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Corneal Nerve Regeneration after Collagen Cross-linking for Keratoconus: a Five Year Longitudinal Study

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ABSTRACT

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**Importance:** It is unknown whether a neurotrophic deficit or pathologic nerve morphology persists in keratoconus in the long term after corneal collagen cross-linking (CXL) treatment. Nerve pathology could impact long-term corneal status in keratoconus patients.

**Objective:** To determine whether CXL treatment for keratoconus results in normalization of subbasal nerve density and architecture up to five years after treatment.

**Design:** Observational study of keratoconus patients with longitudinal follow-up to five years postoperatively (2009-2015), including healthy comparator group.

**Setting:** Primary care center, university hospital ophthalmology department.

**Participants:** Nineteen consecutive patients with early-stage keratoconus indicated for a first CXL treatment and nineteen age-matched healthy volunteers.

**Exposures:** Keratoconus patients underwent standard epithelial-off UVA-riboflavin CXL treatment with 30 min UVA exposure at 3 mW/cm² irradiance.

**Main Outcome Measures:** Central corneal subbasal nerve density and subbasal nerve architecture by laser-scanning in vivo confocal microscopy. Subbasal nerve analysis by two masked observers, a fully-automated method, and wide-field mosaics of subbasal nerve architecture by an automated method. Ocular surface touch sensitivity by contact esthesiometry.

**Results:** Relative to healthy corneas, subbasal nerve density in stage I-II keratoconus was reduced 51-56% (mean difference 10.7 mm/mm², 95% CI: 6.8 to 14.6 mm/mm², t-test, P < 0.001). After CXL, nerves continued to regenerate up to five years, but nerve density remained significantly reduced relative to healthy corneas at final follow-up (mean reduction of 8.5 mm/mm², 95% CI: 4.7 to 12.4 mm/mm², t-test, P < 0.001) despite recovery of touch sensitivity to normal levels by 6 months. Preoperatively, more frequent nerve loops (P < 0.001), crossings (P = 0.03), and greater crossing angles (P = 0.02) were observed, relative to healthy corneas. Postoperatively, nerve looping frequency increased, crossings were more frequent, and nerve tortuosity increased. Wide-field mosaics indicated persistent disrupted orientation of the regenerating subbasal nerves five years after CXL.

**Conclusions and Relevance:** Keratoconus is characterized by a neurotrophic deficit and altered nerve morphology that CXL treatment does not address, despite providing a positive biomechanical effect in the stroma. Given the widespread use of CXL in keratoconus management, progression of abnormal innervation post-CXL should be recognized.
Introduction

Corneal collagen cross-linking (CXL) has emerged as a promising treatment to strengthen the cornea in conditions such as corneal ectasia and keratoconus.\(^1\) Results from longer-term clinical studies\(^2-7\) suggest a lasting benefit of CXL treatment in halting the progression of keratoconus, thereby avoiding the need for transplantation. At the tissue level, knowledge of the effect of cross-linking has been gained from rabbit studies,\(^8-11\) and use of in vivo confocal microscopy (IVCM) in patients.\(^3,12-21\) Patient investigations have revealed not only a cross-linking effect in the corneal stroma, but also an effect of the procedure on corneal epithelial nerves.\(^12,14-17,19-21\) In epithelium-off CXL, epithelial nerves are completely removed in the treatment zone, typically an 8-9 mm diameter region of the central cornea. Analysis of the subbasal nerve plexus by IVCM has indicated gradual regeneration of these nerves postoperatively.\(^15,19-21\) Nerve regeneration is important for re-establishment of a healthy epithelium, protective blink reflex and trophic effects on the corneal stroma.\(^22\) Corneal nerves have also been postulated to have a role in the development of keratoconus.\(^23\) Regeneration of subbasal nerves after CXL has been shown to occur within the first postoperative year,\(^12,14,19-21\) but the long-term effect of CXL on corneal nerves has not been reported. It is unknown if corneal nerves reach equilibrium after the first year, whether they continue to regenerate over time, or if the reduced nerve density in keratoconus\(^23-28\) can improve after CXL. It is therefore of interest to investigate whether CXL can restore a healthy subbasal nerve density to the keratoconic cornea in the long term, or if a nerve deficit persists despite clinical success of the treatment. CXL is a relatively new treatment often given to young patients, whereas long-term clinical consequences such as a potential neurotrophic deficit may take decades to manifest.

In addition to reduced subbasal nerve density, several reports have indicated disrupted subbasal nerve patterns in keratoconus, including tortuous, branching, and looping patterns.\(^20,23,25-27,29\) It is not known, however, how prevalent such patterns are in healthy corneas or if CXL can influence these patterns (and by proxy the neurotrophic status) in the regenerated nerve plexus. Because subbasal nerve guidance is closely linked with epithelial
cell migration, subbasal nerves can mirror the epithelial status, which has been shown to be pathologic in keratoconus.

To better understand the regenerative capacity of subbasal nerves in keratoconus and in response to CXL treatment, a prospective study was conducted in a young patient population with early stage keratoconus undergoing CXL treatment.

**Methods**

**Subjects and Examinations**

Prior to recruitment, ethical approval was obtained from the Linköping Regional Human Ethics Review Committee. All study subjects gave voluntary informed consent to participate, and the study followed the tenets of the Declaration of Helsinki. Patients were included if they had documented progressive keratoconus over at least two clinic visits within a 12-month period, defined by decrease in uncorrected visual acuity ≥ 0.1 (decimal), increase in astigmatism ≥ 1D, increase in max K reading ≥ 1D, decrease in minimum corneal thickness (MCT) ≥ 20 µm or combination thereof, in sequential examinations made by an ophthalmologist and/or optometrist. Those with preoperative MCT below 400 µm were excluded. Persons under 18 years of age, those with other ocular pathology or prior ocular surgery, dry eye symptoms, diabetics, and pregnant women were also excluded from the study.

Preoperative examination included determination of uncorrected and best spectacle-corrected visual acuity (BSCVA), measurement of MCT by ultrasound pachymetry (UP; Tomey SP-2000, Japan) and anterior segment optical coherence tomography (ASOCT; Visante®, Carl Zeiss Meditec, Jena, Germany), topographic measurement (Orbscan II; Bausch & Lomb, Rochester, NY, USA), and in vivo confocal microscopy (IVCM; HRT3-RCM, Heidelberg Engineering, Heidelberg, Germany).

Study visits were conducted on seven separate occasions: preoperative, 1-6 m, 7-12 m, 13-24 m, 25-36 m, 37-48 m, and 49-60 m postoperative. Postoperatively, IVCM, ASOCT, topography, and refraction were performed. Additionally at the final postoperative visit, Schirmer’s test for tear production (without anesthesia) and the tear break-up time test were performed. Ocular surface sensitivity was measured by contact esthesiometry (Cochet-
Bonnet; Luneau Ophthalmologie, Chartres, France) preoperatively and at the 3, 6, and 12 month postoperative visits and at the final study visit.

Additionally a comparison group of age-matched healthy subjects was recruited. After obtaining informed consent, general medical status was taken and a full ophthalmic examination (including refraction, slit lamp biomicroscopy, ASOCT, and intraocular pressure measurement) was conducted to exclude systemic or ocular pathology. Only asymptomatic, healthy subjects with a clear cornea on slit lamp examination were included. IVCM and ASOCT examinations were performed for this group.

**UVA-Riboflavin Collagen Cross-linking Treatment (Epithelium-off Method)**

Standard epithelium-off CXL was performed as follows. The epithelium was removed in an 8-9 mm diameter central zone using alcohol. Riboflavin 0.1% with 20% dextran or a hypotonic riboflavin 0.1% solution was given topically, one drop every three minutes for 30 min (hypotonic solution for MCT < 430 µm). After confirming penetration of riboflavin into the anterior chamber, UVA irradiation was applied at 5 cm distance from the corneal surface with a 9 mm aperture for 30 minutes, during which time one drop of riboflavin was administered every three minutes. Preoperatively, the UVA source (with potentiometric voltage regulator; UV-X, IROC AG, Zürich, Switzerland) was calibrated (UV Light Meter, Model: YK-34UV, Lutron Electronic Enterprise Co., Ltd. Taipei, Taiwan) to give 3.0 mW/cm² at the corneal surface at 365 nm wavelength.

After treatment, patients received topical antibiotics (Oftaquix 5mg/ml, SantenPharma AB, Solna, Sweden) 4 times daily for 7 days. Starting day 5 postoperatively, dexamethasone (Maxidex 0.1%, Alcon, Stockholm, Sweden) was applied 3 times daily for 3 weeks. Patients were also given analgesics (e.g., acetaminophen and diclofenac) and tear substitutes (e.g., Viscotears, Laboratoires Thea, Clermont-Ferrand, France).

**In Vivo Confocal Microscopy**

IVCM was performed according to an established protocol. A motorized joystick was used to locate the subbasal nerve plexus layer, and images were acquired in sequence scan mode as the field of view was scanned over the subbasal nerve plexus. Two experienced observers selected images of subbasal nerves based on an earlier protocol taking into account...
contrast, absence of artifacts, no overlap and central location. Three images meeting these criteria were selected randomly for each subject and time point, and were coded to mask subject group and postoperative time. The resulting set of images was used for manual and automated nerve tracing analysis. For manual analysis, nerves were traced independently by the observers using NeuronJ, and main subbasal nerve crossings (excluding thinner secondary branches) were defined as two nerve branches continuing in an unaltered path after intersection. The narrowest crossing angle was measured using the angle tool in the software Fiji. Presence of nerve loops was noted, defined as main subbasal nerves with at least 180° change in path direction within a single image frame.

Automated analysis consisted of fully automatic image pre-processing, nerve recognition and tracing, and post-processing to remove false recognitions, all without human intervention. Automated analysis yielded subbasal nerve density and tortuosity using a previously reported index.

Generation of Subbasal Nerve Mosaics

At final follow-up, IVCM data from six patients was used for wide-field mosaic reconstruction. Mosaicking was performed by a fast, fully-automated algorithm described previously. Briefly, the algorithm iteratively compared pairs of images to determine image positioning in the mosaic space, and were registered by translation, rotation, and affinity transformations. Blending based on pixel intensity weighting provided a merged mosaic with homogeneous luminosity and contrast.

Statistical Analysis

The 95% limits of agreement for inter-observer and inter-method differences in subbasal nerve density were determined by the Bland-Altman method. Frequency of nerve loops across groups were tested with the z-test for proportions. MCT, nerve crossings and angles, and nerve density between specific groups were compared with independent t-tests, and the Mann-Whitney test for non-normal data. Tortuosity and time-dependence of nerve density were assessed using one-way ANOVA on ranks with Dunn’s method for post-hoc comparison. For longitudinal corneal sensitivity, one-way repeated measures ANOVA was used with the Holm-Sidak post-hoc method. With the exception of post-hoc tests, a two-
tailed alpha level of < 0.05 was considered significant. Statistics were performed using SigmaStat for Windows (Systat Inc, Chicago, IL, USA).

# Results

## Patient Characteristics

Patient characteristics (eTable 1) indicated thinner corneas in the keratoconus group (P < 0.001) while astigmatism, MCT, and K readings in the patient cohort represented early-stage keratoconus. Twelve keratoconus patients (63%) were classified as stage I, while seven (37%) were stage II, according to the Amsler-Krumeich classification.  

## Comparison of Subbasal Nerve Density

Preoperative subbasal nerve density in the keratoconus cohort was compared to healthy age-matched subjects (Figure 1). Subbasal nerve density in early-stage keratoconus (10.3 ± 5.6 mm/mm², mean ± SD) was reduced (by 51%) relative to the healthy, age-matched group (21.0 ± 4.2 mm/mm²), yielding a mean difference of 10.7 mm/mm² (95% CI: 6.8 to 14.6 mm/mm², t-test, P < 0.001). Automated analysis similarly indicated reduced nerve density in keratoconus, by 56% (8.9 ± 4.1 vs. 20.2 ± 3.6 mm/mm²; mean difference 11.3 mm/mm², 95% CI: 8.3 to 14.3 mm/mm² t-test, P < 0.001).

Inter-observer and inter-method comparisons of nerve density (eTable 2) revealed over/underestimation of nerve density by the manual/automated method, which was more pronounced in keratoconus subjects. Agreement between manual observers was stronger (narrower limits of agreement) than between methods.

## Regeneration of Subbasal Nerves after CXL Treatment

CXL procedures were completed without intra-operative complications. Each patient attended a mean of 5.5 visits during the 0-66 month study period (attendance rate of 79%). Longitudinal analysis of nerve regeneration corresponded to study visits arranged by interval: preoperative, 1-6 m, 7-12 m, 13-24 m, 25-36 m, 37-48 m, and 49-66 m postoperative. Nerve regeneration by manual and automated methods of analysis was time-dependent (P < 0.001 for both, Figure 2). Regardless of method, nerve density was reduced up to 6 months, followed by an increase at 7-12 m (ANOVA, P < 0.001). At 7-12 m, nerve
density did not differ from preoperative; however, median nerve density increased up to 4 - 5 years postoperative. By both analysis methods, final nerve density did not differ from preoperative but remained reduced relative to healthy corneas (Manual: mean reduction 8.5 mm/mm², 95% CI: 4.7 to 12.4 mm/mm², t-test, P < 0.001; Automated: 8.4 mm/mm², 95% CI: 5.0 to 11.8 mm/mm², t-test, P < 0.001).

Ocular surface sensitivity (Figure 2) was normal preoperatively (59 ± 3 mm), declined to 52 ± 13 mm at 3 months (P = 0.017), and recovered to preoperative, healthy levels at 6 months (60 ± 0 mm), with no further change at 12 months or at five years relative to preoperative. At final follow-up, tear production by the Schirmer test was 21 ± 6 mm in 5 min (range: 12 – 30 mm), and tear break-up time was 14 ± 4 sec (7 – 20 sec).

**Subbasal Nerve Morphology**

Preoperatively, reduced nerve density, nerve loops and crossings were evident (Figure 3). Regenerated nerves also exhibited loops and crossings, some following tortuous paths. No looping nerves and rare crossings were observed in healthy corneas, where dense nerves had mainly parallel orientations (Figure 3). Nerve loops were present in 0% of images from healthy subjects, 30% of preoperative images, and in 56% of images at final follow-up. A greater proportion of looping nerves was present in the keratoconus corneas compared to healthy corneas (P < 0.001, z-test). Crossings of main subbasal nerves were observed three times more frequently in keratoconus than in healthy subjects (Figure 4). The mean number of crossings per image frame was 0.27 for healthy subjects, 0.76 preoperatively (P = 0.03 relative to healthy), and 0.89 one year or longer post-CXL (P = 0.002). The mean crossing angle of subbasal nerve trunks was 57° ± 18° in healthy subjects, 70° ± 15° preoperatively (P = 0.02), and 65° ± 16° postoperatively. Tortuosity differed among healthy, preoperative, and final follow-up (ANOVA P = 0.008; Figure 4) with an increase after the first year post-CXL relative to healthy corneas.

**Architecture of Regenerated Subbasal Nerves**

At the five year follow-up, wide-field mosaics of the subbasal nerve plexus were constructed in six patients (Figure 5). As standard epi-off CXL removes the subbasal nerve plexus while leaving intact the nerve fiber bundles within and underneath Bowman’s layer, patterns of
nerve regeneration were examined by observing subbasal nerve paths starting at the penetration points (Figure 5A, E, and F, black arrows) into the subbasal layer. Nerves adopted radial, circumferential, or mixed orientations as they regenerated. Predominantly circumferential paths were observed in Figure 5A and F while Figure 5B, C, D, and E depicted all orientation types. Radial paths originated in the central cornea and were directed towards the periphery in straight lines. Mixed paths alternated between radial and circumferential orientations. Different orientation types appeared to give rise to the nerve patterns observed in single-image analysis. Crossings (black arrowheads in Figure 5B and E) were intersection points between radial and circumferential paths. Likewise, nerve loops appeared as paths alternating between circumferential and radial (white arrowheads in Figure 5B, C, E, and F). The dominance of one orientation over another appeared to give rise to abrupt or more gradual directional changes, resulting in sharp (Figure 5F) or smooth (Figure 5C and E) looping structures. Highly tortuous regenerated nerves were also apparent, representing frequent path alternations on a smaller scale than those giving rise to nerve loops (white arrows in Figure 5A, D, and E).

**Effect of Contact Lens Wear on Nerve Parameters**

Four and six patients had a history of pre- and postoperative contact lens wear, respectively. When stratified by contact lens wear, no difference in subbasal nerve density or the number of nerve crossings, respectively, was found preoperatively ($P = 0.82$, $P = 0.62$) or postoperatively ($P = 0.77$, $P = 0.79$).

**Stromal Status Five Years Post-CXL**

The full stromal thickness was scanned by IVCM in patients at final follow-up. Isolated zones devoid of keratocytes were evident, with apparent cellular debris and linear needle-like structures indicative of kerocyte apoptosis (eFigure 1). Outside these narrow zones (typically spanning a depth range of 10 – 20µm), normal-appearing keratocytes were visible.

**Discussion**

This study reports subbasal nerve regeneration after CXL over the longest follow-up period to date. Nerve density in the long-term remained reduced (by over 50%) relative to age-matched healthy corneas. Despite clinical success of CXL in halting keratoconus progression
and recovery of touch sensitivity,\textsuperscript{19,41} subbasal nerves did not regenerate beyond the original level even five years after CXL. Earlier studies have highlighted a poor correlation of subbasal nerve density and mechanical touch sensitivity;\textsuperscript{42,43} however, the root cause of abnormally sparse innervation of the subbasal plexus in keratoconus is clearly not addressed by the CXL treatment.

Another major finding was impaired nerve guidance resulting in loops, crossings, and tortuous paths seldom observed in healthy corneas. Moreover, abnormal nerve migration tended to progress after CXL treatment. Subbasal nerves forming open or closed loops have been noted qualitatively in keratoconus,\textsuperscript{23,25,29} and images indicating nerve path crossings are visible in several studies,\textsuperscript{20,23,25,26} but were not specifically noted or recognized as pathologic or characteristic of keratoconus. Additionally, subbasal nerve tortuosity has been noted to be subjectively increased in keratoconus.\textsuperscript{23,29} Quantifying these features for the first time and comparing to a healthy age-matched group, we report an increased frequency of nerve loops, crossings, right-angled crossings, and elevated tortuosity in early-stage keratoconus. Imaging these nerve features by IVCM could aid in the detection of early-stage keratoconus.

Besides analysis at the single-image level, reconstructed wide-field mosaics provided striking evidence that the CXL-treated cornea does not possess normal subbasal nerve architecture. While the normal spiraling architecture of corneal subbasal nerves\textsuperscript{44} has been shown to be perturbed in keratoconus,\textsuperscript{25} examination of mosaics after removal of the plexus during CXL presents a unique opportunity to examine subbasal nerve guidance. Balanced circumferential and radial forces resulting in a spiral pattern in the healthy cornea are dramatically disrupted in keratoconus. Preoperatively and long after clinical halting of progression, some nerves migrate only radially while others migrate only circumferentially (leading to inevitable right-angled crossings). Still other nerves receive mixed signals, changing orientation to form loops and tortuous paths.

Recent clinical studies indicate that the gross morphology of the corneal stroma is stabilized for at least 4-5 years after CXL,\textsuperscript{2,6} but it is unlikely that the pathologic expression of proteins and enzymes in the keratoconic eye is altered by the treatment. Corneal subbasal nerves (axons originating outside the stroma) may instead reflect the underlying disease process in
the long term. Clinical signs of a neurotrophic deficit (such as inflammation, modified tear film, or development of dry eye) were absent in this study; however, accumulation of dendritic cells was noted in several patients and a detailed investigation of the epithelium was not undertaken. Additional long-term study of these parameters is warranted. Within the stroma, persistent zones devoid of keratocytes, accompanied by features indicative of earlier apoptosis was an unexpected secondary finding also requiring further investigation.

Fully-automated nerve analysis led to the same conclusions as manual analysis, despite wider limits of agreement and a tendency to underestimate nerve density when fewer nerves were present (such nerves were often thinner with reduced contrast). Nevertheless, automation minimizes human bias and could enable near real-time analysis in the clinic.

It is pertinent to highlight limitations of the present study. The proportion of patients wearing contact lenses was low, which could mask a possible impact of contact lens wear on nerve regeneration after the CXL treatment in this small subset of subjects. Also, the cohort size was relatively small; larger prospective, long-term studies are warranted to confirm the present findings and establish more precise estimates of subbasal nerve parameters after CXL treatment. Finally, the present study focused only on early-stage keratoconus and not severe, advanced cases - including these patients could yield additional insight into progressive changes in corneal nerve parameters and morphology in keratoconus.

In summary, CXL treatment did not improve the nerve deficit in keratoconus and nerve disorientation persisted, reflecting the progressive condition. In CXL treatment for keratoconus and other corneal pathologies, the unlikelihood of improving neurotrophic status should be recognized.

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manuscript or decision to submit the manuscript for publication. NL had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. Author Contributions: conception or design: NL, TBW, TPU; acquisition, analysis, or interpretation of data: MP, SR, EP, PG, AR, SF, TBW, TPU, NL; drafting the manuscript: NL, MP, AR; critical review of manuscript: MP, SR, EP, PG, AR, SF, TBW, TPU, NL; statistical analysis: NL, MP; obtaining funding: NL, MP; administrative, technical or material support: MP, SR, EP, PG, AR, SF, TBW, TPU, NL; supervision: AR, TPU, NL.


**Figure Legends**

**Figure 1.** Subbasal nerve density in the central cornea in early-stage keratoconus (preoperative) versus healthy age-matched subjects (19 subjects per group).

**Figure 2.** Subbasal nerve regeneration up to five years after CXL treatment for progressive keratoconus. Top: manual analysis of subbasal nerve density indicated a significant reduction in the early postoperative period (asterisk). Center: Automated analysis yielded a similar pattern of nerve regeneration as manual analysis. Bottom: Mean and 95% confidence interval for corneal sensitivity, indicating a significant but minor reduction in sensitivity at 3 months postoperative (P = 0.017).

**Figure 3.** Nerve architecture in keratoconus. Left column: subbasal nerve plexus with roughly parallel nerve fiber bundles, low tortuosity, and rare crossings (black arrows). Centre column: looping nerves (white arrows) and increased crossings (black arrows). Right column: persistent looping nerves (white arrow), crossings (black arrows) and tortuous nerve paths (white arrowheads). All images are 400 µm x 400 µm.

**Figure 4.** Quantitative analysis of subbasal nerve morphology. Top: the number of subbasal nerve crossings per image frame. Center: the minimum crossing angle of subbasal nerves in cases of crossings. Bottom: nerve tortuosity.

**Figure 5.** Nerve plexus mosaics in 6 different patients five years after corneal collagen cross-linking treatment for keratoconus. (A) Circumferential nerve paths emerging from penetration points (black arrows), and tortuous paths (white arrows). (B) Crossings (black arrowheads) at intersections of radial and circumferential nerves, and loops (white arrows).
(C) Loops (white arrowheads) varying between radial and circumferential orientations. (D) Tortuous paths (white arrows). (E) Nerves penetrate (black arrows) and orient radially. Crossings (black arrowheads) where radial and circumferential nerves intersect. Also, tortuosity (white arrow) and loops (white arrowheads). (F) After penetration (black arrows), abrupt orientation changes (white arrowheads) form loops. All images, bar = 400µm.
Subbasal nerve density (mm/mm²)

P < 0.001

keratoconus  healthy  keratoconus  healthy

manual  automated
Healthy preop >12m post CXL subbasal nerve crossings per frame

- P = 0.03
- P = 0.02

Subbasal nerve crossing angle (degrees)

- P < 0.05

Tortuosity index

- P < 0.05
Online Only Material

The Online Only material consists of the following elements:

eTable 1. Subject Characteristics

eTable 2. Inter-observer and inter-method comparison of subbasal nerve density.

eFigure 1. IVCM images of the corneal stroma in 4 different patients taken 58 months after standard epithelium-off collagen cross-linking treatment.
**eTable 1.** Subject Characteristics.

<table>
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<tr>
<th></th>
<th>Healthy corneas n = 19</th>
<th>Keratoconus n = 19</th>
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<tbody>
<tr>
<td><strong>Sex, n (%)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>12 (63)</td>
<td>17 (89)</td>
</tr>
<tr>
<td>Female</td>
<td>7 (37)</td>
<td>2 (11)</td>
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<tr>
<td><strong>Age (y)</strong></td>
<td>29.9 ± 6.8</td>
<td>27.5 ± 7.1</td>
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<tr>
<td>Range (y)</td>
<td>20 - 45</td>
<td>19 - 44</td>
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<tr>
<td><strong>MCT (µm)</strong></td>
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<td>428 ± 36</td>
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<tr>
<td>Range (µm)</td>
<td>487 - 559</td>
<td>372 – 497</td>
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<tr>
<td><strong>Astigmatism (D)</strong></td>
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<td>Range (D)</td>
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<tr>
<td><strong>Max K (D)</strong></td>
<td>50.5 ± 4.9</td>
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<tr>
<td>Range (D)</td>
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<tr>
<td><strong>Min K (D)</strong></td>
<td>44.9 ± 4.9</td>
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<tr>
<td>Range (D)</td>
<td>38.3 - 59.8</td>
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</tbody>
</table>

*n*, number of subjects. Values for the keratoconus group are preoperative.
**Table 2.** Inter-observer and inter-method comparison of subbasal nerve density.

<table>
<thead>
<tr>
<th></th>
<th>Healthy</th>
<th>Keratoconus</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>$n = 57$</td>
<td>$n = 226$</td>
</tr>
<tr>
<td>Inter-observer (Obs 1 - Obs 2)</td>
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<td></td>
</tr>
<tr>
<td>Mean density difference (mm/mm²)</td>
<td>-0.30</td>
<td>0.06</td>
</tr>
<tr>
<td>95% LOA (mm/mm²)</td>
<td>± 1.58</td>
<td>± 1.22</td>
</tr>
<tr>
<td>Inter-method (Automated – Manual)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean density difference (mm/mm²)</td>
<td>-0.26</td>
<td>-1.74</td>
</tr>
<tr>
<td>95% LOA (mm/mm²)</td>
<td>± 3.13</td>
<td>± 4.41</td>
</tr>
</tbody>
</table>

$n$ refers to the number of confocal microscope images analyzed from each group. Three images were analyzed for each healthy reference subject and for each keratoconus subject at preoperative and postoperative time points. Obs 1, Obs 2 refer to the two trained human observer values from manual nerve tracing of images. 95% LOA are the 95% limits of agreement according to the Bland-Altman method.38
**Figure 1.** IVCM images of the corneal stroma in 4 different patients taken 58 months after standard epithelium-off collagen cross-linking treatment. In certain depth zones, the central corneal stroma was devoid of keratocytes and populated by apparent cellular debris (white arrows) and linear needle-like structures (black arrows), features indicative of kerocyte apoptosis. Depths of the images from the corneal surface (0µm) are 91, 185, 214, and 391µm for A-D, respectively. All images are 400 × 400µm.