Corneal Nerve Regeneration After Collagen Cross-Linking Treatment of Keratoconus
A 5-Year Longitudinal Study

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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 patients with keratoconus.

OBJECTIVE To determine whether CXL treatment of keratoconus results in normalization of subbasal nerve density and architecture up to 5 years after treatment.

DESIGN, SETTING, AND PARTICIPANTS Observational study of 19 patients with early-stage keratoconus indicated for a first CXL treatment with longitudinal follow-up to 5 years postoperatively (examinations were performed from 2009 to 2015; analysis was performed from February to May 2015) and 19 age-matched healthy volunteers at a primary care center and a university hospital ophthalmology department.

EXPOSURE The patients with keratoconus underwent standard epithelial-off UV-A/riboflavin CXL treatment with 30-minute UV-A exposure at 3 mW/cm² irradiance.

MAIN OUTCOMES AND MEASURES Central corneal subbasal nerve density and subbasal nerve architecture by use of laser-scanning in vivo confocal microscopy; subbasal nerve analysis by 2 masked observers and by use of a fully automated method; wide-field mosaics of subbasal nerve architecture by use of an automated method; and ocular surface touch sensitivity by use of contactesthesiometry.

RESULTS Mean (SD) age of the 19 patients with keratoconus was 27.5 (7.1) years (range, 19-44 years), and minimal corneal thickness was 428 (36) μm (range, 372-497 μm). Compared with the mean (SD) preoperative subbasal nerve density of 21.0 (4.2) mm/mm² in healthy corneas, the mean (SD) preoperative subbasal nerve density of 10.3 (5.6) mm/mm² in the corneas of patients with stage 1 or 2 keratoconus was reduced 51% (mean difference, 10.7 mm/mm² [95% CI, 6.8-14.6 mm/mm²]; P < .001). After CXL, nerves continued to regenerate for up to 5 years, but nerve density remained reduced relative to healthy corneas at final follow-up (mean reduction, 8.5 mm/mm² [95% CI, 4.7-12.4 mm/mm²]; P < .001) despite recovery of touch sensitivity to normal levels by 6 months. Preoperatively, more frequent nerve loops, crossings, and greater crossing angles were observed in the corneas of patients with keratoconus compared with healthy corneas. Postoperatively, the frequency of nerve looping increased, crossings were more frequent, and nerve tortuosity increased. Wide-field mosaics indicated persistent disrupted orientation of the regenerating subbasal nerves 5 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 the management of patients with keratoconus, the progression of abnormal innervation after CXL should be recognized.
Corneal collagen cross-linking (CXL) has emerged as a promising treatment to strengthen the cornea in conditions such as corneal ectasia and keratoconus. Results from longer-term clinical studies 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 and use of in vivo confocal microscopy (IVCM) in patients. Patient investigations have revealed a cross-linking effect on the corneal stroma but also an effect of treatment on corneal epithelial nerves. In epithelium-off CXL, epithelial nerves are completely removed in the treatment zone, typically an 8- to 9-mm diameter region of the central cornea. Analysis of the subbasal nerveplexus by use of IVCM has indicated gradual regeneration of these nerves postoperatively. Nerve regeneration is important for reestablishment of a healthy epithelium, a protective blink reflex, and trophic effects on the corneal stroma. Corneal nerves have also been postulated to play a role in the development of keratoconus. Regeneration of subbasal nerves after CXL has been shown to occur within the first postoperative year, but the long-term effect of CXL on corneal nerves has not been reported. It is unknown whether corneal nerves reach equilibrium after the first year, whether they continue to regenerate over time, or whether the reduced nerve density in keratoconus 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 whether a nerve deficit persists despite clinical success of the treatment. Collagen cross-linking is a relatively new treatment often given to young patients, and its 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. It is not known, however, how prevalent such patterns are in healthy corneas or whether CXL can influence these patterns (and by proxy the neurotrophic status) in the regenerative 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, we conducted a prospective study in a young patient population with early-stage keratoconus undergoing CXL treatment.

### Methods

**Participants and Examinations**

Prior to recruitment, ethical approval was obtained from the Linköping Regional Human Ethics Review Committee in Linköping, Sweden. All study participants provided written 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 2 clinic visits within a 12-month period, defined as a decrease in uncorrected visual acuity of 0.1 or more (decimal), an increase in astigmatism of 1 diopter (D) or more, and an increase in the maximum keratometry reading of 1 D or more, a decrease in minimum corneal thickness (MCT) of 20 μm or more, or combination thereof, in sequential examinations made by an ophthalmologist and/or optometrist. Those patients who underwent prior ocular surgery, persons with other ocular pathology or who underwent a prior ocular surgery, persons with diabetes mellitus, and pregnant women were also excluded from our study.

The preoperative examination included determination of uncorrected and best spectacle–corrected visual acuity, measurement of MCT by ultrasonographic pachymetry (Tomey SP-2000) and anterior segment optical coherence tomography (ASOCT; Visante; Carl Zeiss Meditec), topographic measurements (Orbscan II; Bausch & Lomb), and IVCM (HRT3-RCM; Heidelberg Engineering). Study visits were conducted on 7 separate occasions: before treatment and 1 to 6 months, 7 to 12 months, 13 to 24 months, 25 to 36 months, 37 to 48 months, and 49 to 60 months after treatment. Postoperatively, IVCM, ASOCT, topography, and refraction were performed. In addition, at the final postoperative visit, the Schirmer test for tear production (without anesthesia) and the tear breakup time test were performed. Ocular surface sensitivity was measured by contact esthesiometry (Cochet-Bonnet; Luneau Ophtalmologie) preoperatively, at the 3-, 6-, and 12-month postoperative visits, and at the final study visit.

In addition, a comparison group of age-matched healthy volunteers was recruited. The general medical status of all healthy volunteers was determined, and a full ophthalmic examination (including refraction, slitlamp biomicroscopy, ASOCT, and intraocular pressure measurement) was conducted in order to exclude patients with systemic or ocular pathology. Only asymptomatic, healthy volunteers with a clear cornea determined by slitlamp examination were included. Examinations using IVCM and ASOCT were performed for this healthy group.

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At a Glance

- How the neurotrophic status of the cornea in early-stage keratoconus is impacted by collagen cross-linking (CXL) treatment in the long term is unknown, but it may have implications for the progression of the disease.
- By use of in vivo confocal microscopy, a neural deficit of more than 50% was noted in patients with early-stage keratoconus that persisted 5 years after CXL treatment.
- Pathologic nerve patterns were more frequent in the corneas of patients with keratoconus than in the corneas of healthy volunteers, and they continued to progress in the long term after CXL treatment.
- Although only a small cohort of patients with early-stage keratoconus was examined, these findings suggest that CXL treatment is limited in its ability to improve the corneal neural status in patients with keratoconus.
UV-A/Riboflavin CXL Treatment (Epithelium-Off Method)
Standard epithelium-off CXL was performed as follows. The epithelium was removed in an 8- to 9-mm diameter central zone using alcohol. Riboflavin, 0.1%, with dextran, 20%, or a hypotonic riboflavin, 0.1%, solution was given topically, 1 drop every 3 minutes for 30 minutes (hypotonic solution for MCT < 430 μm). After confirming penetration of riboflavin into the anterior chamber, we applied UV-A irradiation at 5-cm distance from the corneal surface with a 9-mm aperture for 30 minutes, during which time 1 drop of riboflavin was administered every 3 minutes. Preoperatively, the UV-A source (with potentiometric voltage regulator [UV-X; IROC AG]) was calibrated (UV Light Meter, model YK-34UV; Lutron Electronic Enterprise Co, Ltd) to give 3.0 mW/cm² at the corneal surface at 365-nm wavelength.

After treatment, patients received topical antibiotics (5 mg/mL of levofloxacin) 4 times daily for 7 days. Starting day 5 postoperatively, dexamethasone sodium phosphate was applied 3 times daily for 3 weeks. Patients were also given analgesics (eg, acetaminophen and diclofenac sodium) and tearsubstitutes (eg, Viscottears; Laboratoires Théa).

In Vivo Confocal Microscopy
In vivo confocal microscopy was performed according to an established protocol. 33 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, 33 taking into account contrast, absence of artifacts, no overlap, and central location. Three images meeting these criteria were selected randomly for each participant and time point, and were coded to mask participant 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, 34 and main subbasal nerve crossings (excluding thinner secondary branches) were defined as 2 nerve branches continuing in an unaltered path after intersection. The narrowest crossing angle was measured using the angle tool in the software Fiji. 35 The presence of nerve loops was noted, and these loops were defined as main subbasal nerves with at least a 180° change in path direction within a single image frame.

Automated analysis consisted of fully automatic image preprocessing, nerve recognition and tracing, and postprocessing to remove false recognitions, all without human intervention. 33, 36 Automated analysis yielded subbasal nerve density and tortuosity using a previously reported index. 37

Generation of Subbasal Nerve Mosaics
At final follow-up, the IVCM data from 6 patients were used for wide-field mosaic reconstruction. Mosaicking was performed by a fast, fully automated algorithm described previously. 38 In brief, the algorithm iteratively compared pairs of images to determine image positioning in the mosaic space, and these images 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 interobserver and intermethod differences in subbasal nerve density were determined by use of the Bland-Altman method. 39 Frequencies of nerve loops across groups were tested for proportions by use of the χ² test. The MCTs, nerve crossings and angles, and nerve densities between specific groups were compared by use of independent t tests, and the Mann-Whitney test was used for nonnormal data. Tortuosity and time dependence of nerve density were assessed using 1-way analysis of variance (ANOVA) on ranks, with use of the Dunn method for post hoc comparison. For longitudinal corneal sensitivity, 1-way repeated-measures ANOVA was used with the Holm-Sidak post hoc method. With the exception of post hoc tests, a 2-tailed level of less than .05 was considered statistically significant. Statistics were performed using SigmaStat for Windows (Systat Inc).

Results
Patient Characteristics
Mean (SD) age of the 19 patients with keratoconus was 27.5 (7.1) years (range, 19-44 years), and minimal corneal thickness was 428 (36) μm (range, 372-497 μm). Patient characteristics (eTable 1 in the Supplement) indicated thinner corneas in the group of patients with keratoconus (P < .001), and the astigmatisms, MCTs, and keratometry readings in this patient cohort represented early-stage keratoconus. Of the 19 patients with keratoconus, 12 (63%) were classified as having stage 1 keratoconus according to the Amsler-Krumeich classification, 40 and 7 (37%) were classified as having stage 2 keratoconus.

Comparison of Subbasal Nerve Densities
Compared with the mean (SD) preoperative subbasal nerve density of 21.0 (4.2) mm/mm² in healthy corneas (determined by manual nerve-tracing analysis), the mean (SD) preoperative subbasal nerve density of 10.3 (5.6) mm/mm² in the corneas of patients with stage 1 or 2 keratoconus was reduced 51% (mean difference, 10.7 mm/mm² [95% CI, 6.8-14.6 mm/mm²]; P < .001, determined by use of the t test) (Figure 1). Compared with the mean (SD) preoperative subbasal nerve density of 20.2 (3.6) mm/mm² in healthy corneas (determined by automated nerve-tracing analysis), the mean (SD) preoperative subbasal nerve density of 8.9 (4.1) mm/mm² in the corneas of patients with stage 1 or 2 keratoconus was reduced 56% (mean difference, 11.3 mm/mm² [95% CI, 8.3-14.3 mm/mm²]; P < .001, determined by use of the t test) (Figure 1).

Interobserver and intermethod comparisons of nerve density (eTable 2 in the Supplement) revealed an overestimation and an underestimate of nerve density by the manual and the automated methods, respectively, which was more pronounced in the patients with keratoconus. Agreement between manual observers was stronger (narrower limits of agreement) than between methods.
Regeneration of Subbasal Nerves After CXL Treatment

Collagen cross-linking procedures were completed without intraoperative complications. Each patient attended a mean of 5.5 visits during the 66-month study period (with an attendance rate of 79%). Longitudinal analysis of nerve regeneration corresponded to study visits arranged by interval (before treatment and 1-6 months, 7-12 months, 13-24 months, 25-36 months, 37-48 months, and 49-66 months after treatment). Nerve regeneration by manual and automated methods of analysis was time-dependent ($P < .001$ for both) (Figure 2A and B). Regardless of method, nerve density was reduced at 6 months, followed by an increase at 7 to 12 months ($P < .001$, determined by ANOVA). At 7 to 12 months, nerve density did not differ from preoperative levels; however, the median nerve density increased for up to 4 to 5 years after treatment. By both analysis methods, the final nerve density did not differ from the preoperative nerve density but remained reduced relative to healthy corneas (manual method: mean reduction, 8.5 mm/mm$^2$ [95% CI, 4.7-12.4 mm/mm$^2$]; $P < .001$, determined by use of the $t$ test; automated method: mean reduction, 8.4 mm/mm$^2$ [95% CI, 5.0-11.8 mm/mm$^2$]; $P < .001$, determined by use of the $t$ test).

The mean (SD) ocular surface sensitivity (Figure 2C) was normal preoperatively at 59 (3) mm, decreased to 52 (13) mm at 3 months ($P = .02$), and recovered to preoperative, healthy levels at 6 months (ie, 60 [0] mm), with no further change at 12 months or at 5 years relative to preoperative levels. At final follow-up, the mean (SD) tear production by use of the Schirmer test was 21 (6) mm in 5 minutes (range, 12-30 mm), and the mean (SD) tear break-up time was 14 (4) seconds (range, 7-20 seconds).
Subbasal Nerve Morphology

Preoperatively, reduced nerve density and reduced number of nerve loops and nerve crossings were evident (Figure 3). Regenerated nerves also exhibited loops and crossings, some following tortuous paths. No looping nerves or 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 volunteers, 30% of preoperative images from patients with keratoconus, and in 56% of images from patients with keratoconus at final follow-up. A greater proportion of looping nerves was present in the keratoconic corneas compared with the healthy corneas ($P < .001$, determined by use of the $z$ test). Crossings of main subbasal nerves were observed 3 times more frequently in keratoconic corneas than in healthy corneas (Figure 4). The mean number of crossings per image frame was 0.27 for healthy volunteers, 0.76 for patients with keratoconus before CXL treatment ($P = .03$ relative to healthy volunteers), and 0.89 for patients with keratoconus 1 year or longer after CXL treatment ($P = .002$). The mean (SD) crossing angle of subbasal nerve trunks was $57°(18°)$ for healthy volunteers, $70°(15°)$ for patients with keratoconus before CXL treatment ($P = .02$), and $65°(16°)$ for patients with keratoconus after CXL treatment. Tortuosity differed among healthy corneas and keratoconic corneas before CXL treatment and at final follow-up ($P = .008$, determined by ANOVA) (Figure 4), with an increase in tortuosity 1 year after CXL treatment relative to healthy corneas.

Architecture of Regenerated Subbasal Nerves

At the 5-year follow-up, wide-field mosaics of the subbasal nerve plexus were constructed for 6 patients (Figure 5). Because standard epithelium-off CXL removes the subbasal nerve plexus while leaving intact the nerve fiber bundles within and underneath the Bowman layer, patterns of nerve regenera-
tion were examined by observing subbasal nerve paths starting at the penetration points (Figure 5A, E, and F) into the subbasal layer. Nerves adopted radial, circumferential, or mixed orientations as they regenerated. Predominantly circumferential paths are observed in Figure 5A and F, whereas all types of orientation are depicted in Figure 5B, C, D, and E. Radial paths originated in the central cornea and were directed toward 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 (Figure 5B and E) were intersection points between radial and circumferential paths. Likewise, nerve loops appeared as alternating circumferential and radial paths (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 (Figure 5A, D, and E).

Effect of Contact Lens on Nerve Parameters
Four patients with keratoconus wore contact lenses before CXL treatment, whereas 6 patients with keratoconus wore contact lenses after CXL treatment. When stratified by contact lens wear, no difference in subbasal nerve density or number of nerve crossings, respectively, was found preoperatively ($P = .82$ and $P = .62$) or postoperatively ($P = .77$ and $P = .79$).

Stromal Status 5 Years After CXL Treatment
The full stromal thickness was scanned by use of IVCM for patients at final follow-up. Isolated zones devoid of keratocytes were evident, with apparent cellular debris and linear needle-like structures indicative of keratocyte apoptosis (eFigure in the Supplement). Outside these narrow zones (typically spanning a depth range of 10-20 μm), normal-appearing keratocytes were visible.

Discussion
Our study reports of subbasal nerve regeneration after CXL over the longest follow-up period to date. Nerve density in the long term remained reduced (by >50%) in keratoconic corneas relative to age-matched healthy corneas. Despite the clinical success of CXL in halting keratoconus progression and with regard to the recovery of touch sensitivity,19,41 subbasal nerves did not regenerate beyond the original level even 5 years after CXL. Earlier studies42,43 have highlighted a poor correlation of subbasal nerve density and mechanical touch sensitivity; 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 the 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,23,25,29 and images indicating nerve path crossings are visible in several studies20,24,44 but were not specifically noted or recognized as pathologic or characteristic of keratoconus. In addition, subbasal nerve tortuosity has been noted to be subjectively increased in keratoconic corneas,23,29
Figure 5. Nerve Plexus Mosaics in 6 Different Patients 5 Years After Corneal Collagen Cross-Linking Treatment of Keratoconus

A. Circumferential nerve paths and tortuous paths

B. Crossings and loops

C. Loops

D. Tortuous paths

E. Nerve crossings, tortuosity, and loops

F. Abrupt orientation changes after penetration

A, Circumferential nerve paths emerging from penetration points (black arrowheads), and tortuous paths (white arrowheads). B, Crossings (magenta arrowheads) at intersections of radial and circumferential nerves, and loops (yellow arrowheads). C, Loops (yellow arrowheads) varying between radial and circumferential orientations. D, Tortuous paths (white arrowheads). E, Nerves penetrate (black arrowheads) and orient radially. Crossings (magenta arrowheads), where radial and circumferential nerves intersect, tortuosity (white arrowhead), and loops (yellow arrowheads). F, After penetration (black arrowheads), abrupt orientation changes (yellow arrowheads) form loops. Scale bars represent 400 μm.
Quantifying these features in patients with keratoconus for the first time and comparing them with those features in healthy age-matched volunteers, we report an increased frequency of nerve loops, crossings, and right-angled crossings and elevated tortuosity in patients with early-stage keratoconus. Imaging these nerve features by use of 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 has been shown to be perturbed in keratoconic corneas, the 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 the keratoconic cornea. Progressively 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 to 5 years after CXL, 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 our study; however, the 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 kerocytes, accompanied by features indicative of earlier apoptosis, were 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 the limitations of the present study. The proportion of patients wearing contact lenses was low, which could mask a possible effect of contact lens on nerve regeneration after the CXL treatment in this small subset of patients. 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; including patients with severe, advanced stages of keratoconus could yield additional insights into the progressive changes in corneal nerve parameters and morphology that occur in keratoconic corneas.

Conclusions

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