothelial cell density (cells/mm²)</strong></td>
<td>2945 ± 115</td>
<td>2932.2 ± 269.7</td>
<td>2965.8 ± 216.9</td>
<td>2947.7 ± 105</td>
<td>2978.2 ± 65.3</td>
<td>2984 ± 67.3</td>
<td>2981.3 ± 60.1</td>
</tr>
<tr>
<td>P value</td>
<td>0.84</td>
<td>0.63</td>
<td>0.93</td>
<td>0.23</td>
<td>0.12</td>
<td>0.11</td>
<td></td>
</tr>
<tr>
<td><strong>Total length of sub-basal nerves (µm)</strong></td>
<td>588 ± 97</td>
<td>NA</td>
<td>NA</td>
<td>300.6 ± 161</td>
<td>397.9 ± 145.7</td>
<td>487.7 ± 119.8</td>
<td>554.6 ± 137.5</td>
</tr>
<tr>
<td>(cells/mm²)</td>
<td>(419.6–724.8)</td>
<td></td>
<td></td>
<td>(280.7–529.8)</td>
<td>(332.9–600.6)</td>
<td>(187.9–671.8)</td>
<td>(237.6 to 712.7)</td>
</tr>
<tr>
<td>P value</td>
<td>3.03 ± 10^-14</td>
<td>3.03 ± 10^-14</td>
<td>1.22 ± 10^-5</td>
<td>7.01 ± 10^-3</td>
<td>0.01</td>
<td>0.35</td>
<td></td>
</tr>
<tr>
<td><strong>Length density of sub-basal nerves (µm/mm²)</strong></td>
<td>9610.6 ± 1325.9</td>
<td>NA</td>
<td>NA</td>
<td>5245.2 ± 931.8</td>
<td>5983.4 ± 1327.9</td>
<td>8085.4 ± 2875.9</td>
<td>9921.9 ± 1173.6</td>
</tr>
<tr>
<td>(cells/mm²)</td>
<td>(7865–11765)</td>
<td></td>
<td></td>
<td>(3829–6463)</td>
<td>(3127–7489)</td>
<td>(2293–11232)</td>
<td>(7865 to 11977)</td>
</tr>
<tr>
<td>P value</td>
<td>1.82 ± 10^-15</td>
<td>1.82 ± 10^-15</td>
<td>4.46 ± 10^-8</td>
<td>6.66 ± 10^-7</td>
<td>0.05</td>
<td>0.51</td>
<td></td>
</tr>
<tr>
<td><strong>Total number of sub-basal nerves (no.)</strong></td>
<td>6 ± 2.5 (2–10)</td>
<td>NA</td>
<td>NA</td>
<td>4.5 ± 1.8 (2–9)</td>
<td>4.8 ± 1.3 (3–7)</td>
<td>6 ± 1.8 (3–8)</td>
<td>6.2 ± 1.9 (3 to 10)</td>
</tr>
<tr>
<td>P value</td>
<td>4.75 ± 10^-8</td>
<td>4.75 ± 10^-8</td>
<td>0.1</td>
<td>0.16</td>
<td>1</td>
<td>0.68</td>
<td></td>
</tr>
<tr>
<td><strong>Total number of stromal nerves (no.)</strong></td>
<td>2.1 ± 1 (1–4)</td>
<td>NA</td>
<td>NA</td>
<td>1.9 ± 1.1 (0–4)</td>
<td>2.2 ± 1.1 (1–4)</td>
<td>2.4 ± 1 (1–4)</td>
<td>2.3 ± 1 (1 to 4)</td>
</tr>
<tr>
<td>P value</td>
<td>1.47 ± 10^-7</td>
<td>1.47 ± 10^-7</td>
<td>0.58</td>
<td>0.75</td>
<td>0.48</td>
<td>0.66</td>
<td></td>
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</tbody>
</table>

**TABLE 2.** In Vivo Confocal Microscope Values and P Values for Comparison of Preoperative and Postoperative Results at Each Time Point in Eyes Treated by Photorefractive Keratectomy Plus Cross-Linking for Progressive Keratoconus

*Mean ± standard deviation (range).
postoperatively but never regained preoperative values. Sub-basal and stromal nerve densities were not evaluable at the first and third postoperative month examinations but gradually returned to the preoperative values by the fourth year postoperatively. By the eighteenth postoperatively (top row), anterior stroma showed dishomogeneous hyper-reflectivity without recognizable cells and nerve fibers; mid stroma was edematous and hyporeflective with rare activated keratocytes; the boundary of the cross-linked area was outlined by a reticular hyper-reflective pattern with elongated keratocytes and hyper-reflective needle-like structures; deeper, posterior stroma showed keratocyte nuclei. Three months postoperatively (middle row), anterior stroma showed a honeycomb appearance, and mid stroma hyporeflectivity without detectable cellular nuclei; the boundary of the treated area was underlined by hyper-reflective bands, while the deeper stroma was unchanged. Six months postoperatively (bottom row), structural changes are similar to those in the third month, but tiny nerve fibers exhibiting a fragmented appearance are recognizable in the anterior-mid stroma.

FIGURE 1. Corneal confocal images 1, 3 and 6 months after the photorefractive keratectomy plus cross-linking procedure in a 32-year-old patient with keratoconus. (Top row) anterior stromal (left), mid stromal (middle left) boundary between treated and untreated stromal (middle right) and posterior stromal (right) layers 1 month after surgery. (Intermediate row) anterior stromal (left), mid stromal (middle left) boundary between treated and untreated stromal (middle right) and posterior stromal (right) layers 3 months after surgery. (Bottom row) anterior stromal (left), mid stromal (middle left) boundary between treated and untreated stromal (middle right) and posterior stromal (right) layers 6 months after surgery. One month postoperatively (top row), anterior stroma showed dishomogeneous hyper-reflectivity without recognizable cells and nerve fibers; mid stroma was edematous and hyporeflective with rare activated keratocytes; the boundary of the cross-linked area was outlined by a reticular hyper-reflective pattern with elongated keratocytes and hyper-reflective needle-like structures; deeper, posterior stroma showed keratocyte nuclei. Three months postoperatively (middle row), anterior stroma showed a honeycomb appearance, and mid stroma hyporeflectivity without detectable cellular nuclei; the boundary of the treated area was underlined by hyper-reflective bands, while the deeper stroma was unchanged. Six months postoperatively (bottom row), structural changes are similar to those in the third month, but tiny nerve fibers exhibiting a fragmented appearance are recognizable in the anterior-mid stroma.

Computer-assisted manual measurement of cell and nerve density on confocal microscope images is a time-consuming and subjective method, with obvious concerns about intra- and interexaminer repeatability. Not only is the evaluation of each image parameter affected by the skill of the operators, but the analysis of each corneal layer raises additional issues. With regard to stromal keratocyte density, cell nuclei with higher contrast and sharper edges are more easily identifiable and countable than those with lower contrast and blurred edges.11,12 In 2010, McLaren and associates13 developed a program to detect automatically stromal cell nuclei in Confoscan 4 images, but no one has yet used this consistently in clinical studies. Moreover, the confocal microscope is unable to distinguish keratocytes from bone marrow-derived cells and other cells that are present in the corneal stroma, and these cell types will affect estimates of keratocyte density. With regard to endothelial cell density, in this study, semiautomated image analysis was performed. In this way, the operator verifies the cell border outline and can make manual corrections. A greater accuracy of endothelial cell density analysis has been reported using the semiautomated method.14 Moreover, methods of objective measurement of the light backscatter by corneal layers have also been developed, but none of these has been used extensively in clinical studies.15,16 Since 2010, solutions to the problem of standardization of the measurements of brightness of light backscattered by the cornea have been proposed so as to ensure
comparable results at different times and in different laboratories.\textsuperscript{16,17} In this study, however, only a qualitative assessment of the backscattered light was made to detect the possible corneal layers involved. For the nerve-density parameters, we used the semiautomated Nerves Tracking Tool of NAVIS software, which requires interaction with the operator in discriminating visible nerves from background data. To the best of our knowledge, there are no published data regarding the repeatability of the Nerves Tracking Tool of NAVIS software. Despite all limitations related to the measurement methods, the results of this study unequivocally show a depletion of the anterior and mid stromal keratocytes that persists over time as well as a reduction of the nerve density parameters but with restoration of the baseline values by 18 months after surgery.

Various authors have described corneal changes by confocal microscopy after the standard CXL procedure. Structural changes involve the superficial and mid corneal stroma, but the reports are conflicting. In fact, after CXL some authors have reported a full-thickness stromal keratocyte repopulation as soon as 1 year postoperatively,\textsuperscript{3} whereas other authors have noted a lower density of keratocytes after variable follow-up times. Knappe and associates\textsuperscript{4} pointed out that until 12 months postoperatively, the number of hyper-reflective keratocyte nuclei in the anterior stroma was reduced as compared to before treatment. Croxatto and associates\textsuperscript{5} reported an incipient repopulation of keratocytes in the superficial and mid stroma, together with a normal pattern of the deep stroma as soon as 1 month after cross-linking, but examination at 36 months disclosed hypocellularity of the anterior and mid corneal stroma with a moderate hyper-reflectivity of the extracellular matrix as compared with the preoperative findings.

\textbf{FIGURE 2.} Corneal confocal microscope images 12, 18 and 48 months after the photorefractive keratectomy plus the cross-linking procedure in a 32-year-old patient with keratoconus. (Top row) anterior stromal (left), mid stromal (middle left) boundary between treated and untreated stromal (middle right) and posterior stromal (right) layers 12 months after surgery. (Intermediate row) anterior stromal (left), mid stromal (middle left) boundary between treated and untreated stromal (middle right) and posterior stromal (right) layers 18 months after surgery. (Bottom row) anterior stromal (left), mid stromal (middle left) boundary between treated and untreated stromal (middle right) and posterior stromal (right) layers 48 months after surgery. Twelve months postoperatively (top row), the anterior and mid stroma showed a dishomogeneous hyper-reflectivity with recognizable nerve structures (trunks and branches); the boundary of the treated area can be identified by the presence of hyper-reflective bands that retained cell nuclei; the deep stroma was normoreflective and populated by cells. Eighteen months postoperatively, too (middle row), anterior and mid stroma were unformed and widely acellular, while the sub-basal nerve plexus was restored to the preoperative state; at the boundary of the treated area hyper-reflective band-like structures remained; the deep stroma was unchanged. Four years postoperatively, the sub-basal nerve plexus formed an interconnected network; anterior and mid stroma remained largely acellular and dishomogeneously hyper-reflective; hyper-reflective bandlike structures with keratocytes through mesh were evident between the cross-linked and untreated areas; deep stroma was unchanged.
After the PRK plus CXL procedure, full-thickness corneal stromal keratocyte repopulation has not been demonstrated until 12 months.

Our study confirms previous confocal reports in terms of incomplete keratocyte repopulation and deeper acellular stromal thickness after the PRK plus CXL procedure, and it adds a longer follow-up time (4 years). The PRK plus CXL procedure includes ablation of Bowman membrane together with the epithelium, so perhaps this could lead to a deeper penetration of riboflavin into the corneal stroma. It has been shown that in animals without Bowman membranes (such as rabbits, in which the mean corneal thickness is 407 ± 20 μm), 24 hours after the standard epithelium-off CXL there is extensive keratocyte and endothelial cell death. All this invites speculation about the biomechanical role of the Bowman membrane. The biomechanical role of the Bowman membrane in human was the object of reports in the 1990s. In fact, Seiler and associates claimed that the Bowman membrane made no significant contribution to the mechanical stability of the cornea, and Wilson and associates asserted that the Bowman layer had no critical function in corneal physiology, being only an indicator of stromal-epithelial interactions. Otherwise, indirect confirmation of the nonsubstantial function of the Bowman membrane in corneal biomechanics was gained from data about the risk for ectasia after refractive surgery. In photorefractive keratectomy, the Bowman membrane, together with a certain amount of anterior stroma, is removed by excimer laser, but the risk for postsurgical keratectasia is significantly less than after LASIK and is attributable to many factors.

After CXL, the increase in rigidity of the human cornea was estimated to be about 300%. We hypothesize that an increased biomechanical strength of the cornea, induced by CXL, together with the effect of a deeper penetration of riboflavin into the corneal stroma, could explain the pronounced keratocyte depletion of the stromal cornea postoperatively. Perhaps after an initial apoptosis period, keratocytes have great difficulty in repopulating and penetrating the corneal stroma because of a greater compactness and stiffness induced by cross-linking. In fact, in our experience, in a minority of the corneas examined (23.5% at 12 months [n = 4]; 35.2% at 18 months [n = 6]; 41.1% at 48 months [n = 7]) at various times after surgery, there was keratocyte repopulation only in the anterior portion of the cornea, immediately beneath the epithelium. In this minority of corneas, the cross-linked area appears almost completely acellular and is preceded and followed by stroma-containing cells that are probably unable to make their way into the cross-linked stroma. There are 2 indirect confirmations of this hypothesis. First, the keratocyte repopulation in epikeratoplasty lenticules was noted to be of variable extent and more common in the anterior than in the posterior region of central lenticules. Second, by contrast, biosynthetic corneas made of recombinant human collagen, synthesized in yeast and chemically cross-linked, and the sponge skirt surrounding a central core in AlphaCor (Argus Biomedical, Pty. Ltd., Perth, Australia), are cell-free when implanted, and they allow biointegration by in-growth of endogenous cells, nerves and collagen deposition due to emulion of the scaffolding function of the natural extracellular matrix of the cornea.

Keratocytes produce the collagen and proteoglycans necessary to maintain the turnover of corneal tissue so, theoretically, a keratocyte depletion could play a role in the health of the cornea. However, in our experience so far, the corneal keratocyte depletion appears to have no consequences on corneal clarity or curvature.

An acute turnover of corneal collagen occurs rapidly after the first 2 weeks of wound healing, but the rate of collagen turnover in healthy and keratoconic corneas is not yet known. Cornea is presumed to be a very slow-turnover tissue. A potential limitation of this study is that follow-up is still not long enough to provide a clear answer to the question of the effects over time of stromal keratocyte depletion.

After CXL, transitory corneal opacities similar to haze have been described in a minority of eyes, mainly in cases with preoperative confocal evidence of hyperactivated, hyperdense keratocyte nuclei in the anterior stroma, or deep stromal dark striae. After the PRK plus CXL procedure, Kymionis and associates found a posterior linear stromal haze in 46.42% of the treated eyes up to 1 year after surgery. This posterior haze gradually moved anteriorly and became less dense during the follow-up period. In our study, at slit-lamp examination, 14 eyes (82%) demonstrated a specific CXL demarcation line and 7 eyes (50%) had central subepithelial haze formation (grade 0.5 to 1) by 12 months after treatment. Confocal microscopy examination helped to better localize and characterize the sources of backscatter (haze) after the PRK plus CXL procedure. In our experience, confocal microscope examination in all patients showed 2 regions of high corneal backscatter: at the boundary between the cross-linked and not treated area and in the subepithelial location. Even at 4 years after surgery, the baseline morphology of the corneal stroma had not been restored. The visual impact of subepithelial haze and abnormalities in the corneal stroma has yet to be determined. Corneal backscatter per se does not affect vision because backscattered light does not affect retinal image quality, but we speculate that it might be a factor that could limit the quality of vision.

It is well known that sub-basal nerve density is lower in keratoconic corneas, and a positive correlation between sub-basal nerve density and the severity of keratoconus has also been demonstrated. Also, in the early weeks or months after CXL a sub-basal nerve depletion has been reported in confocal microscope studies, based on qualitative assessment only, with a return to the preoperative status several months after the procedure. In our experience, after the PRK plus CXL procedure, sub-basal and stromal...
nerves were not identified up to 6 months after surgery because they were absent or because of edema, which limits the analysis to the confocal microscope. This is further complicated by the fact that changes in backscattered light after the PRK plus CXL procedure could limit visibility of the nerve fibers because of a masking effect, making early isolated and short nerve fibers invisible on confocal microscope images during the first months after surgery. From the eighteenth postoperative month, nerve density remained stable over time. The ability of the sub-basal nerve plexus to restore the preoperative density had already been noted after CXL, but in this study we were able to visualize nerve structures only from the sixth postoperative month onward. The first nerve fibers appeared in the anterior-mid stroma and then in the subepithelial region.

This study suffers from some limitations, in addition to those related to computer-assisted manual assessment of cell and nerve densities. First, because of the thick optical section produced by the Confoscan as compared to the Heidelberg retinal tomograph, accurate identification and localization of specific cell layers could be difficult. Second, examinations were confined to the central 3–4 mm of the cornea, although not in identical regions each time because confocal microscopy employs magnification up to $\times 500$. As a result, positional repeatability is low, and the scans represented random, full-thickness samples of the central cornea. This is complicated by the fact that the eye may move with respect to the tip of the lens during scanning, so that even spacing between images is not necessarily ensured.

In conclusion, the results of our study indicate that the PRK plus CXL procedure affects corneal innervation transiently and keratocyte density significantly, up to 4 years of follow-up. Further studies with larger patient samples and longer follow-up periods are needed to confirm our findings.

ALL AUTHORS HAVE COMPLETED AND SUBMITTED THE ICMJE FORM FOR DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST, and none were reported. Involved in Conception and design of study (G.A., M.L., C.F., C.S., M.G.L.); Collection of data (M.L.); Analysis and interpretation of data (M.G.L., M.L.); Preparation and writing of manuscript (M.G.L.); Critical revision of manuscript (G.A., M.G.L., C.F.); Final approval of manuscript (C.S.); Data collection and literature search (M.L.); and Final revision (M.G.L., M.L.).

REFERENCES


Biosketch

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